



THE INFLUENCE OF SELECTED SULPHUR CONTAINING COMPOUNDS ON RETINAL CELL DEATH

Aman Shah B. Abdul Majid

Green Templeton College
University of Oxford

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THESIS ABSTRACT

Ganglion cell death in glaucoma is caused by a variety of insults that include ischemia, insufficient neurotrophic support and oxidative stress. Experimental studies were therefore conducted on cell cultures to determine how serum deprivation (to mimic insufficient neurotrophic support) or oxidative glutamate toxicity (GB), cause oxidative stress and induce retinal cell death. Moreover, studies were carried out to deduce whether selected sulphur containing compounds can blunt any negative influences to cells in culture and/or a defined ischaemic insult to the rat retina in situ, as this might suggest their use for the treatment of glaucoma.

Serum deprivation and GB caused generation of reactive oxygen species (ROS) and apoptosis-like death to transformed retinal ganglion cells (RGC-5 cells). RGC-5 cells were more susceptible to the detrimental effects of GB than serum deprivation. RGC-5 cells subjected to serum deprivation appear to die by mechanisms that resemble classical apoptosis more closely than that caused by GB and the phase between the maximal generation of ROS and cell death were different. Cell death caused by serum deprivation was caspase-dependent but this was not the case for GB. Moreover, of the two sulphur compounds sulbutiamine and N-acetyl cysteine (NAC), sulbutiamine blunts the effect of serum deprivation more effectively. In addition, the pan caspase inhibitor z-VAD-fmk attenuated the negative effect of serum deprivation to RGC-5 cells while the necroptosis inhibitor (necrostsatin-1) counteracted solely the insult of GB.

The sulphur containing compounds, ACS1 and ACS 67 which release hydrogen sulphide (H_2S) slowly and NAC (a pro-cysteine GSH precursor) attenuated GB-induced cell death of RGC-5 cells. In contrast, sulbutiamine (a lipophilic thiolic thiamine derivative) was particularly effective in protecting RGC-5 cells from an insult of serum deprivation. Moreover, all of the sulphur compounds directly sequestered different types of ROS but with varying efficiency.

Common features by which all tested sulphur containing agents seem to elicit a mode of action include the stimulation of GSH and the antioxidant enzyme glutathione-S-transferase (GST) as well as to scavenge excess free radicals. Moreover, the slow release of H_2S from the ACS compounds appears to protect cells from oxidative stress through increasing the level of GSH, modulation of the cystine uptake transporter xCT, stimulation of the oxidative stress related transcription factor Nrf2 and the stimulation of pro-survival signalling pathways.

The slow releasing H_2S sulphur compound ACS67 also counteracts a number of detrimental influences to the rat retina in situ because of ischemia/reperfusion that includes damage to their ganglion cells. This suggests that such sulphur compounds might find a use in the treatment of glaucoma where ischemia probably plays a part in the disease process.

DEDICATIONS

To my mother Wan Bee bt. Che Din

The merciful mum, shining into my life as a sun. I can never repay you

To my father Abdul Majid b. Bahadur Khan

The symbol of devotion, inspiring and supporting me without limitation. I am proud of you and lucky to have you

To my grandmother Fatimah bt. Pawanche

You love me enough to teach me kindness and compassion, and gave me the strength to overcome all hurdles in life





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ABBREVIATIONS

ADTOH (ACS1)…….. 5-(4-hydroxyphenyl)-3*H*-1,2-dithiole-3-thione
ADP……..Adenosine diphosphate
AEC……..3-amino-9-ethylcarbazole
AIDS…….Acquired immunodeficiency syndrome
AIF……....Apoptosis inducing factor
Apaf-1……...Apoptosis activating factor 1
Ape-1…….....Apurinic endonuclease-1
AQP4…….Aquaporin-4
ARVO……...Association for Research in Vision and Ophthalmology
ATP……...Adenosine triphosphate
Bak……....Bcl-2 homologous antagonist/killer
Bax……....Bcl-2-associated X protein
BBB……..Blood brain barrier
BDNF……....Brain-derived neurotrophic factor
BER……...Base excision repair
bFGF-2……..Basic fibroblast growth factor
Bid…….BH3 interacting domain death agonist
BSA……...Bovine serum albumin
BSO……...DL-buthionine sulfoxime
CAT……...Catalase
Ca^{2+} ……..Calcium
ChAT…….Choline acetyltransferase
CM-H_2DCFDA…….5-(and-6)-chloromethyl-2', 7'-dichlorodihydrofluorescein diacetate, acetyl ester
CNS……...Central nervous system
CNTF…….Ciliary neurotrophic factor
CO_2……....Carbon Dioxide
COX……...Cyclooxygenase
CREB…….cAMP response element binding protein
CSF…….Cerebrospinal fluid
Cu^{2+}……Copper
Cyt c……...Cytochrome C
dATP……..Deoxyadenosine triphosphate
dCTP……..Deoxycytidine triphosphate
DCFH-DA……..Dichlorofluorescin diacetate
DEPC…….....Diethylpyrocarbonate
dGTP……..Deoxyguanosine triphosphate
DHE……...2’, 7’-dihydroethidium
Dibutyryl cAMP……....N^6, 2'-O-dibutyryladenosine 3':5'-cyclic monophosphate sodium
DISC……..Death-inducible signaling complex
DMSO……Dimethyl sulfoxide
DNA……...Deoxyribonucleic acid
DNase I……..Deoxyribonuclease I
dNTPs……Deoxynucleotide triphosphate
DTT……Dithiothreitol
dTTP……...Deoxythymidine triphosphate

dUTP……Deoxyuridine triphosphate
EDTA……...Ethylene diamine tetraacetic
EGCG……..Epigallicatechin gallate
EGFR……...Epidermal growth factor receptor
ELISA……..Enzyme-linked immunosorbent assay
Endo G……Endonuclease G
eNOS……....Endothelial nitric oxide synthase
EP1……...Prostaglandin E1 receptor
EP2……...Prostaglandin E2 receptor
EP3……...Prostaglandin E3 receptor
EP4……...Prostaglandin E4 receptor
ER……Endoplasmic reticulum
ERG……Electroretinogram
ERK……Extracellular signal-regulated kinase
FAD……..Flavin adenosine dinucleotide
FADD……...Fas-associated death domain
$FADH_2$……1,5-dihydroflavin adenine dinucleotide
FASL……Fas ligand
FBS……...Foetal bovine serum
FCS……...Foetal calf serum
Fe^{2+} ……....Iron
FITC……Fluorescein isothiocyanate
FLICE……..Fas-like ICE
FSC……..Forward scatter
GAAD……Granzyme A-activated DNase
GABA……..Gamma-amino-butyric acid
GAP-43……....Growth-associated protein-43
GAPDH……...Glyceraldehyde phosphate dehydrogenase
GCL……Ganglion cell layer
GEF……..Guanin Nucleotide exchange factor
GFAP……...Glial fibrillary acidic protein
GLAST……L-glutamate/L-aspartate transporters
GPI……...Glycosylphosphatidylinositol
Gpx……...Glutathione peroxidase
GrA……...Granzyme A
Grx……....Glutaredoxin
GS……Glutamine synthetase
GST……...Glutathione-S-transferase
GSH……..Glutathione (reduced)
GSSG……....Glutathione disulfide
H_2O_2……..Hydrogen peroxide
H^+……..Hydrogen
HEPES……..N-2-hydroxyl piperazine-N'-2-elthane sulfonic acid
HST……...High saline Tris buffer
HtrA2/Omi……High–temperature requirement protein A2
HO-1……Haemeoxygenase-1
IAPs……....Inhibitor of apoptosis proteins
IDH_m……....Mitochondrial isocitrate dehydrogenase
IEB……...Immunoelectroblotting
IFN……...Interferon

IgG…….Immunoglobulin G
IMM……...Inner mitochondrial membrane
INL……Inner nuclear layer
iNOS……..Inducible nitric oxide synthase
IOP …….....Intraocular pressure
IPL…….Inner plexiform layer
ipRGCs……..Intrinsically photosensitive retinal ganglion cells
JAK…….Janus tyrosine kinases
JNK…….Jun kinase
K^+……...Potassium
LMP……Lysosomal membrane permeabilization
MAG……..Myelin-associated glycoprotein
MAPK……Mitogen-activated protein kinase
MEM……..Modified Eagle Medium
Mg……..Magnesium
MLS……Mitochondrial localization sequences
M-MLV……..Mononey murine leukaemia virus
MOMP……Mitochondrial outer membrane permeabilization
MPT……Mitochondrial permeability transition
MPTP …….1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
mtDNA……...Mitochondrial deoxyribonucleic acid
MTT…….3, 4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide
Na^+……...Sodium
NAD……Nicotinamide adenosine dinucleotide
NADH…….Nicotinamide adenosine dinucleotide (reduced)
NADP……..Nicotinamide adenosine dinucleotide phosphate
NADPH……...Nicotinamide adenosine dinucleotide phosphate(reduced)
NaHS……....Sodium Hydro Sulphide
NAC…….N-Acetyl-L-Cysteine
NFL……..Nerve fibre layer
NF-L…….Neurofilament light form
NLS……..Nuclear localization sequence
NMDA…….N-methyl-D-aspartate
nNOS……...Neuronal nitric oxide synthase
NO……...Nitric oxide
NOS…….Nitric oxide synthase
Nrf-2……Nuclear factor (erythroid-derived 2)-like 2
NU1025……...8-hydroxy-2-methyl-quinazolin-4-[3H] one
O_2•-…….Superoxide anion
OD……...Optical density
OFL…….Optic fibre layer
OH•-……...Hydroxyl radical
Oligo$[dT]_{30}$……..Oligo-dithymidylate$_{30}$
OLM…….....Outer limiting membrane
OMM……...Outer mitochondrial membrane
ONH……Optic nerve head
ONL…….Outer nuclear layer
OPL …….Outer plexiform layer

PAGE……...Polyacrylamide gel electrophoresis
PARG……....Poly(ADP-ribose) glycohydrolase
PARP…….Poly (ADP-ribose) Polymerase
PBS……....Phosphate buffered saline
PCD……...Programmed cell death
PGD_2…….Prostaglandin D2
PGE_2…….Prostaglandin E2
$PGF_{2\alpha}$……....Prostaglandin F2α
PGI_2……..Prostaglandin I2
PGJ_2……..Prostaglandin J2
PHAP……Putative HLA-DR-associated protein
PI3-K……Phosphatidylinositol 3-kinase
PIDD……p53-induced protein with a death domain
PKA……..Protein kinase A
PKC……..Protein kinase C
PLA_2 ……Phospholipase A_2
PMSF……Phenylmethylsulfonyl fluoride
PN1……...Postnatal day 1
pp32……..Protein phosphatase 2A
PR…….Photoreceptor
Prx…….Peroxiredoxin
PTPC…….Permeability transition pore complex
PUFA……Polyunsaturated fatty acid
qPCR…….Real Time/Quantitative-Polymerase Chain Reaction
RAIDD…….RIP-associated ICH-1 homologous protein with a death domain
RGC……..Retinal ganglion cell
RIP1……..Receptor-interacting protein kinase 1
RRA……..Resazurin Reduction Assay
ROS……...Reactive oxygen species
RPE……...Retinal pigment epithelium
rRNA…….Ribosomal ribonucleic acid
RT-PCR…….Reverse Transcription-Polymerase Chain Reaction
SD……..Serum deprivation
SDS…….. .Sodium dodecyl sulphate
SEM……...Standard error of the mean
Smac……...Second mitochondrial activator of caspases
SNAP…….S-nitroso-N-acetyl-DL-penicillamine
SNP……Sodium nitroprusside
STAT3……....Signal transducers and activators of transcription 3
STR……Scotopic threshold response
SOD……...Superoxide dismutase
TBARS……..Thiobarbituric acid reactive species
TBE……Tris-borate-ethylene-diamino-tetra-acetic acid
TBS……Tris-buffer saline
TBS-T…….....Tris-buffer saline containing 0.1% Tween-20
TdT…….Terminal deoxynucleotidyl transferase
TEMED……..N,N,N’N’-tetramethylethylenediamine
TH……..Transhydrogenase
TLK……....Toll-like receptor

TNF-α…….....Tumour necrosis factor-alpha
TNFR……..Tumor necrosis factor receptor
TRAIL…….....TNF related apoptosis inducing ligand
TRAILR……..TNF related apoptosis inducing lignad receptor
Trk……...Tyrosine kinase
tRNA……..Transcriptional ribonucleic acid
Trx……...Thioredoxin
TrxR……Thioredoxin reductase
Tx…….....Thromboxane
UV……..Ultraviolet
VDAC…….....Voltage-dependent anion channel
WST-1…….....4-[3-(-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetzolio]-
1,3 benzene disulfonate
XIAP……...X-linked inhibitor of apoptosis protein
Z-VAD-FMK..……Benzoylcarbonyl-Val-Ala-Asp-fluoromethyl ketone

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Chapter 1

General Introduction



" and when the sky is torn apart so it was like a red rose, like ointment"

1.1 Introduction

Neurodegenerative diseases are chronic diseases of the nervous system that includes the brain and retina. Although for the most part the causes and mechanisms of the various types of neurodegenerative diseases remain elusive, they are generally associated with ageing. Moreover, different neurodegenerative diseases have similar yet distinctive characteristics. Specific populations of neurons become dysfunctional and die at different times, producing signs and symptoms of that disease. Often a genetic link is associated with specific neurodegenerative diseases. Significantly, it is known that in neurological degenerative diseases, over-production of oxidative free-radical compounds (oxidative stress) that cause lipid, protein and genetic structural changes are involved. Other risk factors, such as nutritional deficiencies, trauma, specific toxins, autoimmune immunological responses, specific vascular abnormalities, ischaemia/hypoxia, change in specific neurotransmitter concentrations, deprivation of neurotrophic factors and mitochondrial malfunctions play a part in characterizing a particular neurodegenerative disease.

Brain neurodegenerative diseases are numerous and include Amyotrophic Lateral Sclerosis (ALS), Alzheimer's disease, Multiple Sclerosis, Parkinson's disease and Huntington's disease. In contrast, neurodegenerative diseases of the retina are restricted to photoreceptor and ganglion cells being affected, as in Retinitis Pigmentosa and the optic neuropathies, respectively. Glaucoma is an example of the optic neuropathies. A characteristic of many of the neurodegenerative diseases, including glaucoma, is that neurone-types that die, do so at a variable rate. This provide an opportunity for therapeutic action, to protect surviving neurones. There are various ways with to achieve this, including the use of pharmacological agents to counteract specific negative events causing neuronal death. Such an aim can be achieved where

the pathogenesis of the neurodegenerative disease is known and the disease mechanism can be targeted. One way forward is to test hypotheses of pathogenesis and use this information as a blue-print for the development of therapeutic agents.

This thesis embodies these ideas in relation to the pathogenesis of glaucoma (Primary Open-Angle Glaucoma (POAG)) and the potential use of certain agents that might be applicable for the treatment of POAG.

1.2 Overview of the pathogenesis of POAG

About 82% of all blind persons are 50 years of age and older (Resnikoff et al. 2004) and glaucoma is the second leading cause of blindness worldwide (Quigley 1996). Glaucoma, of which there are many type is classified as a neurodegenerative disease involving the loss of retinal ganglion cells (RGCs) and producing a characteristic pattern of optic neuropathy (Foster et al. 2002). Raised intraocular pressure (IOP) is a significant risk factor for developing glaucoma, and there is no set threshold of IOP to initiate the disease. One person may develop glaucomatous nerve damage at a relatively low pressure, while another person may have high eye pressure for years and yet never develop glaucoma (Tielsch et al. 1991). However, untreated glaucoma eventually results in progressive visual field loss and finally blindness. Although there are different forms of glaucoma, the most common is POAG. Visual loss as a consequence of POAG predominantly affects the elderly and symptoms such as loss of peripheral vision, seeing halos around lights, difficulty focusing on close work and frequent changes of prescription glasses are warning signs (Quigley 1993).

The elevation of IOP in POAG is thought to be caused by abnormal resistance of aqueous outflow through the trabecular meshwork (TM), a specialized tissue lining the outflow pathway of the eye (Seiler and Wollensak 1985; Loewen et al. 2001). Elevated IOP is associated with decreased blood perfusion pressure at the optic nerve head, which is thought to increase the risk of progressive neuropathy and ganglion cell death in the neural retina. Persistent uncontrolled increase in eye pressure therefore damages the optic nerve, causing ganglion cells to die that eventually results in blindness (Gordon et al. 2002; Feiner and Piltz-Seymour 2003). How the glaucomatous optic nerve appears at different stages of POAG in comparison with normal human optic nerve is shown in Figure 1.1. Peripheral vision is first affected in POAG and may not be noticed until significant vision loss has already occurred. Loss of central vision occurs at later stages in POAG (Tsai 2008).

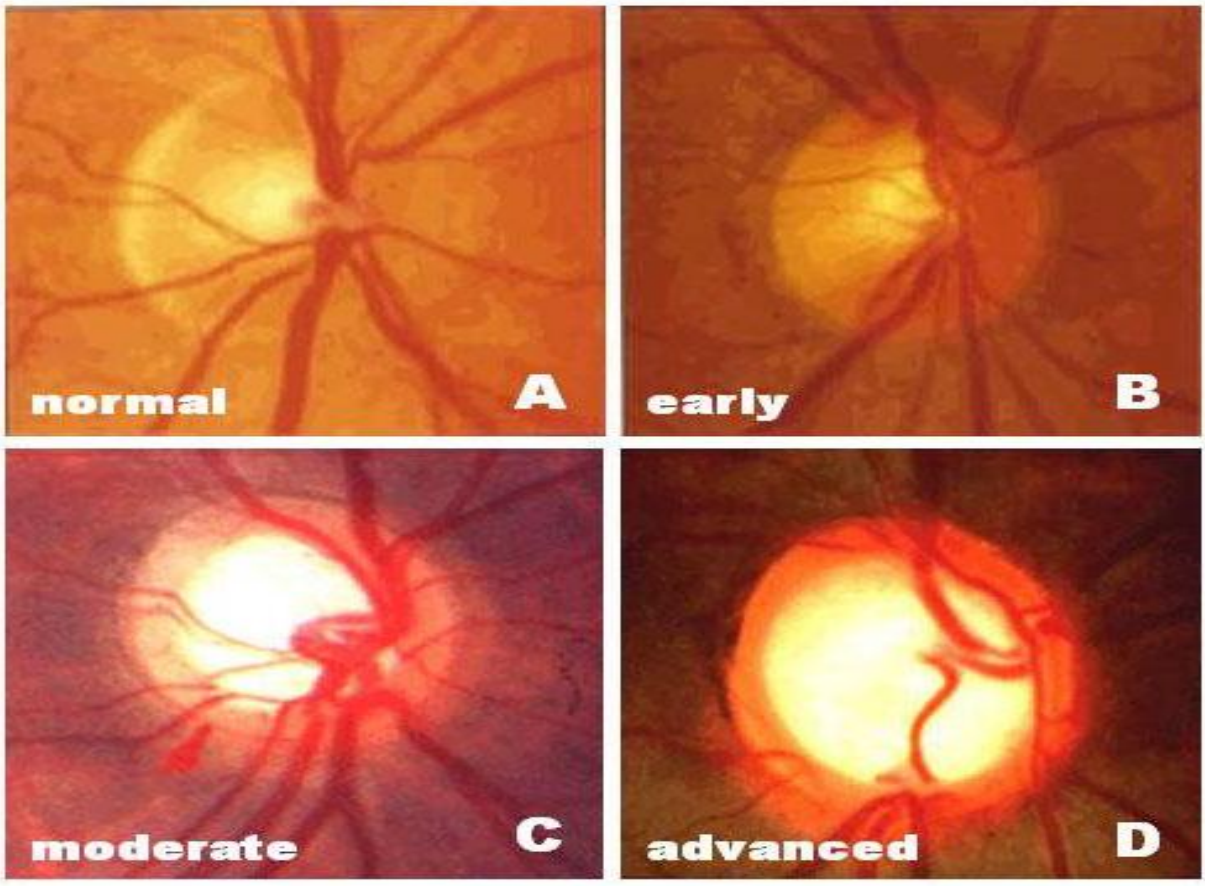


Figure 1.1: Structural changes in the human optic nerve in POAG. In moderate and advanced stages the disc appears pale with cupping evidenced by the changes in the orientation of the retinal vessels. The loss of the retinal rim is also clearly noticeable. (Adapted from O'Neill EC et al. (2009) The optic nerve head in hereditary optic neuropathies. Nat Rev Neurol 5:277-87)

It is generally thought that age- and disease-related loss of RGCs and eventual axonal degeneration is the cause behind visual loss in glaucoma. Loss of TM cells, followed by substitution with the extracellular matrix, which contributes to an increased resistance to aqueous outflow and the subsequent increase in IOP, has been suggested as a cause for the initiation of POAG in some patients (Alvarado et al. 1981; Potau et al. 2000; Lutjen-Drecoll 2005; Lütjen-Drecoll 1989). Other possible causes for initiating POAG include mutations of specific genes, such as the myocilin (*MYOC*) 19 and optineurin (*OPTN*) 20 gene and vascular abnormalities to the optic nerve head including systemic arterial hypotension, bleeding, and atherosclerosis (Flammer et al. 1999; Chung et al. 1999; Pache and Flammer 2006). More recently, the abnormally high trans-lamina cribrosa pressure difference brought about by a decrease in cerebrospinal fluid pressure and changes in IOP have also been implicated in the pathogenesis of POAG (Wostyn et al. 2009). Thus, the term 'ischaemia', which is a pathological situation where the cellular demands are compromised due to affected blood delivery, is often implicated in the initiation of POAG (Osborne et al. 2004). This can be sustained or intermittent, with episodes of reduced perfusion due to IOP fluctuation and thus would have varying effects on the optic nerve head (Osborne 2010). The significant up-regulation of hypoxia-inducible factor-1α (HIF-1α) usually due to the lack of oxygen in the glaucomatous human retina (Tezel and Wax 2004a) also suggests the dual role of ischaemia and/or hypoxia in POAG.

Oxidative stress has also been implicated in the pathogenesis of POAG (Tezel 2006, Izzoti 2003). The term implies that the homeostatic balance for free radicals in the different cellular compartments no longer exist for one reason or another and this leads to a cascade of biochemical events where the outcome is unfavourable to cell survival (Osborne 2010a). The

roles of mitochondria are critical, as they maintain cellular homeostasis in numerous metabolic functions that include oxidative energy metabolism, maintaining intracellular calcium levels and ROS production (Osborne 2010a). Mitochondrial energy metabolism (Boveris and Navarro 2008) is affected by age and this may also play a part in the pathogenesis of POAG, as ganglion cells are heavily dependent on their mitochondria (Osborne 2010).

The retina especially, has a high metabolic demand and depends heavily on normal mitochondrial function to sustain a continuous metabolic requirement, which includes the production of free radicals at normal physiological levels (Szabo et al. 1991; Szabo et al. 1992). For example, ROS are reported to trigger a number of events that lead to cell death (Bonne et al. 1998). Based on cell culture studies, ROS are thought to affect TM cells of certain POAG patients (He et al. 2008) leading to a reduction of local antioxidant activities, reducing the aqueous outflow, and promoting the activities of ROS-scavenging enzymes, such as superoxide dismutase (SOD) and glutathione peroxidase (GPx) (Ferreira et al. 2004; Kahn et al. 1983). It is possible that such pathological changes could also affect RGCs in a similar fashion, leading to axonal degeneration and eventual visual disturbance in POAG patients (Izzotti et al. 2003). The association of oxidative damage in POAG has also been further supported by the finding that glaucoma-affected patients have a significant depletion of total antioxidant potential in the aqueous humour (Ferreira et al. 2004), an increase in serum antibodies against the detoxifying enzyme glutathione-S-transferase (GST) (Yang et al. 2001), a decrease in plasma glutathione (GSH) level, (Gherghel et al. 2005) and an increase in lipid peroxidation products in the plasma (Yildirim et al. 2005). Such observations point to a role for oxidative stress in the pathogenesis of POAG and provide new insights into the development of novel neuro-protective

pharmacological therapies aimed at reducing the oxidative load and its negative effects on retinal cell survival in POAG.

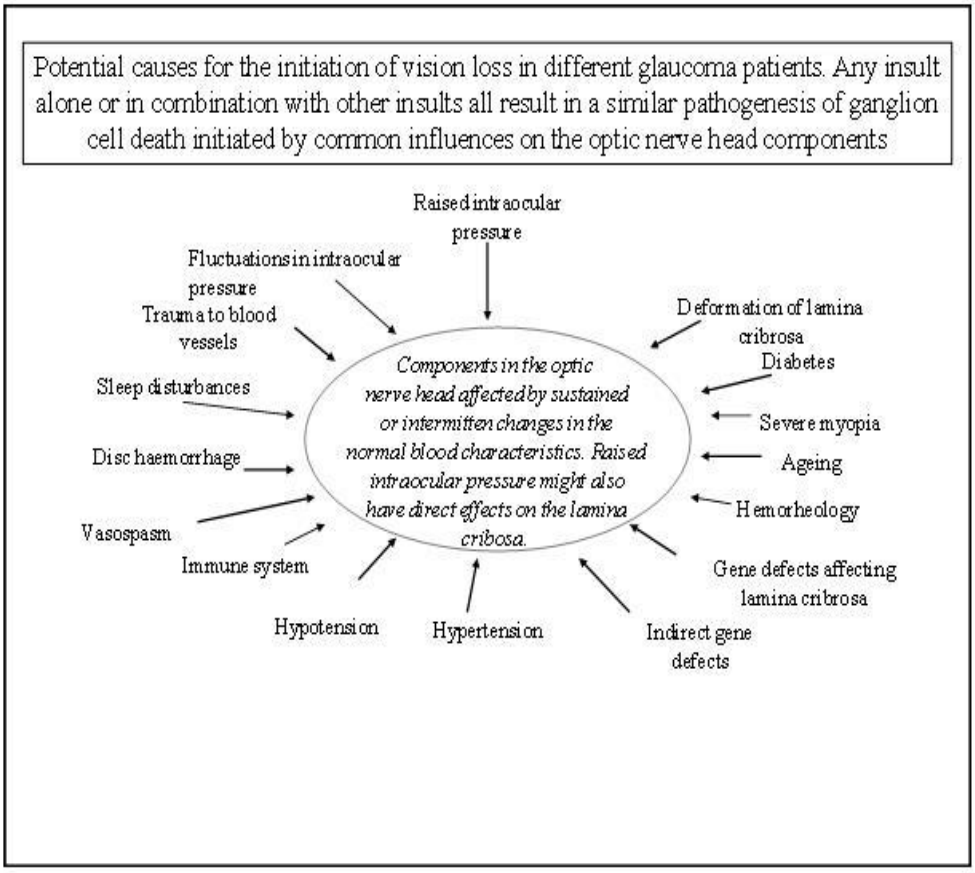


Figure 1.2: Summary of some probable events that result in optic nerve head injury as occurs in POAG. Intermittent or sustained ischaemia plays a major role, which leads to ganglion cell mitochondrial dysfunction due to deprivation of essential substrates for energy metabolism. This renders the ganglion cells susceptible to secondary insults. This is proposed to vary from cell to cell because of differences in receptor profile and mitochondrial content of individual cells. This hypothesis provides an explanation of the cause of ganglion cells dying at different times following the onset of POAG (Adapted from Osborne (2009)).

1.3 Basic structure and functions of the eye

The basic pattern of vertebrate eyes is much the same. Figure 1.3 is a diagram based on the anatomy of the human eye. The cornea, the transparent tissue in the anterior (front) part of the eye is a permanently fixed lens cover. The lens focuses an image on the retina, which integrates this information to be sent to the brain for vision to occur. In primates, the focusing of an image onto the retina is effected by regulating the thickness of the lens by the ciliary muscle. At rest, the lens is less convex. Metabolic requirements for complex retinal functions are catered for by the pigment epithelium, the heavily pigmented unicellular layer located behind the retina. The space between the lens and retina is filled with a viscous transparent substance called the vitreous humour or vitreous body. The vitreous body is important for the eye to maintain its shape. The outside surface of the cornea is protected by a thin film of tear. The lens and the inside surface of the cornea are maintained clear and functional by the aqueous humour that is produced by the ciliary body and fills in the anterior chamber. The iris contracts or expands and regulates the amount of light entering the eye. As the iris contracts or expands, more light is allowed to enter and the pupil increases in diameter. The pupil is not a specific tissue but a space, a part of the retina visible from outside.

The eye is an inflated, spherical shell, whose outer layer is formed by a tough, collagen-rich, corneosclera. The normal (IOP) is about 15mmHg and is maintained by a balance of aqueous humour formation by the ciliary body and the outflow through the TM into the canal of Schlemm (Figure 1.4). Pathological changes can occur to the TM, thus decreasing the aqueous outflow and, in the process, causing an elevation of the IOP. Increase of the normal IOP can affect the

optic nerve head region and subsequently RGC survival. Raised IOP is known to distort the structure of the lamina cribrosa and reduce the vascular perfusion pressure at the optic nerve head.

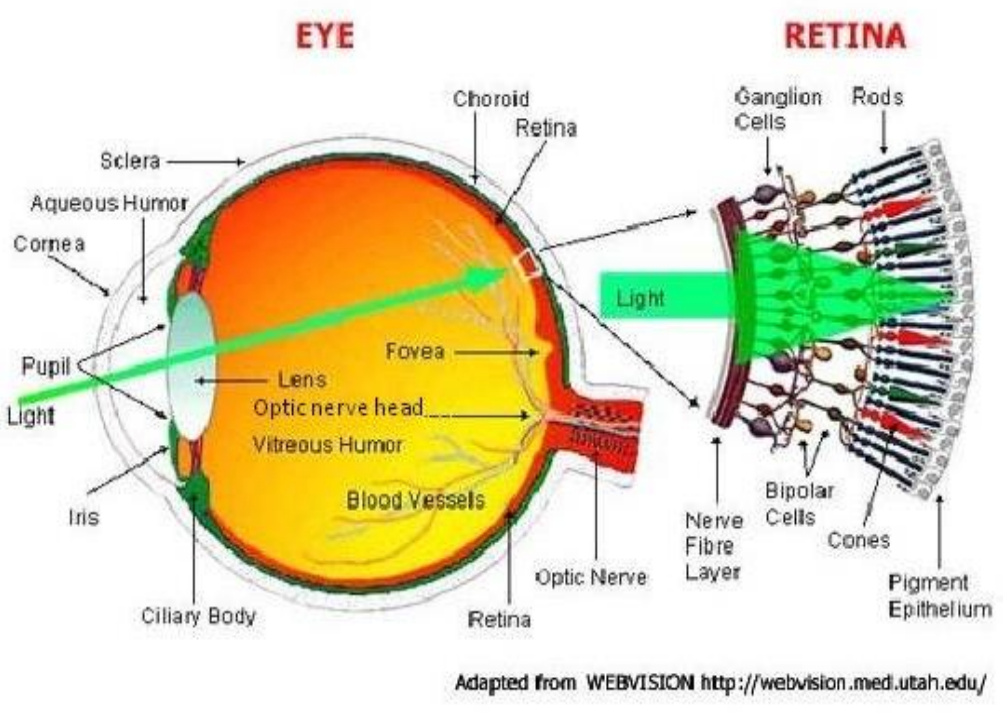


Figure 1.3 Schematic representation of the human eye. With permission from Ghazwa Al-Doori, Chemical Education Section, University of Pennsylvania, PA, USA

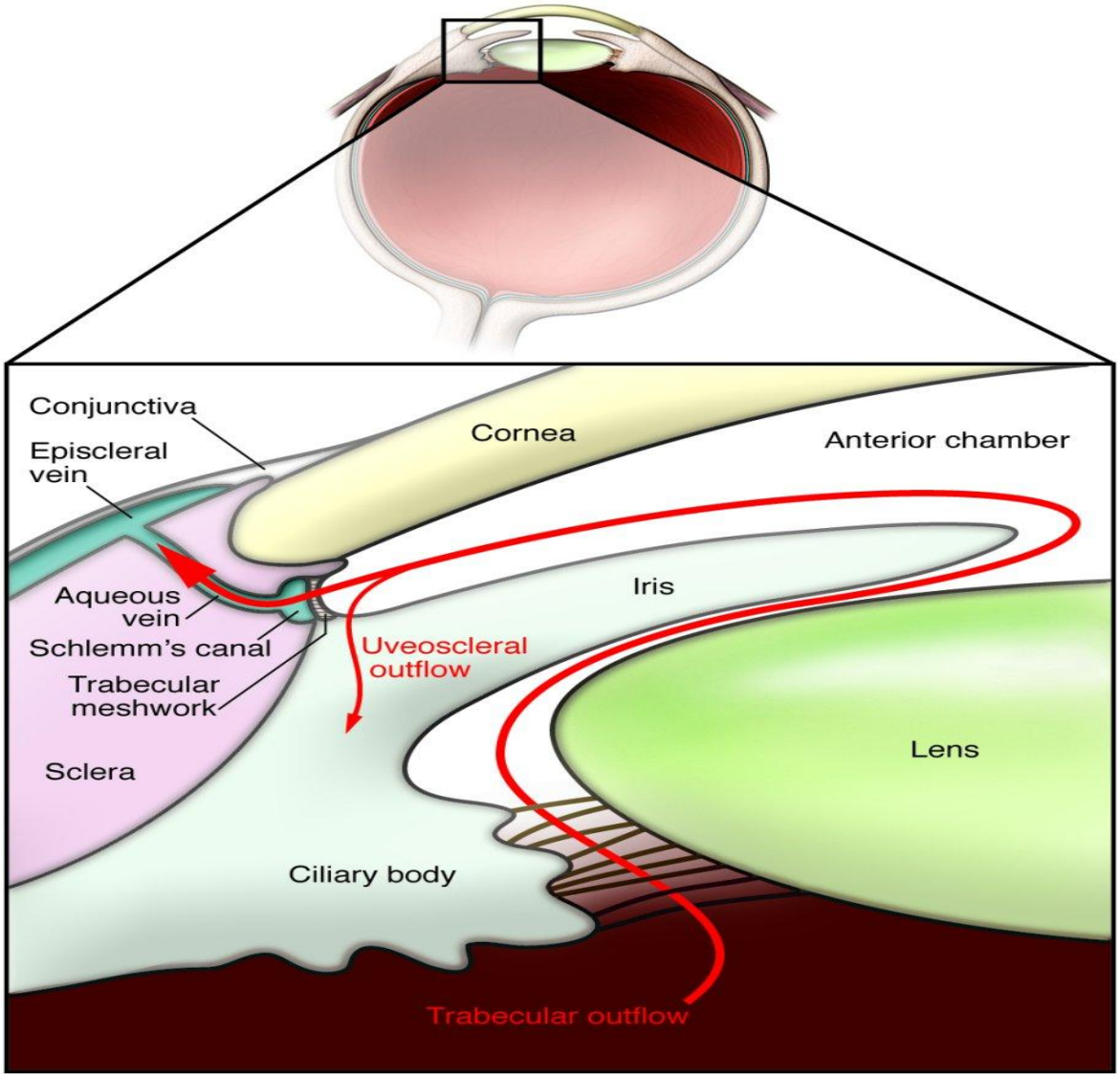


Figure 1.4 Schematic representation of aqueous outflow through the TM and Schlemm's canal in the human eye. The TM, acts as a drain that allows fluid to exit from the eye. Pathological changes to the TM, reduces the outflow of fluid and can lead to increased pressure as in glaucoma. The ciliary body and ciliary muscles are ring-shaped structures that circle the eye behind the iris. The ciliary body consists of a series of small projections (ciliary processes) that produce the aqueous fluid. The aqueous fluid flows around the pupil margin made by the iris border and fills the anterior portion of the eye. Aqueous fluid is drained from the eye at a defined rate through the TM. The ciliary muscles allow for control in near vision or for an increase in aqueous outflow.

(adapted from Berwick Eye and Surgicentre (www.berwickeye.com.au/glaucoma.htm)

1.3.1 Basic Anatomy of the retina

Light microscopy of the sectioned vertebrate retina can reveal ten organized layers (Figure 1.5). In most primates the retina is about 200-250 micrometers thick. The organized layers of the retina from the outermost surface to innermost surface are as follows:

1. A layer of photoreceptor cells contains the outer and inner segments of the rod and cone photoreceptors.
2. The outer limiting membrane is not a layer in itself but serve to anchor Müller cells and photoreceptors by a series of terminal bars (tight junctions).
3. The outer Nuclear Layer (ONL) contains cell bodies of rod and cone photoreceptors.
4. The outer Plexiform Layer (OPL) is where rod and cone axons, horizontal cell processes, bipolar processes form synapses.
5. The Inner Nuclear Layer (INL) consists of nuclei of horizontal, bipolar, amacrine and Müller cells.
6. The Inner Plexiform Layer (IPL) is where axons of bipolar cells, processes of amacrine cells and dendrites of ganglion cells form synapses.
7. The Layer of Ganglion cells (GCL) contains the nuclei and cell bodies of the ganglion cells and displaced amacrine cells as well as astroglia and microglia.
8. The Layer of optic nerve fibres consists of fibers from ganglion cells traversing the retina to leave the eyeball at the optic disk as the optic nerve.
9. The inner limiting membrane is formed by tight junctions between Müller cell end feet, and the vitreous cortex with its fibrils.

10. The above layers form the neuroretina. The outermost layer of the retina is the retinal pigment epithelium (RPE), which has a close association with photoreceptor outer segments.

The seven main classes of cell types are present in the vertebrate retina (rod and cone photoreceptors, bipolar cells, ganglion cells, amacrine cells, horizontal cells and Müller glia) and are organized into three distinct cellular layers. When stimulated by light of various intensities and wave-length, rods and cones transmit their signals to the ganglion cells through bipolar cells, whose cell bodies are found in the INL. From the ganglion cell bodies, these signals make their way along the optic nerve to the brain. Horizontal and amacrine cells in the ONL and INL respectively make lateral connections and modulate the direct signalling pathway from photoreceptors to ganglion cells. Most amacrine cells are in the INL but are also present in the GCL and are referred to as displaced amacrine cells. Müller glial cell bodies lie in the centre of the INL and their processes span all three cellular layers of the retina. These specialized glial cells are believed to be involved in the protection and/or repair of retinal neurons (Dyer and Cepko 2001).

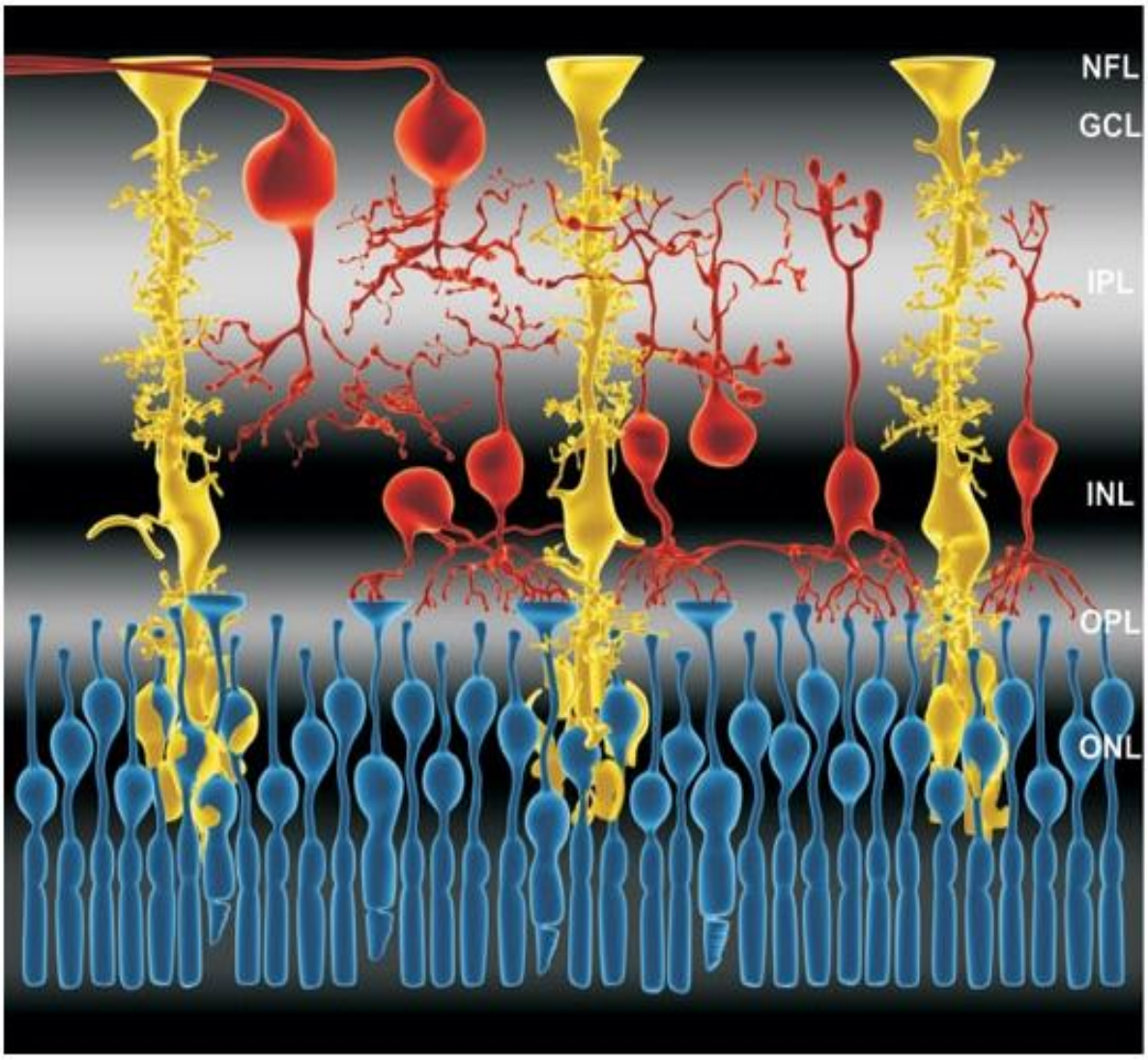


Figure 1.5: Schematic 3D drawing of a retinal cross-section. NFL, nerve fiber layer; GCL, ganglion cell layer; IPL, inner plexiform layer; INL, inner nuclear layer; OPL, outer plexiform layer; ONL, outer nuclear layer of the retina; yellow, Müller cells; red, interneurons and ganglion cells; blue, photoreceptor cells.(adapted from Lu YB et al., (2006) Viscoelastic properties of individual glial cells and neurons in the CNS. Proc Natl Acad Sci U S A. 103:17759-17764)

1.3.2 Anatomy and physiology of the retinal neurons

In addition to photoreceptors, the retina has five other basic types of retinal neurons: horizontal, bipolar, amacrine, inter-plexiform and ganglion cells. Horizontal cells lie along the outer margin of the INL, while bipolar cell perikarya are located mainly in the middle of the INL. Amacrine and inter-plexiform cell perikarya are arranged along the proximal border of the INL. The perikarya of the ganglion cells make up the most proximal layer, known as the GCL.

1.3.2.1 RGCs

Ganglion cells are the final output neurons of the vertebrate retina to the visual cortex. They collect information about the visual world initially recorded by the photoreceptors in the form of electrical signals sensed by receptors on the ganglion cell membrane. These transmembrane receptors, in turn, transform the chemical and electrical messages into intracellular signals. These are then integrated within ganglion-cell dendrites and cell body and converted into nerve spikes. Nerve spikes are a time-coded digital form of electrical signalling used to transmit nervous system information over long distances, in this case through the optic nerve and into brain visual centres. Ganglion cells are probably the most complex information-processing systems in the vertebrate retina and in humans there are approximately 1.5 million of them (Curcio and Allen 1990). Ganglion cell axons terminate in brain visual centres, principally the lateral geniculate nucleus and the superior colliculus. Ganglion cell axons are connected to specific visual centres in the brain depending on the characteristic of the visual signals they encode. It is the optic nerve that collects all the axons of the ganglion cells. Various types of ganglion cells have been discovered, varying in size, shape, orientation, connectivity and function.

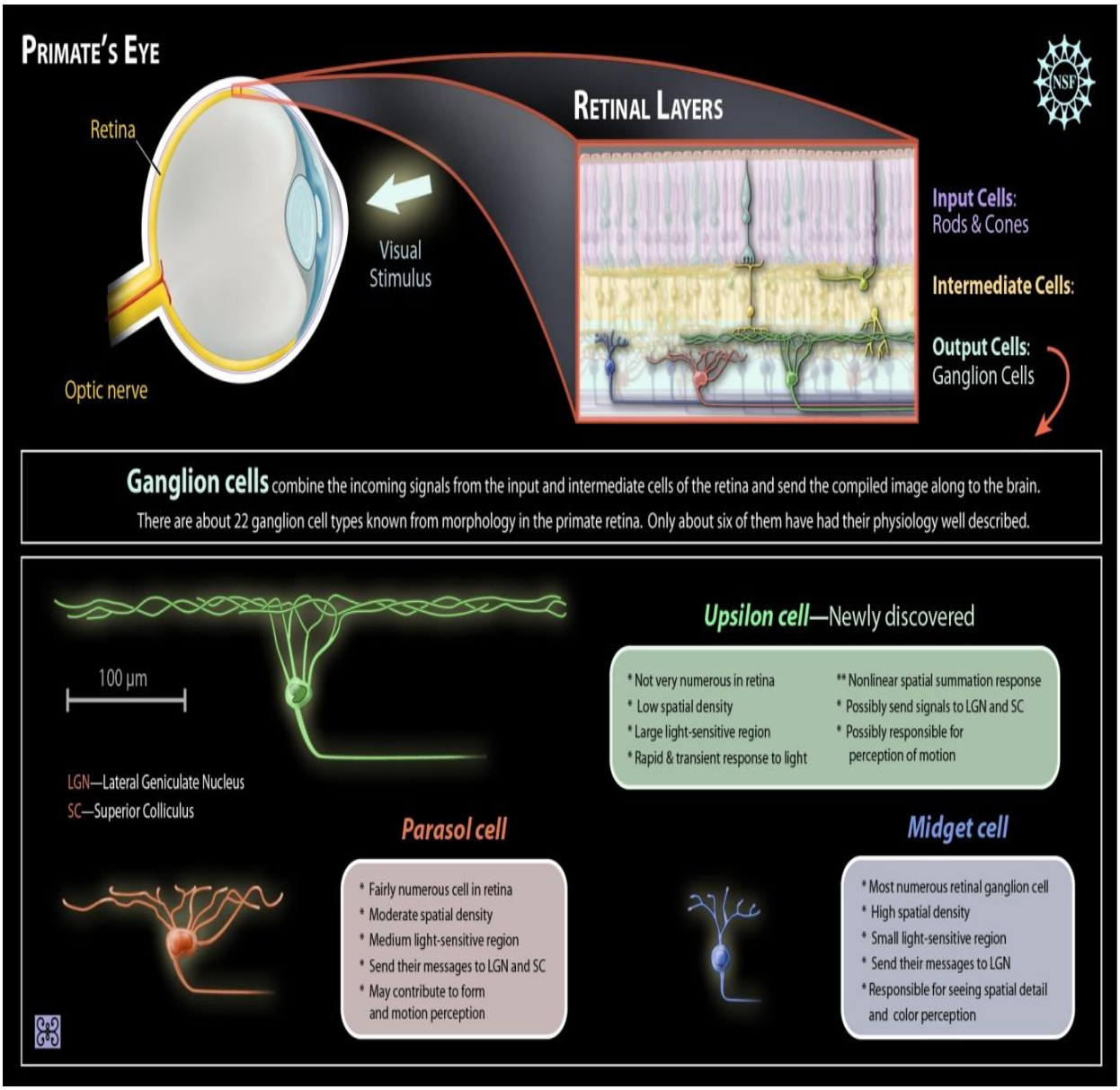


Figure 1.6 The basic types of ganglion cells in the primate retina. There are about 22 ganglion cell- types known in the primate retina but only about six of them have been well described physiologically. Each cell type has a characteristic branching pattern and level of stratification. Recently, researchers have been able to describe the electrophysiology of the upsilon cell. (Adapted from National Science Foundation www.nsf.gov/news/news_images.jsp)

The ganglion cells process features of the visual world by converting then to signals in different frequencies and pass these electronic signals along optic nerve axons to various visual brain centres. The first step in this process is the binding of amacrine and bipolar cell neurotransmitters onto specialized receptor proteins embedded in the ganglion cell dendritic membrane. These receptor molecules are concentrated at sites of synapses with amacrine and bipolar cells. Once the neurotransmitter is bound, ion selective channels in the synaptic membrane open. Charged ionic species rush through these channels, driven by electrochemical gradients. As a consequence, current flows into the cell, altering the membrane potential. The ganglion cell rate of action potential thus can be regulated and signalled to the brain visual centres via the complex communication between dendrites and voltage sensitive ion channels (Fohlmeister and Miller, 1997).

An important characteristic of the verterbrate visual system is the ON and OFF signal pathways in response to light (Schiller 1991). In the mature retina, dendrites of ON- and OFF-centre RGCs stratify in different sublaminae of the IPL, and are differentially innervated by two types of bipolar cells that depolarize and hyperpolarize on exposure to light (Wässle and Boycott 1991). Electrical recordings of single optic nerve fibre responses show diverse 'discharges of impulses', or 'action potentials' in response to light stimulation. The patterns of light response are described in Figure 1.7.

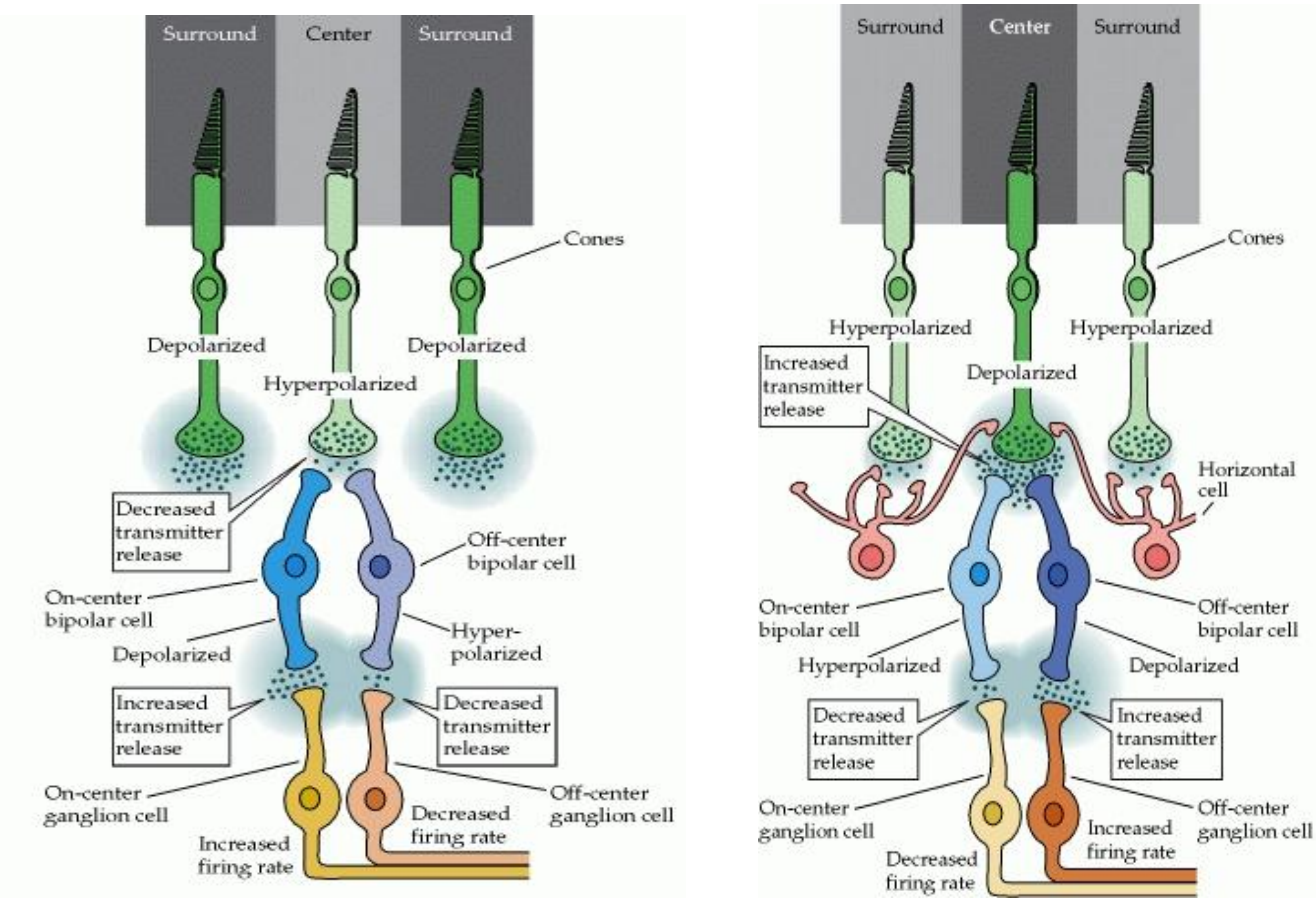


Figure 1.7: A schematic diagram showing the unique pattern of light response of ganglion cells. Circuitry responsible for generating receptive field centre responses of retinal ganglion cells. In this case, the centres of the on- and off-centre ganglion cell receptive fields are illuminated without illumination of the surrounds. Light falling on the receptor leads to a reduction in transmitter release that has opposite effects on the two populations of bipolar cells: on-centre bipolar cells depolarize, and off-centre bipolar cells hyperpolarize. This, in turn, leads to an increase in the firing rate of on-centre ganglion cells and a decrease in the firing rate of off-centre ganglion cells. 'ON' type fibres respond with a transient burst to light onset, and a sustained elevated discharge rate throughout photic stimulation. 'ON-OFF' fibres' however respond with discharge bursts at the onset and cessation of light stimuli, but otherwise quiet. 'OFF' fibres are quiet until the stimulus light is turned off, whereupon they respond with a sustained burst of impulses. Central illumination of an ON-centre ganglion cells depolarizes the cell membrane. Much of the normally inside negative resting potential is lost. The action excites nerve impulse activity. There is an increase in membrane conductance with a reversal potential near zero mV, typical of an open ion channel selectively permeable to cations. Central illumination of OFF-centre ganglion cells hyperpolarizes the cell membrane. The inside cell potential, normally negative, increases in magnitude. This action reduces nerve impulse activity and causes the cell to become quiet. Concomitantly there is an increase in membrane conductance with a reversal potential more negative than the resting potential (from Neuroscience. 2nd edition. Purves D et al., (2001) Sunderland (MA): Sinauer Associates)

Certain large-field, sparsely branching ganglion cell types are known to project to the hypothalamic suprachiasmatic nucleus (SCN) in the brain, which is the primary circadian oscillator in mammals and is essentially the biological clock allowing resetting of bodily functions to local time. The melanopsin ganglion cells, involved in entraining the circadian clock, project from the eye to the SCN via the retinohypothalamic tract (RHT). They apparently contain the neuropeptide pituitary adenylate cyclase-activating polypeptide (PCAP) as well as glutamate. They receive input in the inner plexiform layer from amacrine cells and cone bipolar cell axons (Belenky 2003). These ganglion cells contain the photoreceptive pigment melanopsin in their dendrites, proximal axons and cell membranes and thus can signal luminance levels independently of the photoreceptor bipolar chain of input (Foster 1998). The responses of these melanopsin ganglion cells are slow and sluggish depolarizations in contrast to the faster hyperpolarizing responses of rods and cones. The action spectrum of the melanopsin ganglion cells appears to be due to an opsin-like pigment but it is most sensitive at 484 nm compared with rods at 500 nm and cones at 510 (green cones) and 359 nm (uv cones) in the mice (Berson 2003). The melanopsin ganglion cells are also thought to be involved in the pupillary light reflex for they also project to the lateral geniculate nucleus through the olivary pretectal nucleus and on to the Edinger Westaphal nucleus for control of the pupillary light reflex.

The first retinal cells to differentiate fully are RGCs (Snow and Robson 1994; Belecky-Adams et al. 1996; Rapaport et al. 1996) migrating to the ganglion cell layer and extending their axons into the optic nerve even before forming dendrites (Maslim and Stone 1986). Synaptic connection is first established with amacrine cells, and later with bipolar cells. Two major factors have been proposed to regulate the dendritic development of RGCs (intrinsic genetically programmed or

extrinsic factors). External influences that affect the development of RGCs includes target-derived factors such as neurotropins (Von Bartheld 1998; Cui et al. 1998), interactions with surrounding environments and dendro-dendritic contact with adjacent RGCs. Thy-1, Brn3a and NF-L are known to be specific cellular markers for ganglion cells (Shaw and Weber 1981; Barnstable and Drager 1984; Perry et al. 1991; Xiang et al. 1996).

1.3.2.2 Horizontal cells

Horizontal cells derive their name from the extensive horizontal extensions of their cell processes. There are two subtypes of horizontal cells: type A is an axonless cell with dendrites that synapse only with cones; type B has dendrites that make contact with cones, in addition to which it has an axon that synapses with multiple rod spherules. In humans, several types of horizontal cells have been found. However, rats, mice and guinea pigs have a single type (Ahnelt et al. 1994). Horizontal cells have an integrative role in retinal processing and release inhibitory neurotransmitters, mainly gamma-amino-butyric acid (GABA).

1.3.2.3 Bipolar cells

The bipolar cells are the output neurones that carry visual information from OPL to IPL, while the interplexiform cells carry information from the IPL to the outer OPL. In human retina 11 different bipolar cell types are revealed by Golgi staining (Boycott and Wassle 1991; Kolb et al. 1992; Mariani 1983). Ten are for cones and one type is for rods. The rod bipolar is typically a stout bipolar with cell body situated middle to high in the INL and producing a tuft of dendrites

entering the OPL and reaching up to different levels between cone pedicles to reach the stacked rod spherules. Rod bipolar cells are ON cells, receiving inputs from a number of rods, and transmit signals to ganglion cells or rod amacrine cells (Wassle et al. 1991; Kolb 1994). The cone bipolar cells come in a variety of morphologically distinct types and make up approximately half of the total bipolar cell population (Euler and Wassle 1998). Hyperpolarizing and depolarizing bipolar cell terminals segregate in sub-lamina a (the outer part) and sub-lamina b (the inner part) of the (IPL) (Schiller 1996). Bipolar cells use glutamate to signal ganglion cells (Kalloniatis and Fletcher 1993) where the segregation of ON and OFF pathways is maintained (Famiglietti and Kolb 1976). Bipolar cell responses underlie several electroretinogram components, including the b-wave, which is a sensitive indicator of retinal ischaemia (Hanitzsch et al. 1996).

1.3.2.4 Amacrine cells

Amacrine cells, while predominantly located in the proximal (or vitreal aspect) of the INL, are also found in the IPL where they are known as interstitial cells. Nine morphologically distinct subtypes have been identified in the rat retina (Kolb 1979; Fletcher et al. 1996). Amacrine cells display great morphologic diversity and contain a broad range of neurotransmitters. Recently, amacrine cells have been implicated in the generation of the oscillatory potentials of the ERG (Zhou et al. 2007). Importantly, different classes of amacrine cells show varying levels of susceptibility to metabolic insult and ischaemia (Tzekov et al. 1999; Osborne et al. 2004).

1.3.2.5 Interplexiform cells

Interplexiform cells are either GABAergic or dopaminergic and send their processes to both outer and IPL carrying feedback signals between these layers.

1.3.3 Anatomy and physiology of the retinal non-neuronal cells

There are three basic types of glial cells in the human retina: Müller cells, astroglia cells and microglia cells.

1.3.3.1 Müller cells

Müller cells are the principal glial cell of the retina. They form architectural support structures stretching radially across the thickness of the retina and forming the limits of the retina at the outer and inner limiting membrane respectively. Müller cell bodies sit in the INL and project irregularly thick and thin processes in either direction to the outer limiting membrane and to the inner limiting membrane. Müller cell processes insinuate themselves between cell bodies of the neurons in the nuclear layers and envelop groups of neural processes in the plexiform layers. In fact, retinal neural processes are only allowed direct contact at their synapses, without enveloping Müller cell processes. The junctions forming the outer limiting membrane are Müller cells and photoreceptor cells consist of sturdy desmosomes or zonula adherens. In many species, gap junctions (specialized membrane associations and channels that allow small molecules and ions to pass through) or tight junctions are part of these Müller cell junctions (Miller and Dowling 1970). The surface of the Müller cells facing the pigment epithelium and subretinal space are expanded by many projections of the Müller cell membranes or apical villi. The inner limiting membrane, on the other hand, is formed by conical endfeet of the Müller cells but

without specialized junctions. Müller cells also form endfeet on to large retinal blood vessels at the inner surface of the retina. The surface of the Müller cell membrane facing the vitreous, are covered with a basement membrane. Müller cells contain intermediate filaments which are immunoreative for vimentin and to some extent to glial fibrillary acidic protein (GFAP). The latter filaments are normally in the inner half of the Müller cells and their endfeet, but following trauma to the retina such as retinal detachment, both vimentin and GFAP are massively up-regulated and found throughout the cell (Guerin et al. 1990). Müller cells have a range of functions all of which are vital to the health of the retinal neurons and function in a symbiotic relationship with the neurons. Müller cell functions include:

1. Supplying end-products of anaerobic metabolism (breakdown of glycogen) to fuel aerobic metabolism in the nerve cells.
2. Mopping-up neural waste products such as carbon dioxide and ammonia and recycle spent amino acid transmitters.
3. Protecting neurons from exposure to excess neurotransmitters such as glutamate using well developed uptake mechanisms to recycle this transmitter. They are particularly characterized by the presence of high concentrations of glutamine synthase.
4. They may be involved in both phagocytosis of neuronal debris and release of neuroactive substances such as GABA, taurine and dopamine.
5. They are thought to synthesize retinoic acid from retinol (retinoic acid is known to be important in the development of the eye and the nervous system) (Edwards 1994).
6. They control homeostasis and protect neurons from deleterious changes in their ionic environment by taking up extracellular K+ and redistributing it.
7. They contribute to the generation of the ERG b-wave (Miller and Dowling 1970;

Newman and Odette 1984), the slow P3 component of the ERG (Karwoski and Proenza 1977) and the scotopic threshold response (STR) (Frishman and Steinberg 1989) by regulation of K^+ distribution across the retinal vitreous border, across the whole retina and locally in the IPL of the retina.

1.3.3.2 Astroglia cells

Astrocytes are not glial cells of the retinal neuroepithelium but enter the developing retina from the brain along the developing optic nerve (Stone and Dreher 1987). They have characteristic morphology of a flattened cell body and a fibrous series of radiating processes. Intermediate filaments fill their processes and thus they stain well with antibodies against GFAP (Schnitzer 1988). Astrocyte cell bodies and processes are almost entirely restricted to the nerve fibre layer of the retina. Their morphology changes from the optic nerve head to the periphery, from extremely elongated near the optic nerve and symmetrical stellate in form in the far peripheral retina (Schnitzer 1988). Astrocytes are sparsely distributed in the ganglion cell and IPL. However, they are the major glial cell type in the optic nerve head and are vital for RGC survival including providing mechanical support to nearby neurons (Hernandez 2000), as their processes contact ganglion cells and endothelial cell walls, contributing to the blood-retinal barrier. Optic nerve head astrocytes, like Müller cells, aid in maintaining the extracellular levels of glutamate through the Na^+ dependent L-glutamate transporters (GLAST and GLT-1) (Matsuura et al. 2002; Rothstein et al. 1996; Watase et al. 1998) and regulate the extracellular levels of K^+ and H^+ ions (Ransom 1992). Astrocytes have also been reported to provide neurotropic support for neurons

and serve as an antioxidant defence system for the CNS (Makar et al. 1994; Raps et al. 1989; Sagara et al. 1993).

1.3.3.3 Microglia cells

Microglia cells are of mesodermal origin and thus are not neuroglial like the astrocytes and Müller cells. They enter the retina coincident with the mesenchymal precursors of retinal blood vessels in development (Chan-Ling 1994). Microglial cells are ubiquitous in the human retina being found in three separate retinal layers: GCL, (including the NFL), IPL and OPL (Chen et al. 2002; Santos et al. 2008). Microglia cells may be of two types. One form is thought to enter the retina at earlier stages of development from the optic nerve mesenchyme and lie dormant in the retinal layers for much of the life of the retina. Under pathologic conditions such as CNS injury, microglia release compounds such as glutamate, free radicals, proteases, cytokines, leukotrienes, and nitric oxide (NO) (Godbout et al. 2004; and Kremlev et al. 2004).

1.3.3.4 RPE

The RPE cell layer is also an important non-neuronal component of the retina. It constitutes a monolayer in the outer layer of the neural retina. The RPE cells are known to play a key role in maintaining the normal function of neurosensory retina. They form part of the blood-retinal barrier via their tight junctions (Boulton 2001; Boulton et al. 2004). RPE cells can phagocytose and degrade the shed tips of photoreceptor rod outer segments, then promote the photoreceptor

membrane turnover (Schraermeyer and Heimann 1999; Boulton 2001; Boulton et al. 2004). The RPE is also involved in the maintenance of retinal adhesion and the recycling of outer segment discs. Moreover the cells are involved in retinoid metabolism. 11-cis-retinaldehyde is generated in RPE cells. When photoreceptors react to the light, 11-cis-retinaldehyde undergoes photoisomerization and reduction, and it is changed into all-trans retinol. Then all-trans retinol is transferred from photoreceptors to RPE cells, and undergoes reisomerization to 11-cis-retinaldehyde. The 11-cis-retinaldehyde will travel back to the photoreceptors to participate in another "visual cycle" (Fishkin et al. 2005). RPE cells contain melanin granules that have significant physiological functions. The melanin plays an important role in protecting retina from damage caused by light. Being a powerful electron acceptor, melanin acts as a free-radical sink and diminishes cytotoxic lipid peroxidation. Melanin can store and release zinc which is necessary for activation of SOD or carbonic anhydrase. Furthermore, it has been suggested that the melanin protects the pigment cells as well as the adjacent tissues by absorbing potentially harmful substances and then releases them slowly at a non-toxic rate. RPE contains transporters for many neuroactive agents including glutamate, GABA, taurine and dopamine as well as retinoic acid (Peterson and Miller 1995).

1.4 Pathophysiology of cellular degeneration

1.4.1 Oxidative stress

The term 'oxidative stress' can be generally described as a state of elevated oxidant production in cells characterized by the release of free radicals or ROS and resulting in cellular degeneration, which occurs when there is an excess of free radicals, a decrease in antioxidant

levels, or both. However, in reality, the roles of free radicals, antioxidant levels and their dynamic interactions have proven to be very complex and are still not well understood (Figure 1.8). Oxidative stress can also be described as a shift towards the pro-oxidant state from a balance between the pro-oxidant/antioxidant state, because of increases in oxidative metabolism. Factors contributing to this phenomenon in humans are thought to occur because of excessive alcohol, medication, cold, trauma, toxins, strenuous physical activity, infections, radiation and poor diet (Halliwell 1996a; Halliwell 1996b; Singh, 2004).

Intracellular generation of ROS originate mainly from the mitochondria, where partial reduction of molecular oxygen in the electron transport chain results in the formation of the superoxide anion (Boveris and Chance, 1973) and a range of hydrogen peroxide (H_2O_2)-producing enzymes, such as monoamine oxidase and amino acid oxidase. Also, phospholipase A2 leads to the formation of superoxide via the subsequent metabolism of its product, arachidonic acid, by lipoxygenases and cyclooxygenases (Chan and Fishman 1980). Autooxidation of endogenous compounds such as catecholamines results in the formation of quinines and H_2O_2 (Graham et al. 1978). H_2O_2 can give rise to the formation of hydroxyl radicals (OH^-), the most reactive species, a process markedly accelerated in the presence of ferric ions via the Fenton reaction (Halliwell 1992). Cytotoxic mechanisms of oxidative stress include lipid peroxidation, damage to proteins, DNA strand breakage, and alterations of the deoxyribose and of purine and pyridine bases, with the latter resulting in activation of poly (ADP-ribose) polymerase, with subsequent depletion of NAD, reduction in ATP levels and an increase in intracellular Ca^{2+} concentration with concomitant activation of Ca^{2+}-dependent proteases (Halliwell 1992).

Cellular mechanisms to protect from oxidative stress include the antioxidant enzymes (SOD), catalase, and GPx as well as endogenous nonenzymatic antioxidants such as ascorbic acid, α-tocopherol, and GSH. Two forms of SOD (cytosolic copper/zinc-containing SOD and mitochondrial manganese-containing SOD) catalyze the dismutation of the superoxide radical to H_2O_2 (Fridovich 1989), which in turn can be converted to H_2O by catalase and GPx, thereby reducing the risk of OH^- formation. Catalase activity is mostly detected in peroxisomes (Ben-Yoseph 1996), whereas GPx appears in both cytosol and mitochondria (Vitorica et al. 1984). However, the brain expresses only moderate activities of SOD and catalase and GPx appear to be the most important antioxidative enzyme (Jain et al. 1991).

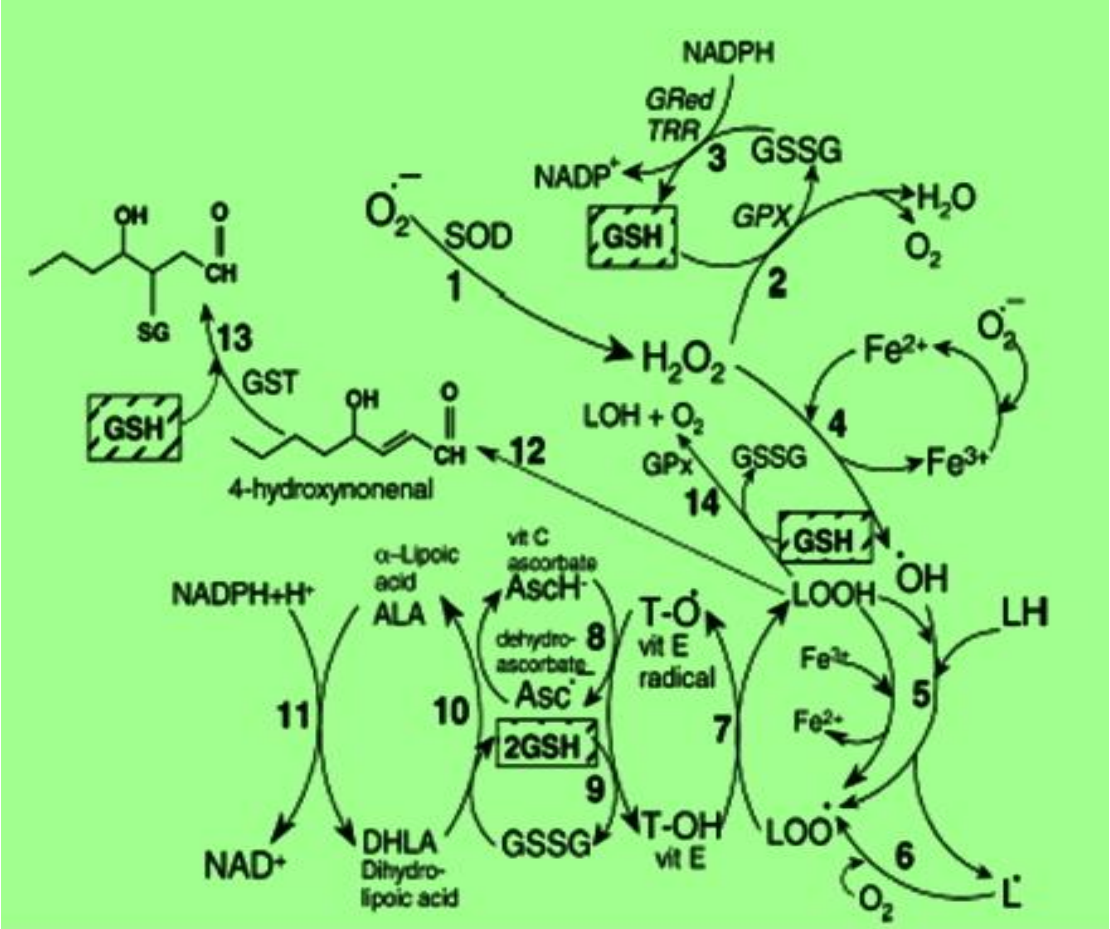


Figure 1.8: Some of the components in oxidative stress response in cells. H_2O_2, formed either directly or by dismutation of superoxide radicals by SOD (1), can be degraded by GPx (2) at the expense of conversion of GSH to GSSG. Regeneration of GSH by glutathione reductase (3) requires NADPH produced by the pentose phosphate pathway enzymes glucose 6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase (not shown). Alternatively, H_2O_2 can be converted to H_2O by catalase (2) or reduced by ferric ions to produce cytotoxic hydroxyl radicals via the Fenton reaction (4). Also shown is the glutathione-S-transferase detoxification reaction with the lipid peroxidation product 4-hydroxynonenal (13). Other important antioxidants in oxidative stress response include vitamin E (7), vitamin C (8) and alpha lipoic acid (10) (Modified from Nordberg and Arner (2001) Reactive oxygen species, antioxidants, and the mammalian thioredoxin system. Free Radic Biol Med. 31:1287-312)

Mitochondria produce the main source of cellular ROS and adenosine triphosphate (ATP), and is the key regulator in the control of cell survival and death (Lane 2006, Abu-Amero et al. 2006). It is therefore of no surprise to find that mitochondria play a pivotal role in both the ageing process and neurodegenerative diseases such as glaucoma. It has recently been reported that several genetic mitochondrial abnormalities are associated with certain POAG, implying that in these patients a decrease in mitochondrial respiratory activity exists (Abu-Amero et al. 2006). This highlights the possible role of mitochondrial dysfunction in the pathogenesis of glaucoma. Other studies have also suggested a causal relationship between mitochondrial dysfunction and many of the degenerative abnormalities associated with ageing (Hajnóczky and Hoek 2007; Kujoth et al. 2005; Dillin et al. 2002, Lin and Beal 2006). This relationship is supported by evidence that mitochondrial abnormalities can reduce the ability of the mitochondria to meet cellular ATP requirements (Acuña-Castroviejo et al. 2001), and therefore disruption of ATP synthesis can impair the oxidative balance of tissues by deregulating cellular energetics to disrupt cell metabolism, which eventually leads to cell death (Kroemer et al. 1998, Lemasters et al. 1999, Cai and Jones 1999)

It has been reported that there is a significant increase in the vitreous glutamate concentration in human and monkey glaucoma eyes (Dreyer et al. 1996). Retinal ganglion cells are very sensitive to the effects of the excitatory amino acid glutamate and N-methyl-D-aspartate (NMDA) and *in vivo* studies have also shown that glutamate toxicity can produce a dose-dependent cell loss of RGCs (Guo et al. 2006). Interestingly, it has also been reported that NMDA receptor-mediated

neurotoxicity depends on the generation of ROS such as NO and superoxide anion, which react to form peroxynitrite (Bonfoco et al. 1995). Peroxynitrite is a powerful oxidant which can cause cell damage or death and is increased by increasing iNOS activity. It has been shown that increased iNOS expression in the TM of patients with POAG, are proportional to the visual field defect and could lead to the increased of nitrotyrosine levels (Durango 2008). Nitrotyrosine levels thus may serve as a marker of oxidative stress in the progression of TM cell death in POAG (Durango 2008). Therefore, oxidative stress leading to free radicals formation could contribute to the final common pathway for neurotoxicity in different types of acute and chronic neurodegenerative diseases (Bonfoco et al. 1995)

There are also other cytologic consequences of a mismatch of the production of free radicals and cell ability to defend against them. When there is an increase in the production of free radicals, there will be a decrease in scavenging of free radicals and/or decreases in repair of oxidatively modified macromolecules. Due to the imbalance of ROS production, there is a build-up of oxidatively modified molecules, which cause cellular dysfunction and also cell death for postmitotic cells such as neurons (Simonian 1996).

1.4.1.1. Role of oxidative stress in glaucoma

The eye is constantly exposed to the external environmental conditions, such as light, and exposure to light of certain wavelengths can induce abnormal cellular metabolic activity and increase the generation of free radicals in the retina. Also, as oxidative phosphorylation increases in the retina in response to cellular or tissue demand, there will be a concomitant increase in free

radicals. Ischaemia, hypoxia, excitotoxicity and an unregulated immune response can also induce production of free radicals. All of the above processes have been implicated in glaucoma.

Most recently, light has been suggested as being a risk factor for oxidative injury in glaucoma (Osborne et al. 2006), where light entering the eye interacts with mitochondria, thereby leading to increased ROS generation in RGCs and their axons. Moreover, when these neurons are in an energetically low state, their antioxidant capacity is exceeded and their survival is compromised (Osborne et al. 2006). A prominent increase in ROS accumulation has been detected in RGC cultures following their exposure to light (Osborne et al. 2006; Wood et al. 2008; Lascaratos et al 2008).

Apart from light-induced injury exacerbating glaucoma, another important factor which leads to over-production of ROS and hence oxidative stress is tissue hypoxia. Hypoxia causes neuronal cells to die as a result of mitochondrial failure and energy depletion. This can induce changes or a disturbance in ionic regulation, activation of HIF-1α and NfkB which can lead to ROS generation (Banasiak et al. 2000; Halterman et al. 1999). Tezel and Wax assessed the effect of hypoxic tissue stress in the glaucomatous optic nerve head and retina. They were able to determine the localization of HIF-1α expression in human donor eyes using immunohistochemistry (Tezel and Wax 2004) and showed an increased immune-labelling of the glaucomatous optic nerve head and retina for HIF-1α compared with age-matched groups. In this particular study, it was concluded that hypoxic tissue stress present in glaucomatous eyes is a likely component of the pathogenic mechanisms of glaucoma. Since hypoxia is a component of ischaemia, the increase in HIF-1α expression in the human glaucomatous retina does not eliminate ischaemia from playing a part in the pathogenesis of this disease.

Oxidative neuronal injury in glaucoma may also be associated with excitotoxic amino acids, including glutamate, which are known to induce ROS generation. This ‘glutamate neurotoxicity' is characterized by increased damage to cell components, especially mitochondria, by ROS (Atlante et al. 2001). In addition, retinal glutamate damage has been shown to be mediated in part by nitric oxide, a highly reactive oxidant (Nucci et al. 2005). Klocker et al. observed that free-radical scavenging, in combination with a nitric oxide synthase inhibitor, potentiated the neurotrophic effect of brain-derived neurotrophic factor (BDNF) on axotomised RGCs (Klocker et al. 1998).

ROS may also be indirectly involved in activating an immune response during glaucomatous neurodegeneration. Glial cells can respond to ROS with a variety of immunologically relevant functions, including activation of the autoimmune response. Glial cells in human glaucomatous eyes exhibit activation of their antigen-presenting ability (Tezel and Wax 2003; Yang et al. 2001) and, moreover, activated glial cells may induce neuronal damage by releasing increased amounts of neurotoxic substances, including TNF-α and nitric oxide (Tezel 2006). Thus, in addition to many other detrimental effects, ROS can also take part in the regulation of immune responses, which is likely to contribute to the glaucomatous neurodegenerative process (Tezel and Wax 2000a, b; Wax et al. 2008).

Results of clinical studies in glaucoma patients also support the contribution of oxidative stress to the pathogenesis of this disease. For example, SOD and GSH activity are elevated in the aqueous humour of glaucoma patients (Ferreira et al. 2004). Also, antibodies against the GSH related antioxidant enzyme glutathione-S-transferase (GST), have been isolated from the sera of glaucoma patients (Yang et al. 2001). Furthermore the activity of GST, among other detoxification enzymes, is determined by the level of ROS *in vivo*. ROS activity directly

influences the biological induction of GST, especially in response to the generation of toxic by-products as a result of oxidative damage (Hayes and Strange 1995). Changes in the aqueous humour activities of detoxification enzymes such as GPx and GST could also serve as an indicator of changes in immunuoreactivities in glaucomatous eyes due to persistent exposure of these enzymes to the effects of oxidative stress (Tezel 2006).

In summary, the role of oxidative stress and ROS in the pathogenic mechanisms leading to the neurodegeneration of RGCs in glaucoma has been the highlight of research over recent years. Light, hypoxia, ischaemia and exitotoxicity have all been implicated in the diseases process. Many *in vivo* and *in vitro* studies have clearly found that ROS generation leads to RGC degeneration, glial dysfunction and activation of the immune response in glaucoma. However, the exact mechanisms of injury caused by oxidative stress and its influence on the neurodegenerative process are still not entirely clear and remain an important topic for future research.

1.4.2 Apoptosis

Programmed cellular degeneration or death is a common characteristic of higher organisms that are genetically determined, allowing for the continuous renewal and repair of tissues and organs. Somatic cells are also continuously dying in order to guarantee the survival of the individual. It is believed that cells activate an intrinsic death programme and thus actively contribute to their own death. This is also termed apoptosis (Kerr et al. 1972; Kroemer 1995), which originally described a particular morphological appearance of cell death, but currently is used as synonym for programmed cell death. This naturally occurring physiological process, by which a cell is directed to undergo suicide, is based on a complex genetic programme that is part and parcel of

the development and function of an organism. In this process, cells that are no longer needed or that will be detrimental to an organism or tissue are disposed of in a highly regulated, selective and orderly manner which prevents the trigger of an inflammatory response, which is often associated with necrotic cell death.

There are at least two broad pathways that lead to apoptosis, the Extrinsic and Intrinsic Pathway. In both pathways, signalling results in the activation of a family of Cys (Cysteine) Proteases (named caspases) that act in a proteolytic cascade to dismantle and remove the dying cell. The extrinsic pathway begins outside a cell, when conditions in the extracellular environment determine that a cell must die. The intrinsic apoptosis pathway begins when an injury occurs within the cell. The injury could result in necrosis and produce an inflammatory response, but the apoptotic machinery is in place to ensure that the damaged cell is packaged and removed cleanly, in order to prevent an inflammatory reaction. Apoptosis in mitochondria is the best known intrinsic apoptosis pathway (Kerr et al. 1972; Kroemer 1995). Apoptosis is, however, characteristically different to another type of cell death, necrosis. Whereas necrosis is caused by physical, chemical, or osmotic damage with consecutive disruption of internal and external membranes, apoptosis involves the active participation of endogenous cellular enzymes in the mediation of death well before membranes lose their integrity. Apoptosis thus is characterized by shrinkage of cell, loss of asymmetry of phosphatidylserine in membrane and nuclear fragmentation (Marin et al. 1995, Koopman et al. 1994). Necrosis entails the liberation of denatured proteins and DNA fragments into the intercellular space, as well as local inflammatory responses. In contrast, cells undergoing apoptosis exhibit near-to-intact external membranes until late phases of the metabolic suicide, allowing adjacent cells to engulf the dying cell and to

eliminate it before its content is released and before local inflammatory reactions are triggered. Acquired dysregulations of apoptosis can cause major pathologies. Failure to undergo apoptosis during organogenesis causes malformations in the nematode *Caenorhabditis elegans* (Ellis et al. 1991), as well as in insects and mammals (Vaux et al. 1994). Similarly, overexpression of the anti-apoptotic proto-oncogene Bcl-2 leads to lymphoid hyperplasia, lymphomas, and self-aggression by self-reactive lymphocytes that are normally deleted via apoptosis. An abnormal susceptibility to apoptosis also has dramatic consequences. Nevertheless, the biochemical mechanisms involved in the execution of cell death are still not fully understood.

Cells respond to aberrant combinations of physiological signals and subnecrotic chemical or physical damage, generally via apoptosis, in order to minimize the risk of perpetuating or expanding mutated, aberrantly differentiated, or transformed cells. Depending on the context, a given second messenger system can either prevent or induce apoptosis, and many different signal transduction pathways may participate in the induction phase of apoptosis (Kroemer 1995). Evidence is accumulating to suggest that the induction phase is followed by an effector phase during which the numerous apoptotic-inducing stimuli converge into a few stereotypical pathways and cells that pass a point of no return and become irreversibly committed to death. During the subsequent degradation phase, numerous vital structures and functions are destroyed, giving rise to the full-blown phenotype of apoptosis, which includes loss of essential cell functions, ultrastructural alterations, endonucleolysis, and cytolysis**.**

Recent studies, however, question the idea that alterations of the nucleus are obligatory events in apoptosis. Sun et al. (1994) were able to provide evidence that dissociates apoptotic morphology

and enzymatic DNA fragmentation. The morphology of nuclear apoptosis is still observed when DNA fragmentation is abolished by inhibitors of DNA-degrading enzymes (Sun et al. 1994). Suppression of DNA degradation delays cytolysis, but it does not prevent ultimate cell death in most systems (Cohen et al. 1992). At least two major differences in the induction mechanisms of apoptosis have to be distinguished. On the one hand, apoptosis can be induced by receptor-mediated physiological stimuli such as the presence of death signals, the absence of rescue signals or combinations of contradictory signals. On the other hand, apoptosis can result from genotoxic insults directly targeted at nuclear chromatin. Obviously, in the latter case, nuclear alterations are the primary events. This is not true for physiological apoptosis, in which alterations affecting mitochondrial and membrane function precede nuclear apoptosis, yet mark a stage of ongoing apoptosis during which cells are irreversibly committed to death. A more detailed summary of the apoptotic pathways is discussed in section 1.4.2.2.

1.4.2.1 RGC apoptosis due to the action of ROS

RGC axons within the globe of the eye are unmyelinated, and are richly provided with mitochondria which produce the necessary energy to conduct action potentials, out of the eye along the myelinated optic nerve to the brain (Wang et al. 200; Koriyama 2009). These mitochondria are likely to be susceptible to ROS exposure. There is also sufficient evidence to show that specific ROS species can lead to distinct cell death mechanism either by apoptosis or necrosis. Thus, distinctive ROS species that form under different conditions might act as initiators or executioners of different types of neuronal death (Kreiger and Duchen 2002).

Although ROS have a major role in the mediation of cell damage, several defence mechanisms reduce their actions or their production. Such defence mechanisms, as previously discussed, include enzymes such as SOD, catalase, and GPx, GST as well as natural lipophylic and hydrophylic antioxidants. Moreover, others include membrane-located or lipoprotein-associated water soluble antioxidants such as vitamin E, the quinones (coenzyme *Q),* and carotenoids. Vitamin C and thiols such as GSH also nullify the negative effects of generated ROS. The notion that ROS participate in apoptosis regulation or execution is based on the following evidence:

1. Excessive ROS or inhibition of antioxidant pathways induces apoptosis. Exogenous sources of ROS such as H_2O_2 and t-butyl hydroperoxide induce apoptosis, provided they are used at subnecrotic doses (Hockenbery et al. 1993; Buttke and Sandstrom 1994). The GSH synthesis inhibitor DL-buthionine-(S,R)-sulfoximine (BSO) increases the susceptibility of cells to apoptosis in response to other agents (Fernandes and Cotter 1994). A similar proapoptotic effect has been described for dimethylmaleate which reduces cellular GSH stores by forming a thioether conjugate in a reaction catalyzed by GST. In spinal cord organotypic cultures, chronic inhibition of SOD transcription or activity causes apoptotic death (Troy and Shelanski 1994). A recent study also showed that unregulated oxidative stress can be controlled by various cellular defense mechanisms such as GSH which can critically counteract the damaging effects of oxidative stress in mammalian cells (Roh et al. 2007). Roh et al. (2007) determined the effects of unregulated oxidative stress induced by GSH depletion on cells in mouse retina. In their study, mice were intraperitoneally injected with BSO at 1.5 g/kg. After 0, 1, 4, and 7 days of BSO administration, retinas were excised and sections were subjected to GSH assay and terminal uridine deoxynucleotidyl nick end labelling (TUNEL) analysis. After four days of BSO

administration, the number of TUNEL positive cells was significantly increased. Their studies suggested that GSH depletion may cause unregulated oxidative stress to the cells in the retina and indeed increased cell death in the retina (Roh et al. 2007)

2. Mutations in Cu^{++}/Zn^{++} SOD gene have also been associated with neurodegenerative disease such as familial ALS, a disease involving apoptotic death of motor neurons (Deng et al 1993, Rosen et al 1993). It has been speculated that high-energy electrons, passing along the mitochondrial electron transport chain, which cause the formation of superoxide anion radicals, which are involved in age related neurodegenerative disorders that are triggered by toxins, hereditary mtDNA mutations, and acquired mtDNA alterations (Coyle and Puttfarcken 1993; Atchison and Hare 1994). When stimulated to undergo apoptosis, cells hyperproduce superoxide anion, as determined with fluorochrome dihydroethidine (Zamzanii et al. 1995). In addition, cell membranes undergo peroxidation, as can be shown by use of the fluorescent dye cis-parinaric acid. Both processes are inhibited by over-expression of Bcl-2 (Hockenbery et al. 1993; Zamzanii et al. 1995). Furthermore TNFα- induced cell death is linked to an increased generation of superoxide anion in mitochondria (Hennet et al. 1993; Schulze-Osthoff et al. 1994), and interference of superoxide generation by respiratory chain inhibitors (Schulze-Osthoff et al. 1993) delays TNFα-induced cell death.

3. Antioxidants and free radical-scavengers can also inhibit the induction of apoptosis. N-acetylcystein (NAC), a thiol antioxidant, is deacylated and converted to GSH to function as both as a redox buffer and a reactive oxygen intermediate scavenger and thus being able to diminish the stimulation of apoptosis. Dimethyl sulfoxide (a membrane-permeable hydroxyl radical

scavenger), thioredoxin (an intracellular thiol reductant), o-phenanthroline (blocks hydroxyl radical formation), N-(2-mercaptoethyl)-1,3-propandiamine (an inhibitor of membrane peroxidation) vitamins C/E and catalase all attenuate the process of apoptosis in some cases (Buttke & Sandstmm 1994). Also, transfection of IL-3-dependent cells in culture with GPx inhibits apoptotic cell death after growth factor deprivation (Hockenbery et al. 1993).

An increase in intracellular ROS can also initiate cell death pathways that involve the activation of both poly (ADP-ribose) polymerase 1 (PARP-1) and apoptosis-inducing factor (AIF). PARP-1 is a ubiquitous human enzyme that catalyses the synthesis of the branched polymer poly (ADP-ribose) by use of NAD^+. Such posttranslational modifications typically occur on glutamate residues of target proteins that include histones, transcription factors and DNA repair proteins, as well as PARP-1. Since poly ADP-ribosylation significantly alters the DNA-binding properties of target proteins, PARP-1 plays a pivotal role in both DNA repair and the regulation of gene transcription. PARP-1 activity is elaborately regulated in the cell by many factors, such as DNA damage, caspases, and the intracellular NAD^+ concentration (Kaufmann et al. 1993; Lautier et al. 1993; De Murcia et al. 1994; Lindahl et al. 1995). For example, the inactivation of PARP-1 by caspase-3 cleavage is one of the key events of apoptosis, while the over-activation of PARP-1 leads to ATP-depletion and subsequent necrosis (Berger 1985). Excessive production of ROS will lead to the production of peroxynitrite which initiates DNA damage and allows for PARP-1 activation. PARP-1 is dependent on NAD^+ for its activation and in poly ADP-ribosylation-dependent processes. These lead to the relocation of mature AIF to the nucleus from mitochondria, where AIF promotes DNA fragmentation and the nuclear condensation results in apoptosis (Yu et al. 2002). AIF is an ancient phylogenetically conserved flavo-protein that has

both NADH oxidizing and apoptosis-inducing activities. AIF is synthesized as a ~67 kDa precursor, imported into mitochondria via its N-terminal prodomain containing two mitochondrial localization sequences and then processed to form a ~62 kDa mature protein (Susin et al. 1999). Under physiological conditions, AIF remains confined to the internal mitochondrial membrane with its N-terminal region oriented towards the matrix and the C-terminal towards the intermembrane space (Susin et al. 1999). AIF has also been proposed to act as a putative ROS scavenger (Klein et al. 2002), but this remains to be established. Upon pathological permeabilization of the outer mitochondrial membrane, mature AIF is further processed to a ~57 kDa form by activated calpains and/or cathepsins (Polster et al. 2005; Yuste et al. 2005). This isoform is then translocated to the nucleus via its C-terminal domain nuclear localization sequence, a hallmark of caspase-independent apoptosis, which then leads to large-scale DNA fragmentation (Susin et al. 1999). AIF is indeed considered as the main molecular effector of apoptosis-like programmed cell death (Joza et al. 2001; Susin et al. 1999; Yu et al. 2002).

Taken together, this may suggest that the cellular redox state and/or the equilibrium between ROS generation and ROS detoxification influence the early stage of apoptosis. ROS might play a role both in the induction and in the effector stages of apoptosis. Thus, the regulation of the cellular redox state is certainly of major importance in determining signal transduction processes (Staal et al. 1994). ROS-mediated membrane damage can disrupt intracellular calcium compartmentalisation and thus cytosolic concentrations of different cations are altered during the apoptotic process. In particular, calcium has been implicated in all phases of apoptosis: as a second messenger during the induction phase, as a Bcl-2-regulated cofactor during the effector

phase (Baffy et al. 1993), and as a pleiotropic activator of proteases and nucleases during the degradation phase (Trump and Berezesky 1995; McConkey et al. 1994). However, given that sustained increases in cytosolic free Ca2+ are not sufficient to elicit apoptosis in cell lines that are susceptible to valinomycin- induced apoptosis, it appears that the role of Ca2+ in apoptosis is more complex than initially suspected. In addition, ROS are likely to be generated during the effector stage of apoptosis in the mitochondrion. Nonetheless, it appears that ROS generation is not obligatory for apoptosis and apoptosis induced by certain stimuli (e.g. cross-linking of CD95/Fas/Apol) is not inhibited by antioxidant drugs (Schulze-Osthoff et al. 1994). Moreover, cells cultured in the absence of oxygen are incapable of generating ROS, yet can be induced to undergo apoptosis via pathways that are inhibited by Bcl-2 and Bcl-xL (Shimizu et al. 1995; Jacobson et al. 1995). In other words, ROS are facultative but not obligatory regulators of apoptosis.

1.4.2.2 Signalling pathways in apoptosis

As mentioned earlier, the biochemical activation of apoptosis occurs through two major pathways, the intrinsic and the extrinsic pathways. The intrinsic pathway is usually initiated by mitochondrial release of cytochrome c, culminating in the activation of caspase 9. In the extrinsic pathway, the triggering of cell-surface receptors such as Fas results in the activation of caspase 8 or caspase 10 (Li et al. 1997). The most common type of cell death in neurodegenerative disease appears to be via the intrinsic mitochondrial apoptotic pathway (Bredesen 2007; Bredesen 2008). Both pathways can converge to cause a cleavage of caspase-3. Cleaved caspase-3 involvement in apoptotic cell death is of major importance. Such apoptotic cell death pathways are therefore caspase-dependent as they involve various members of the caspase family. Apoptosis can also

occur by mechanisms that do not involve the activation of caspases and are termed caspase-independent (Li et al. 1997). The intrinsic mitochondrial pathway of apoptosis functions in response to various types of intracellular stress signals that include growth-factor withdrawal, DNA damage, unfolding stresses in the endoplasmic reticulum. Growth factors can also influence intrinsic mitochondrial apoptosis via PI3K and v-Akt Murine Thymoma Viral Oncogene Homolog (Akt) pathways. Growth factors activate their receptors to stimulate the PI3K pathways that lead to Akt activation. Akt is very important in Bcl2-Antagonist of Cell Death (BAD) regulation, which is a pro-apoptotic member of B-Cell Leukemia-2(Bcl-2) family and is involved in mitochondrial apoptosis (Alladina et al. 2005; Harris and Levine 2005). Protein Kinase-C (PKC) may also play an important role in apoptosis by activating p90RSKs (Ribosomal-S6 Kinases), which inhibits BAD (Kim et al. 2005; Beisner et al. 2005; Alladina et al. 2005). In addition Death Receptor (DR) stimulation i.e. the DR Fas (CD95/Apo1), which is a member of the Tumor Necrosis Factor Receptor (TNFR) family, also plays an important role in cross-talk with the intrinsic mitochondrial apoptotic pathway (Scaffidi et al. 1998). p53 is another sensor of cellular stress and is a critical activator of the intrinsic pathway. p53 results in apoptosis through a three-step process: (1) the transcriptional induction of redox-related genes; (2) the formation of ROS; and (3) the oxidative degradation of mitochondrial components, leading to apoptosis (Polyak et al. 1997). The DNA checkpoints proteins, Ataxia Telangiectasia Mutated protein (ATM), and Checkpoints Factor-2 (Chk2) directly phosphorylate and stabilize p53 and inhibit Mouse Double Minute-2 Homolog (MDM2)-mediated ubiquitination of p53. MDM2 binds p53 and mediates the nuclear export. When bound to MDM2, p53 can no longer function as an activator of transcription. p53 initiates apoptosis by transcriptionally activating pro-apoptotic Bcl2 family members and repressing anti-apoptotic Bcl2 proteins and Cellular

Inhibitor of Apoptosis (CIAPs). Other p53 targets include Bcl2 Associated X-protein (BAX), Noxa, p53-Upregulated Modulator of Apoptosis (PUMA) and BID (Wang et al. 1996; Alladina et al. 2005; Harris and Levine 2005).

The extrinsic pathway involves FasL (Fas Ligand), a homotrimeric protein that acts as a ligand for Fas and causes oligomerization of its receptor on binding. Associated with this is the clustering of the Death Domains and binding of cofactor Fas-Associated via Death Domain (FADD). FADD protein binds via its Death Effector Domain (DED) motif to a homologous motif in procaspase-8. The complex of Fas, FADD and procaspase-8 is called the Death Inducing Signaling Complex (DISC) (Kischkel 1995). Upon recruitment by FADD, procaspase-8 oligomerization drives its activation through self-cleavage. Active caspase-8 then activates downstream caspases (3 and 7). Activated caspase-8 activates caspase-3 through two pathways. In the first pathway, caspase-8 cleaves Bcl2 Interacting Protein (BID) and its COOH-terminal part translocates to mitochondria, where it triggers cytochrome c. Released cytochrome c binds to Apoptotic Protease Activating Factor-1 (APAF1) together with dATP and procaspase-9, and activates caspase-9. Caspase-9 cleaves procaspase-3 and activates caspase-3. In another pathway, caspase-8 cleaves procaspase 3 directly and activates it. Caspase-3 then commits the cell to apoptosis (Budihardjo et al. 1999).

Stress signals can activate pro-apoptotic Bcl2 family proteins, which subsequently interact with inactive anti-apoptotic Bcl2 proteins. The integrity of the mitochondrial outer membrane is regulated by the Bcl-2 family (Cory et al. 2003). Bcl-2 family proteins have been defined both by

their function (either pro- or anti-apoptotic) and overall conservation of sequence motifs known as Bcl-2 homology (BH) domains (Aouacheria et al. 2007). Anti-apoptotic multi-domain members generally share sequence similarity in four conserved BH domains (BH1-4), while pro-apoptotic multidomain members contain only two or three BH domains (BH1-3) (Aouacheria et al. 2007). Anti-apoptotic and pro-apoptotic protein interactions lead to the destabilization of the mitochondrial membranes and release of apoptotic factors that include Cytochrome C, Second Mitochondria-Derived Activator of Caspase (SMAC), Arts and High Temperature Requirement Protein-A2 (Omi/HTRA2). Bcl2L (Bcl2-Like), Bcl-XL and other anti-apoptotic Bcl2 family members reside in the outer mitochondrial membrane and prevent Cytochrome C release (Chao and Korsmeyer 1998). BAX, BID and BIM are initially inactive and must translocate into mitochondria to induce apoptosis, either by forming pores in mitochondria directly or by binding via BH3 domains to Bcl2, Bcl-XL and BFL1, and antagonizing these anti-apoptotic proteins (Alladina et al. 2005).

Other proteins released from damaged mitochondria, SMAC/Diablo, Arts and Omi/HTRA2 (High Temperature Requirement Protein-A2), counteract the effect of Inhibitor of Apoptosis Proteins (IAPs), which normally bind and prevent activation of caspase (Kroemer et al. 2007). The interaction between Bcl family members, IAPs, SMAC and Omi/HTRA2 is central to the intrinsic apoptosis pathway. Endonuclease-G (EndoG), is specifically activated by apoptotic stimuli and is able to induce nucleosomal fragmentation of DNA independently of caspase and DNA-Fragmentation Factor/Caspase-Activated DNAse (CAD). EndoG is a mitochondrion-specific nuclease that translocates to the nucleus and cleaves chromatin DNA during apoptosis (Li et al. 2001). As mentioned earlier AIF has also been attributed a role in apoptosis, becoming

active upon translocation from mitochondria to nucleus, where it initiates chromatin condensation and large-scale DNA fragmentation (Rosenberg 2004). Apart from that endoplasmic reticulum stress also takes part in apoptosis by activating caspase-3 via caspase-12 (Shiraishi et al. 2006).

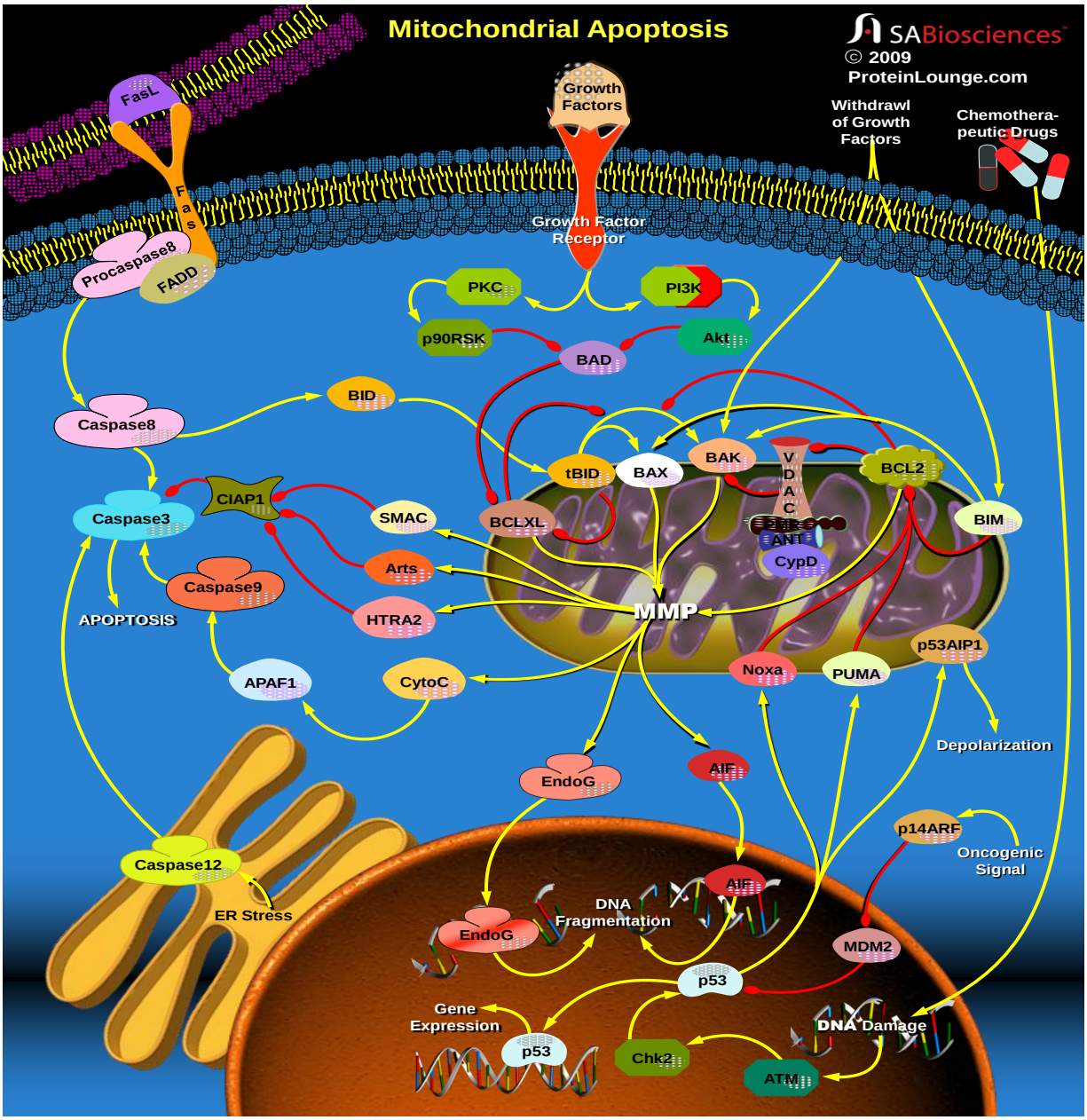


Figure 1.9 Putative extrinsic and mitochondrial pathways of cellular apoptosis from SABiosciences.

1.4.2.3 Neuronal survival pathways

As already mentioned, programmed cell death or apoptosis is a conserved pathway that is appears operative in all metazoans. Apoptosis operates in adult organisms to maintain normal cellular homeostasis. This is especially critical in long-living mammals that must integrate multiple physiological as well as pathological death signals, which for example includes regulating the response to infectious agents and different noxious stimuli. Evidence indicates that stimulation of the apoptotic pathway can lead to cell death such as occurs in acute and chronic degenerative diseases, immunodeficiency, and infertility, whereas insufficient apoptosis can manifest as cancer or autoimmunity (Groenendyk and Michalak 2005; Rosenberg 2004)

As there are many different pathways leading to apoptosis, there are also many involved in neuronal survival. For example, trophic factors that can act as ligands to Trk receptors (TrkA, TrkB and TrkC), which are cell-surface tyrosine kinase receptors. Autophosphorylation of Trk cytoplasmic tails provide docking sites for downstream target molecules. These target molecules include phospholipase C, PI3-K and adaptor proteins which coordinate neuronal survival (Yuan and Yankner 2000). The Trk receptors can be activated by more than one ligand, but with different affinities and potentially different signalling outputs. Thus two ligands may differentially activate the same signalling cascade through the same or different receptors (Yuan and Yankner 2000).

1.4.2.3.1 PI3-K/Akt pathway

Activation of PI3-K mediates numerous cellular functions including proliferation, chemotaxis, metabolic homeostasis and inhibition of apoptosis. PI3-K signalling pathway can be activated by ligands binding to Trk receptors. Activated PI3-K induces the stimulation of Akt through 3'-phosphorylated phosphatidylinositol as well as phosphoinositide-dependent kinase, which in turn phosphorylates and activates Akt (Philpott et al. 1997). Akt promotes cell survival in several different ways: (1) It inhibits the phosphorylation of pro-apoptotic protein BAD and promotes its interaction with the chaperone protein, which results in the inhibition of BAD's pro-apoptotic activity (Datta et al. 1997); (2) it induces phosphorylation of cAMP-response element-binding protein (CREB), which stimulates the transcription of pro-survival factors (Du and Montminy 1998); (3) it also induces phosphorlylation of IkB kinase which in turn phosphorylates the IkB subunits of NfkB•IkB complexes, resulting in activation of NfkB (Kane et al. 1999) (4) it negatively regulates death-promoting signals by phosphorylation of Forkhead family members (Brunet et al. 1999) and (5) it can directly inhibit activation of the apoptosis initiator, caspase-9. PI3-K activation is also the part of endogenous response to ischaemia-reperfusion injury to the retina (Hayashi et al. 1996).

The binding of Trk by neurotophins also activates PI3-K-independent survival pathways (e.g. the mitogen-activated protein (MAP) kinase pathway). This was demonstrated by the observation that PI3-K inhibitors were not able to block neuronal survival, despite the absence of nerve growth factor (NGF) (Philpott et al. 1997). Therefore, it was suggested that neurons can survive in the absence of PI3-K signalling through alternative pathways (Harris et al. 2002).

1.4.2.3.2 MAP kinase pathway

The initial step in the MAP kinase pathway is the blinding of neurotropins to the Trk receptors. In this way, neurotropins can activate the PI3-K pathway but they can also stimulate the docking of the adaptor protein Shc to activated Trk receptors. This triggers the activation of the GTP-binding protein Ras which leads to sequential phosphorylation and activation of Raf, MAP kinase/ERK kinase and extracellular signal-regulated protein kinase (ERK) (Bonni et al. 1999). The effect of the MAP kinase pathway on survival is mediated at least partly by activation of the pp90 ribosomal S6 kinase (RSK) family members (Yuan and Yankner 2000). Similar to Akt, RSK phosphorylates BAD, which results in inhibition of its pro-apoptotic activity. The RSKs also activate the CREB transcription factor, which indirectly stimulates cell survival via Bcl-2 (Bonni et al. 1999). The MAP kinase pathway is also known to control the genes regulating apoptosis, cell proliferation and differentiation. The MAP kinase pathway has anti-apoptotic effects by opposing the pro-apoptotic effects of Jun kinase (JNK) and stress-activated p38 signalling pathways (Xia et al. 1995), and trophic factors induce sustained activation of the MAP kinase pathway (York et al. 2000).

1.4.2.3.3 The role of the Bcl-2 family in neuronal cell survival

The Bcl-2 family of proteins plays a crucial role in the intracellular signal transduction associated with apoptosis (Jacobson 1997; Decaudin et al. 1997). This gene family is composed of both anti-apoptotic (i.e. Bcl-2, Bcl-XL) and pro-apoptotic (i.e. BAX, BAD, BID) proteins (Adams and Cory 1998; Merry and Korsmeyer 1997). The major site of action of these proteins is the mitochondria and they regulate the transmembrane potential and control the release of

apoptosis-inducing cytochrome C into the cytoplasm (Eskes et al. 1998; Jurgensmeier et al. 1998; Kluck et al. 1997). The Bcl-2 and Bcl-xL proteins are known to suppress cytochrome C release (Kluck et al. 1997; Bossy-Wetzel et al. 1998; Yang et al. 1997) while BAX stimulates this event (Eskes et al. 1998; Jurgensmeier et al. 1998; Bossy-Wetzel et al. 1998). Cytochrome C triggers caspase-9 activation through Apaf-1–caspase-9 complex formation, which leads to apoptosis (Zou et al. 1997). The protective effect of the PI3-K/Akt and MAP kinase pathways is mediated, in part, by the upregulation of the anti-apoptotic members of the Bcl family. Transgenic mice expressing the gene for Bcl-2 have been shown to have retinal neurons that are protected from both naturally occurring, developmental programmed cell death and from ischaemia-induced apoptosis (Cenni et al. 1996; Martinou et al. 1994). It is thought that a homeostatic balance exists between pro- and anti-apoptotic factors in the cell, which determines its fate (Tempestini et al. 2003).

1.4.2.3.4 The role of nuclear factor erythroid 2-related factor 2 and neuronal survival

Oxidative stress caused by ROS enhances inflammatory responses during tissue injury, possibly through activation of redox-sensitive chemokines and transcription factor nuclear factor erythroid 2-related factor 2 (Nrf2). Nrf2 has been reported to be a key regulator in cell survival mechanisms where oxidative stress is implicated (Owuor and Kong, 2002) (see Figure 1.10). It induces expression and up-regulation of cytoprotective and antioxidant genes that attenuate tissue injury (Zhang et al. 2005). Under basal conditions, cytoplasmic Nrf2 is bound to the Kelch-like ECH-associated protein 1 (Keap 1), but when cells are exposed to oxidative or xenobiotic stress, Nrf2 dissociates and traverses to the nucleus (Wakabayashi et al. 2003). Nrf2

is considered to be a multi-organ protector (Lee et al. 2005) and is widely viewed as a mediator of neuro-protection. Nrf2 has been shown to reduce ischemic brain injury by protecting against oxidative stress. Furthermore, Shah et al. (2007) found that Nrf2 protects mice from brain damage caused by transient middle cerebral artery occlusion ischaemia followed by reperfusion. He also showed that Nrf-2 modulates neuronal cell death *in vitro* following *tert*-butylhydroperoxide, glutamate and NMDA-induced toxicity (Shah et al 2007). Their data indicate that the induction of Nrf2 translocation is able to reduce cell death caused not only by excitotoxicity but also by oxidative stress. It therefore appears that Nrf2 might trigger more than one protective mechanism when combating stress insults. It is likely that ischaemia and excitotoxcicity trigger different overlapping paths that eventually cause cell death and, where Nrf2 is involved, reduce caspase activity is noticed (Shah et al. 2007). A putative oxidative stress-induced Nrf2 signaling pathway is shown in Figure 1.10.

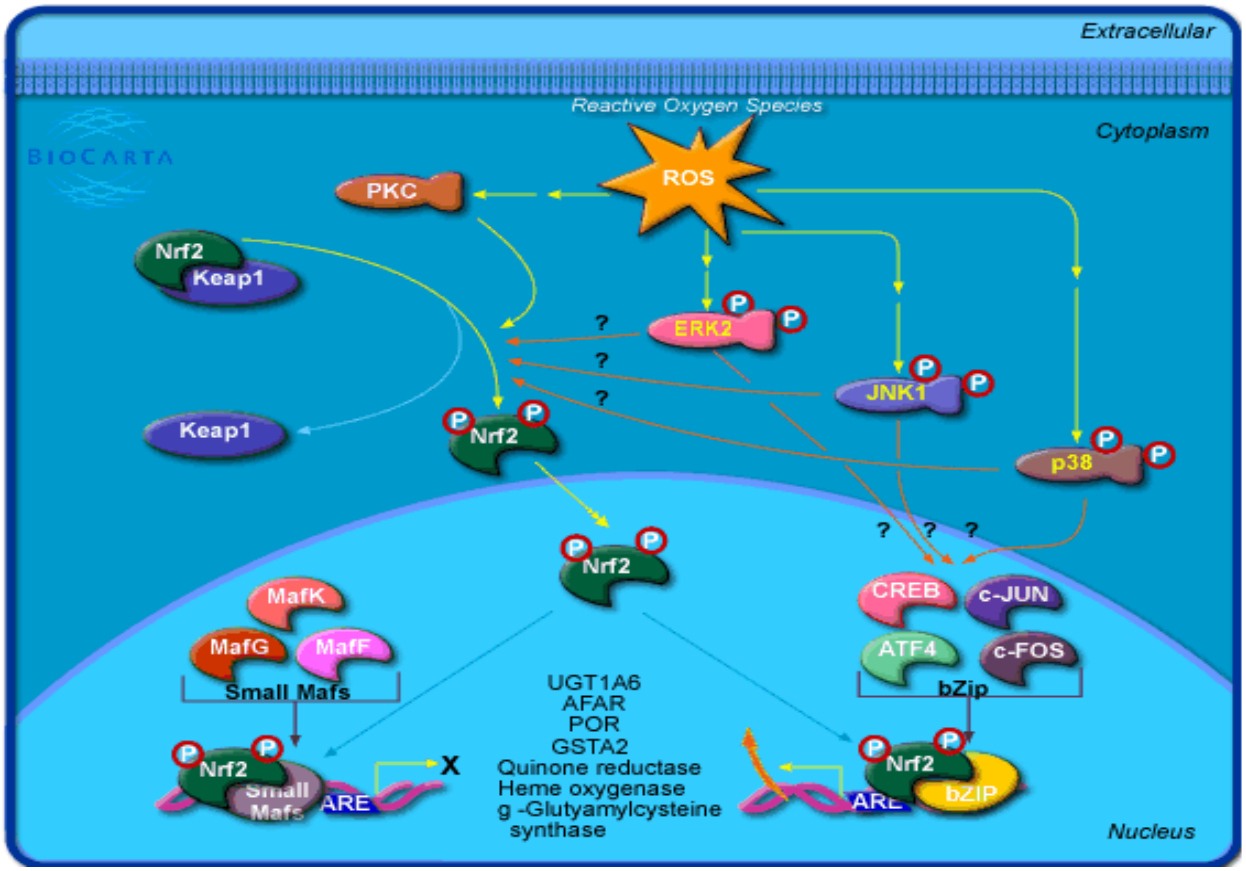


Figure 1.10: Putative signalling pathway of Nrf2 in oxidative stress. The figure shows how Nrf2 might be activated following insults that involve oxidative stress. Electrophilic compounds, xenobiotics and antioxidants are sources of ROS, creating oxidative stress that can harm cells. Enzymes that are involved in the Phase II detoxification of xenobiotics to reduce cellular stress include glutathione transferases, quinone reductase, epoxide hydrolase, heme oxygenase, UDP-glucuronosyl transferases, and gamma-glutamylcysteine synthetase. Expression of these genes protects cells from oxidative damage and can prevent mutagenesis and cancer. Transcription of these enzymes is coordinately regulated through antioxidant response elements (AREs). Nrf2 is a transcription factor that binds to ARE and activate such genes. Inactive Nrf2 is retained in the cytosol by forming a complex with the cytoskeletal protein Keap1. Cytosolic Nrf2 is phosphorylated and translocates into the nucleus in response to protein kinase C activation and elements of the MAP kinase pathways. In the nucleus, Nrf2 activate genes through AREs by interacting with transcription factors in the bZIP family, including CREB, ATF4 and fos or Jun. Nrf2-induced activation of genes is nullified by small Maf proteins (including MafG and MafK) to produce a counterbalance to Nrf2 and to stabilize the oxidative level of the intracellular environment (Source:http://www.biocarta.com/pathfiles).

1.5 Proposed models of RGCs degeneration in POAG

1.5.1 Trophic factor deprivation and glaucoma

It was proposed that deprivation of trophic factor support is one of the primary causes of ganglion cell death in glaucoma (Nickells 1996). The principle underlying this hypothesis is that elevated IOP may interfere with anterograde or retrograde axoplasmic transport by compromising local blood supply or by mechanically pinching the axons at the optic nerve head. By this mechanism, it is thought that a compromising of axoplasmic transport occurs that blocks transport of trophic factors to the cell body leading to death via apoptosis of the affected RGCs (Quigley 1995).

Neurotrophic factors are small peptides grouped in a class of molecules that include growth factors and neurotropins. These small peptides interact with cell-surface receptors, which usually contain protein kinase domains, and activation of these receptors elicits a cascade of events that control cell growth and stasis. The survival of all neurons, including ganglion cells, is dependent on obtaining a fresh and continuous supply of neurotrophic factors. Neurotrophic factors are secreted by other cells that neurons are in contact with, suggesting that these molecules also act as a signalling mechanism which constantly updates the status of neuronal connections (Xu et al. 2003).

Of all the known neurotrophic factors, BDNF is most commonly linked with ganglion cell death in glaucoma. Initially, Johnson et al. reported that BDNF enhanced the survival of RGCs in culture (Johnson et al. 1986). BDNF is released by target neurons in the brain and axons of RGCs which synapse with these target neurons and transport this molecule to the retina in a retrograde manner (Fournier et al. 1997). Thus, it can be thought that any insult which

compromises the transport of BDNF from the target tissue to the retina may cause RGC death, and this cell death mechanism has been proposed to be mainly by apoptosis (Osborne et al. 1999a, d). For example, removal of the superior colliculus resulted in RGC death and BDNF was able to attenuate the RGC death from this insult (Cui and Harvey 1995). The protective effect of BDNF was supported by other studies demonstrating that BDNF can rescue RGCs from death after optic nerve axotomy (Klocker et al. 1998; Peinado-Raman et al. 1996) or ischaemic injury (Unoki and Lavail 1994). Similarly, Ko et al. (2001) reported that intravitreal administration of BDNF enhanced the survival of RGCs in a chronic ocular hypertensive animal model (Ko et al. 2001).

The effect of elevated IOP on the transport of BDNF in RGCs in rats has been reported by Pease et al. (2000). Following elevation of IOP the normal retrograde transport of BDNF from the brain to RGCs was significantly reduced. Also, in a primate model of glaucoma where IOP was chronically elevated, accumulation of BDNF and their Trk receptors was observed to occur at the optic nerve head (Pease et al. 2000). More recent studies have also shown that ganglion cell death can be significantly attenuated when BDNF was provided by a procedure of gene therapy (Martin et al. 2003).

It was initially thought that neurons simply die of passive starvation due to deprivation of trophic factors. However, it was observed that neuronal death caused by nerve growth factor (NGF) deprivation could be reduced by inhibition of RNA and protein synthesis (Martin et al. 1988). Also many studies now support the idea that trophic factor deprivation activates a cellular suicide program, which implies that cell death induced by trophic factor deprivation is an active process (Yuan and Yankner 2000).

The signalling pathways involved by which trophic factor deprivation induces apoptosis is rather complicated, but several studies have shown that caspases play an important role in cell death caused by trophic factor withdrawal (Yuan and Yankner 2000; Haviv et al. 1997; Troy et al. 2001; Charles et al. 2005) Moreover, the observation that caspase inhibitors block neuronal cell death induced by trophic factor deprivation has provided indisputable evidence for a crucial role of caspases in neuronal cell death (Cryns and Yuan 1998).

It is suggested that trophic factor deprivation induces apoptosis via two signalling pathways interconnected with each other (Yuan and Yankner 2000). The first pathway is the nuclear pathway, where trophic factor withdrawal activates JNK and the phosphorylation of c-Jun. Activation of c-Jun induces the expression of DP5/H and subsequently DP5/Hrk activates BAX which results in the release of cytochrome C from the mitochondria. Cytochrome C then forms a complex with Apaf- 1 and procaspase-9, which leads to the activation of caspase-9. The other pathway by which trophic factors induce apoptosis is the non-nuclear pathway, where trophic factor withdrawal directly facilitates formation of the cytochrome C/Apaf-1/caspase-9 complex, resulting in caspase-9 activation.

1.5.2 Retinal ischaemia

The term ‘ischaemia’ can be defined as a decrease in the blood supply to a tissue caused by constriction or obstruction of the blood vessels. It is quite different from anoxia where there is a complete lack of oxygen supply to cells or tissues, or hypoxia where the supply of oxygen to tissues becomes limited or reduced. Although some of the traits of ischaemia often overlap with those of hypoxia and anoxia, one does not imply the other and the terms are not interchangeable.

In ischaemia, cells or tissues become deficient of essential metabolic substrates and oxygen and their ability to remove metabolic by-products simultaneously becomes compromised, leading to the accumulation of toxic metabolic wastes. This can impair normal physiological responses and, over extended periods, leads to malfunction of the affected organ (Osborne et al. 2004). Retinal ischaemia has been implicated as one of the main causes of many vision-impairing conditions or blinding diseases. Various *in vitro* and *in vivo* ischaemia models have been used to study the disease process. *In vivo* studies using ischaemia reperfusion models have been employed to study the effects of raised IOP-induced ischaemia in rat models (Büchi 1992). Ischaemia can also cause an over production of ROS which leads to tissue injury (Shibuki et al. 2000). This type of pathological response to ischaemia overwhelms the cells ability to generate enough antioxidants to counteract the detrimental effects of the free radicals. This is particularly the case during the early reperfusion phase post ischaemic insult (Piantadosi and Zhang 1996). ROS play a critical role in a cell's metabolism but, over-generation of ROS during the early reperfusion phase can result in detrimental effects to the tissue. The principle injurious effects of free radicals are: 1) lipid peroxidation of membranes; 2) oxidative modification of proteins; and 3) reactions with thymine in nuclear and mitochondrial DNA, producing single-stranded breaks. Oxidative phosphorylation is the process whereby ATP is generated in the powerhouse of a cell, i.e. in the mitochondria. In ischaemia this process is severely affected, causing a downregulation of ATP production and hence cell and tissue injury occurs. Since ATP is involved in many metabolic processes, many ATP-dependent metabolic and physiological processes become disrupted, such as the regulation of intracellular and extracelluar ionic balance. For example, the Na^+/K^+ pump is heavily dependent on ATP, which when depleted leads to raised intracellular sodium levels due to an increased influx. This then leads to an alteration in the membrane potential, causing

depolarization of the cell and the increased release of neurotransmitters. Other metabolic disturbances as a result of failure in oxidative phosphorylation include the activation of alternative metabolic processes such as glycolysis and anaerobic metabolism leading to increased lactic acid production. Altered redox status of a cell can also cause the malfunction of many metabolic processes, which require enzymes that function properly only at optimal pH.

1.5.3. Glutamate release and excitotoxicity

The release of neutrotransmitters can be dependent on the membrane potential (Globus et al. 1988; Benveniste et al. 1984). For example, glutamate, a major neurotransmitter in the CNS, is released when a neuron becomes depolarized (Ferreira et al. 1996; Zeevalk and Nicklas 1990). In ischaemia, glutamate activates ionotropic and metabotropic-type receptors with excitotoxicity believed to be caused particularly through the activation of kainate and NMDA glutamate ionotropic receptors. Excitotoxicity refers to the ability of glutamate and structurally related excitatory amino acids to destroy neurons (Olney 1969; Olney and Ho 1970). The negative effect of this process involves the accumulation of sodium and calcium in cells, which leads to repeated depolarizing currents. The influx of Na^+ accompanied by the influx of Cl^- ions creates an increase in osmotic pressure which leads to cellular swelling and damage. This pathological process is accompanied by a second wave of attack as a result of Ca^{2+} influx. This leads to the disintegration of neurons (Choi 1985, 1992). The dysregulation of calcium homeostasis is an important factor in neuronal injury as a result of ischaemia (Nicotera and Orrenius 1992; Robin and Kalloniatis 1992). There are various triggering mechanisms involved in calcium overload:

1) After the membrane of a cell becomes depolarized, voltage sensitive Ca^{2+} channels allow the influx of Ca^{2+} into the cell.

2) The release of glutamate can also induce Ca^{2+} to enter cells via NMDA channels (Iino et al. 1990).

3) Reversal of the membrane Ca^{2+}/Na^{+} transporter, which is normally driven by the transmembrane Na^{+} concentration gradient (Siesjo 1992).

4) Downregulation of ATP generation via oxidative phosphorylation leads to Ca^{2+} accumulation as the cells fail to extrude the ions (Vannucci 1990, 1992).

5) The Ca^{2+} ion can be released from the endoplasmic reticulum by inositol triphosphate formed by metabotropic glutamate receptor stimulation (Choi 1992).

6) The intracellular rise in Ca^{2+} can further down-regulate ATP production by acting directly on the mitochondria (Fiskum 2000; Kruman et al. 1998).

Hence, mechanisms that lead to sustained Ca^{2+} release and overload could potentially alter many important intracellular Ca^{2+}-operated biological components, such as proteases, endonucleases and lipid peroxidases (Hou and MacManus 2002; Zheng et al. 2003).

1.5.4 Oxidative glutamate induced cell death (Oxytosis)

Inhibition of glutamate transporters not only increases extracellular glutamate but also reduces intracellular glutamate. This reduction suppresses uptake of cystine through a cystine/glutamate antiporter (xCT), because intracellular glutamate is directly rate limiting for this antiporter

(Lewerenz et al. 2011). Because cysteine is a component for a cysteine-containing tripeptide, GSH, inhibition of glutamate transporters reduces GSH, which results in ROS production and cell death because GSH is an important antioxidant (Meister 1983). It has been demonstrated that oxidative toxicity of glutamate or oxytosis can occur in C6 glioma cells (Kato et al. 1992), retinal Müller glial cells (Reichelt et al. 1997), neuroblastoma cells (Murphy et al. 1989), immature cortical neurons (Murphy et al. 1990) and RGC-5 cells (Dun et al. 2006). Whether oxidative toxicity of glutamate is distinct from excitotoxicity remains to be established because activation of the NMDA-receptor also produces ROS (Lafon-Cazal et al. 1993; Patel et al. 1996). Several reports have shown that short-term exposure to glutamate induces neuronal death after several hours in normal culture conditions even if glutamate has been removed (Abele et al. 1990), suggesting that short pulse of excessive concentrations of glutamate (as might occur from an overactivated cluster of glutamatergic neurons, *in situ*) is sufficient to cause neuronal damage.

The cystine-glutamate exchanger, (system xc^-), mediates the Na^+-independent electroneutral exchange of cystine and glutamate (Dun et al. 2006; Palacin et al. 1998; McBean 2002). System xc- is a member of the glycoprotein-associated amino-acid transporter family which are exchangers that form heterodimers with a glycoprotein subunit, called the heavy chain, permitting functional surface expression. System xc- comprises the transporter subunit xCT, which is a 502 amino-acid-containing protein with twelve putative transmembrane domains, and the heavy chain 4F2hc (also known as CD98), which is a cell-surface glycoprotein. Under physiological conditions, system xc- transports cystine into cells coupled to the efflux of intracellular glutamate. Once inside the cell, cystine is reduced to cysteine, which is required for the synthesis of the antioxidant GSH, a tripeptide consisting of glutamic acid, cysteine, and

glycine. Cellular synthesis of GSH is limited by intracellular levels of cysteine; thus, system xc- plays a critical role in GSH homeostasis. As GSH is necessary for the protection of cells against oxidative damage, system xc- assumes significance as a transport system closely associated with the cellular antioxidant machinery. Many studies intended to show the link between xc- and cellular antioxidant mechanisms in various cell types under oxidative stress conditions have been carried out (Bridges et al. 2001; Sato et al. 2001; Gukasyan et al. 2003; Hosoya et al. 2002; Tomi et al. 2002; Sasaki et al. 2002). As reviewed by McBean (2002), experimental evidence supports a model in which cystine is taken up by astrocytes for GSH formation, which is then released to supply this antioxidant to neurons. Also, some studies have shown both hippocampal (Allen et al. 2001) and cortical (Danbolt 2001) neurons are able to uptake cystine. Thus, whereas astrocytes are clearly important in providing GSH to neurons, these recent results suggest that at least some neuronal cells in culture are able to efficiently transport cystine and maintain adequate GSH levels, even without astrocytic support (Allen et al. 2001; Shanker et al. 2001).

Since the retina is susceptible to oxidative injury, studies on the system xc- in this tissue is important. Kato et al. (1993) have described system xc- expression in Müller cells, the predominant glial cell of the retina. These findings have been confirmed by Pow (2001) using an antibody against aminoadipic acid, which is a selectively transported substrate for system xc-. System xc- has been studied extensively at the functional and molecular level in Müller cells (Tomi et al. 2003) and also in retinal endothelial cells (Hosoya et al. 2002; Tomi et al. 2002). In addition to these two cell types, system xc- has also been identified in RPE cells and it has been shown that it is upregulated by exposure to NO donors (Bridges et al. 2001) and by the HIV–1 transactivator protein, Tat (Bridges et al. 2004). In the latter study, immunolocalization studies

with affinity purified antibodies against xCT and against 4F2hc (CD98) have suggested the co-localization of these components of system xc- in several retinal cell layers, including neurons of the ganglion cell layer (Dun et al. 2006). These cells are the second order neurons of the visual system and are susceptible to many types of insult, including oxidative stress (Moreno et al. 2004; Boyd et al. 2003) and die in a number of retinal diseases such as glaucoma (Levin and Gordon 2002). Although a system that provides necessary constituents for the synthesis of GSH could arguably be beneficial, it must be kept in mind that, when system xc- is functioning *in vivo*, it exchanges glutamate for cystine. Cystine is being taken up by cells, but glutamate is being released into the extracellular space. Given that glutamate is toxic to ganglion cells mainly via the stimulation of the NMDA receptor (Sucher et al. 1997), this could be a deleterious consequence of the excess activity of system xc-. Therefore, in any retinal condition in which system xc- is up-regulated, retinal Müller cells must be capable of taking up excess glutamate and converting it to glutamine.

Ganglion cell line RGC-5 has been shown to up-regulate this system under situations of oxidative stress similar to those that can occur in retinal conditions and may be relevant to diseases such as glaucoma and diabetic retinopathy (Dun et al. 2006). The involvement of NO has also been implicated, but the mechanism by which NO up-regulates system xc- is not known. Dun et al. (2006) concluded that the evidence that messenger RNA (mRNA) encoding xCT is increased in addition to the protein levels suggests that NO works at the gene level to account for at least some of the marked increase in system xc- activity. NO may be acting directly by nitrosylation of proteins or it could act indirectly by stimulating cGMP (Dun et al. 2006).

1.5.5 Role of NO in RGC degeneration

As well as being an important gaseous mediator throughout the CNS, NO also has numerous important physiological functions in the retina (Goldstein et al. 1996). NO is synthesized intra-cellularly from the amino acid L-arginine via action of the enzyme nitric oxide synthase (NOS). There are three isoforms of NOS indentified so far: the neuronal NOS (nNOS), inducible NOS (iNOS) and endothelilal NOS (eNOS) (Goldstein et al. 1996). Among these, nNOS and eNOS are Ca^{2+}-dependent. iNOS is, however, Ca^{2+}-independent and under normal physiological conditions is not usually detectable (Goldstein et al. 1996). In ischemic injury, NOS expression increases due to the influx of calcium (Cheon et al. 2003). Despite its vital physiological function in the retina, over-production of NO induces nitrosative stress to cells and causes them to degenerate.

1.6 Neuroprotection

Injury to the CNS can cause irreversible functional neuronal loss. Little or no neurogenesis would occur because adult neurons cannot proliferate and repopulate the site of injury. Little or no regeneration occurs in mammalian neurons. Theoretically little or no secondary degeneration will occur unless primary neuronal loss is by necrosis (as in severe ischaemia) and also inflammation. Inflammation combined with release of contents from necrotic cells creates an environment hostile to still surviving neurons (Schwartz, 2003; Reyes et al. 2006).

Normal pressure of the eye is from 12 to 22 millimeter of mercury (mm Hg). The pressure is considered high when it exceeds 22 mm Hg. High pressure alone does not cause glaucoma, but eye-care professionals sometimes refer a person with an elevated IOP as a glaucoma suspect

because elevated eye pressure might lead to glaucoma (Tsai 2008). The current mainstay of glaucoma therapy is still appropriate reduction of IOP as evidenced-based studies support this (Heijl et al. 2002; Vetrungo et al. 2008). However, many patients still progress to develop poor visual acuity due to continuous neuronal loss. Treatment strategies have recently included the possibility of treating IOP-independent mechanisms that may contribute to the neurodegenerative processes. As the optic nerve axons and RGCs are vulnerable to degeneration in POAG, a part of glaucoma treatment's aim is to prevent further neuronal loss, hence the term 'neuroprotection'. Neuroprotection can be defined as the use of pharmacological agents to attenuate the dying process of neurons and/or protect healthy ones from being susceptible to injury or death (Osborne et al. 2001).

1.6.1 Role of Hydrogen Sulphide in the CNS

Hydrogen sulphide (H_2S) has been known solely as a toxic gas with the smell of rotten eggs. However, H_2S is now gradually being considered a broad spectrum toxicant; its major toxic effects are the toxicity of the CNS and the inhibition of the respiratory system (Beauchamp et al. 1984; Guidotti 1996). Recent studies have shown that H_2S is not only a chemical hazard in certain industrial manufacturing processes, but it can also be produced endogenously in mammalian tissues from *L*-cysteine, mainly by three enzymes: cystathionine β-synthetase (CBS), cystathionine γ-lyase (CSE), and 3-mercaptosulfurtransferase (Moor et al. 2003; Hosoki et al. 1997; Kimura et al. 2005; Tang et al. 2006). H_2S signalling pathway is shown in Figure 1.11. The expression of CBS and CSE has been detected in various tissues. H_2S research has recently focused on its physiological effects and significance under disease conditions. Along with NO and carbon monoxide, H_2S has been shown to be the third gaseous transmitter and plays

important roles, both in normal physiological conditions and in the process/progress of several diseases. H_2S is a small molecule and can permeate membranes freely. The H_2S level is 0–46 μmol/L (Zhao et al. 2003) in rat serum and 50–160 μmol/L in the brain (Hosoki et al. 1997). In aqueous solution, a third of H_2S is undissociated and its solubility in lipophilic solvents is 5 times greater than in water (Wang 2002). H_2S is mostly metabolized to sulphate and thiosulphate via the oxidation metabolism in mitochondria, and GSH triggers the reaction. Very little of H_2S can be converted into lower toxic compounds of methylmercaptan and dimethyl sulfate via the methylation metabolism in cytosol. The metabolic product can be eliminated from the kidney and intestinal tract and lungs within 24 hours, so the endogenously generated H_2S under physiological conditions is hardly accumulated or toxic to cells due to the balanced cellular metabolism of the gas (Furne et al. 2001).

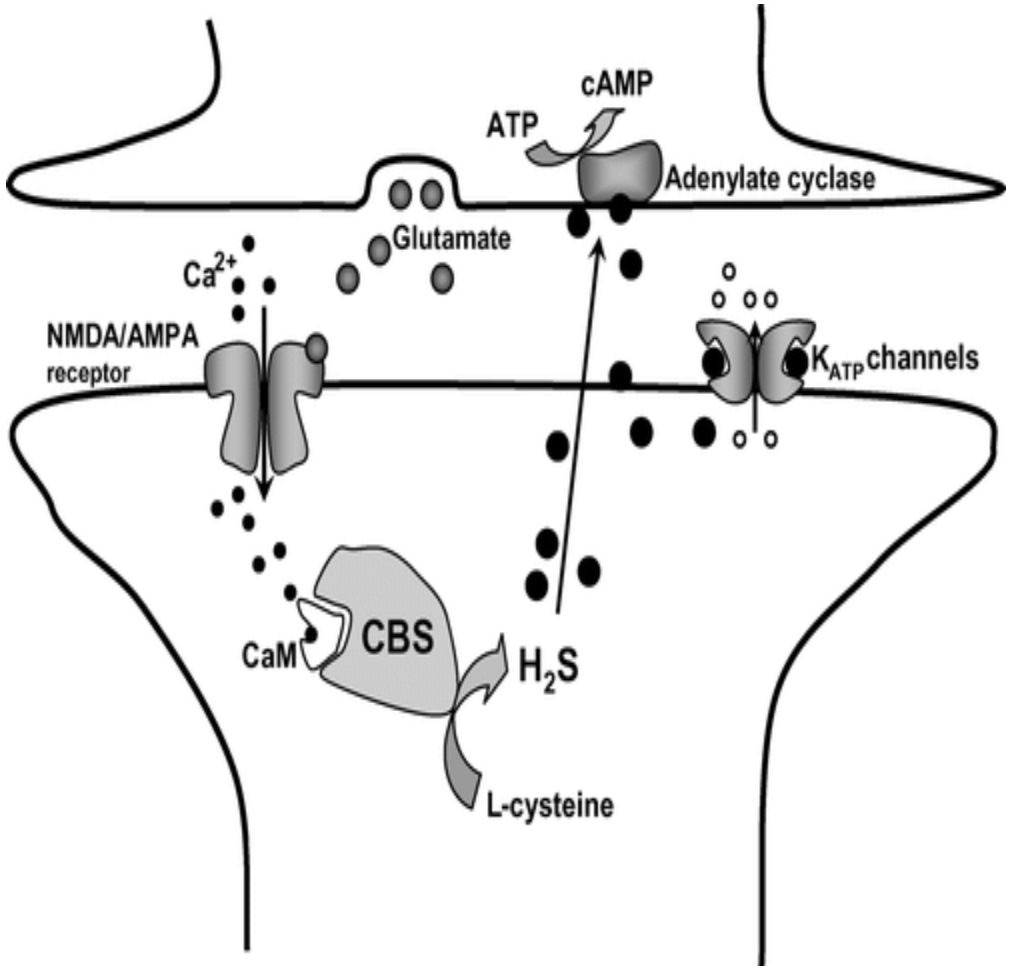


Figure 1.11: H_2S synthesis and targets of action at synapses. Calcium entry through glutamate receptors activates the cysteine metabolizing enzyme cystathionine β-synthase (CBS) via calcium/calmodulin. CBS synthesizes H_2S from L-cysteine. H_2S activates K^+ channels and/or adenylate cyclase. Cysteine, which is metabolized from methionine (not shown) or from and exogenous source, can be converted via CBS/CSE to H_2S. H_2S is mostly metabolized to sulphate and thiosulphate in several steps via the oxidation and metabolism in mitochondria, and GSH is required to start the reaction. (Adapted from Boehning and Snyder (2003) Novel neural modulators, Annu Rev Neurosci 26:105-31)

In the brain, endogenous H_2S is generated mainly by CBS (Abe and Kimura 1996). The transcriptional expression of CBS, but not CSE, in the rat brain (hippocampus, cerebellum, cerebral, and brainstem) was confirmed using the Northern blot assay; a similar conclusion was confirmed in CBS knockout mice. The production of H_2S by CBS in the brain is regulated by Ca^{2+} and calmodulin (Eto and Kimura 2002). It is greatly enhanced by the activation of glutamate receptors and electrical stimulation, which could cause an influx of $Ca^{2+.}$ There are two mechanisms through which CBS could produce H_2S. First, CBS could catalyze the production of H_2S from cysteine by a β-elimination or a α,β-elimination reaction; second, CBS can efficiently catalyze the formation of H_2S via the condensation of homocysteine with cysteine, and the latter is not affected by $Ca2^{+}$ and calmodulin (Chen et al. 2004).

The regulation of CBS activity can affect brain H_2S formation. Eto and Kimnura (2002) found that sodium nitroprusside (SNP), a well known NO donor, could enhance CBS activity by modifying four of 13 cysteine residues of CBS (Eto and Kimnura 2002). A human CBS complete genomic sequence has been determined (Avila et al. 2000) and the transcriptional start sites of five CBS mRNA isoforms, designated CBS-1a–1e, have been mapped (Bao et al. 1998).

Endogenous H_2S produced by CBS in the CNS has multiple physiological functions, including modulation of the NMDA receptor (Kimura 2000). NMDA receptor activation is involved in hippocampal long-term potentiation (LTP) (Harris et al 1984), a synaptic model of learning and memory. Because of the relative high concentration of endogenous H_2S in the brain (50–160 μmol/L), the physiological concentration of H_2S facilitates the induction of LTP by enhancing NMDA receptor-induced currents (Abe and Kimura 1996). This activation could be blocked by

an adenylyl cyclase specific inhibitor, indicating that the modulation of NMDA receptors by H_2S is induced by the enhancement of cAMP production (Kimura 2000). This observation suggests that H_2S is a neuromodulator in the brain. Another study showed that H_2S could increase intracellular Ca^{2+} and induce Ca^{2+} waves in neighbouring astrocytes (Nagai et al. 2004). Therefore, H_2S may mediate signals between neurons and glia and regulate the synaptic activity of neurons. In addition to its role in signal transduction, H_2S can protect neuron cells from oxidative stress, not only by increasing the levels of antioxidant GSH (Kimura and Kimura 2004) but also by activating the K^+ ATP and Cl^- channels (Kimura et al. 2006). In human cultured neuron cells, H_2S could inhibit peroxynitrite ($ONOO^-$), which is an important mediator of human neurodegenerative disease, inducing tyrosine nitration, cell toxicity, intracellular protein oxidation and protein nitration. This antioxidant action of H_2S suggests that it functions as an endogenous $ONOO^-$ scavenger (Whiteman et al. 2005). Thus evidence exists to suggest that H_2S can counteract oxidative stress as occurs in various neurodegerative diseases. Interestingly, the concentration of H_2S in the brain varies in different CNS diseases. Both H_2S and CBS activity is dramatically decreased in the brains of Alzheimer's disease patients, while the level of AdoMet, a CBS activator, is significantly lower (Eto et al. 2002). The CBS activity is also reduced in another disease, homocystinuria (Hu et al. 1993).

Besides its role in the CNS H_2S also have other multiple functions in other organs, especially the cardiovascular system. The novel effects of H_2S on physiological functions such as vasodilation have recently been discovered (Du et al. 2003). Moreover, H_2S also plays a key role in the pathophysiology of endotoxin, septic and haemorrhagic shock (Mok et al. 2004), vasorelaxation

(Hosoki et al. 1997), inflammation (Li et al. 2005, Zanardo et al. 2006) and cardiac function in rats (Geng et al. 2004).

1.6.2 Role of H_2S in the Eye

As already mentioned, endogenous H_2S is generated in mammalian tissues from L-cysteine, a reaction catalyzed by two endogenous pyridoxal-5'-phosphate dependent-enzymes CBS and CSE (Bukovska et al. 1994; Erickson et al. 1990; Stipanunk et al. 1982). Both the enzymes CBS and CSE also occur in ocular tissues (Persa et al. 2006; Pong et al. 2007) and both enzymes exist in significant concentration is the retina suggesting the presence of a functional trans-sulfuration pathway necessary to generate H_2S that may allow it to act as a gaseous retinal neuromodulator. As reviewed by Gerding 1998, CBS deficiency has been associated with several eye disorders that include retinal degeneration, retinal detachment, optical atrophy and glaucoma (Gerding 1998).

1.6.3 Sulphur-containing and H_2S releasing compounds as potential neuroprotectants

Thiols (SH-containing substances) exist as free thiols, thiols bound to proteins and also thiols existing in the disulfide form, such as GSH. These different forms of thiol molecules are involved in many of the important cellular processes necessary for homeostasis, including cell signalling, proliferation and antioxidant responses (Sen 1998; Deneke 2000; Wood et al. 2007; Sen and Packer 2011). Recent studies have shown that thiols play an important role in regulating the redox state of cells. The determination of protein structure, regulation of enzyme activity and control of transcription factor activity and binding is critically determined by the thiol redox

status of intracellular and extracellular compartments (Sen 1998; Deneke 2000; Wood et al. 2007; Sen and Packer 2011). Exogenous thiol compounds can potentially act as antioxidants through a variety of mechanisms. They act for example, as components of the general thiol/disulfide redox buffer system, as metal chelators, as radical quenchers, as substrates for specific redox reactions, and as specific reductants of individual protein disulfate bonds (thioredoxin) (Sen 1998; Deneke 2000; Wood et al. 2007; Sen and Packer 2011). The composition and redox status of the available thiols in a given compartment is highly variable and must play a part in determining the metabolic activity of every cellular compartment.

It is generally beneficial to increase the availability of specific antioxidants under conditions of oxidant stress (Deneke 2000). Cells have devised a number of mechanisms to promote increased intracellular levels of thiols such as GSH and thioredoxin in response to a wide variety of stresses. It has been shown in several types of cell cultures, animal models and human studies that exogenous thiols can increase cell and tissue thiol levels (Sen 1998; Deneke 2000; Wood et al. 2007; Sen and Packer 2011). Increased levels of GSH and other thiols have been associated with tissues/cells showing tolerance to oxidant stresses and in some reports have been shown to benefit treatment of patients (Sen 1998; Deneke 2000; Wood et al. 2007; Sen and Packer 2011). Several thiol-related compounds exist for the adjuvant treatment of certain diseases. These include thiols such as GSH and its derivatives, cysteine and NAC, dithiols such as lipoic acid (which is reduced to the thiol form intra-cellularly), and prothiol compounds such as OTC, which are enzymatically converted to free thiols within the cell (Emonet et al. 1997; Meister et al. 1986; Williamson et al. 1982).

Giustarini et al. (2006), measured plasma levels of both low-molecular-mass sulfhydryls/disulfides and mixed disulfides with proteins in 41 healthy humans aged 21-92 years

with the aim of assessing whether there is a shift in the thiol/disulfide balance during ageing and verifying some of the possible effects of the thiol imbalance (Guistarini et al. 2006). Their data suggest that ageing is strictly correlated to a decrease in plasma GSH and cysteinylglycine with the concomitant increase of most oxidized forms of thiols and a parallel increase in total cysteine and total homocysteine. They further argue that this is probably due to an augmented efflux of these amino acids from various organs. They hypothesized the occurrence of two distinct regulatory systems for plasmatic pools of GSH/cysteinylglycine on the one hand and cysteine/homocysteine on the other hand (Guistarini et al. 2006).

The ability of thiols to function as either anti- or pro-oxidants is determined partly by the type of oxidant stress and the physiological circumstances (Atmaca 2004). In designing or selecting a thiol compound for a specific function (e.g., neuro-protection, protection of lungs from oxidant exposure or protection of organs from ischaemia-reperfusion injury), the effects of these molecules on the rest of the body must also be considered as every drug is different and would have a different potency, efficacy and toxicity profile. For example, large increases in free thiols in the circulation can cause toxic effects (Held et al. 1996; Sen 1998; Deneke 2000). These effects may be the result of thiol radical-mediated reactions but could also be due to destabilizing effects of increases in thiol/disulfide ratios in the plasma, which normally is in a more oxidized state than intracellular compartments. Changes in the thiol redox gradient across cells could also adversely affect any transport or cell signaling processes, which are dependent on formation and rupture of disulfide linkages in membrane proteins (Sen 1998; Deneke 2000). However, therapeutic thiol administration has been shown to have great potential, and its efficacy should be increased by selecting compounds and methods of delivery that will minimize perturbations in the thiol status of regions external to the targeted areas.

One recent study on SN56 cholinergic neurons demonstrated that the inclusion of certain exogenous thiol compounds (i.e. cysteamine) can confer neuro-protection against the effect of a defined insult, i.e. aldehyde-induced cytotoxicity. The study also showed that thiols such as cysteine, NAC, 2-mercaptoethanesulfonic acid, mercapto-propionyglycine, and cysteamine can directly sequester aldehydes. It was also found that all these thiolic compounds augment intracellular cysteine levels via disulfide interchange reactions. Cysteamine and cystamine also augmented basal intracellular cysteamine levels (Wood et al. 2007). This was the first study to demonstrate the importance of intracellular thiols in sequestering the toxic reactive aldehyde products of lipid peroxidation and polyamine metabolism. Some other studies also have begun to elucidate the role of other selected exogenous thiol compounds and their influence on several cellular processes (Wolz and Krieglstein 1996; Packer et al. 1997). However, the mechanisms of action of these compounds remain controversial. This includes demonstrating that cystine uptake into cells can be stimulated by the addition of extracellular thiol compounds such as NAC. It was shown that the increased uptake of cystine was not affected by the presence of glutamate or homocysteate which both inhibited the cystine uptake of control cells (Issels et al. 1988). Further determination of both reduced GSH and oxidized cellular GSSG showed a two-fold increase in cysteamine (MEA) or NAC-treated Chinese hamster ovary (CHO) cells (Issels et al. 1988). BSO, an inhibitor of GSH biosynthesis, completely blocked the promotion of cystine uptake by MEA and NAC. By further analysis using reversed-phase HPLC of cell extracts, more than 90% of the [^{35}S] radioactive cystine taken up by the cells could be recovered within the pool of GSH (Issels et al. 1988). The results demonstrate that exogenous sulphur compounds such as NAC can promote uptake of cystine from the culture medium as well as its rapid utilization for cellular GSH biosynthesis (Issels et al. 1988).

In conclusion, an impressive body of evidence exists to support the hypothesis that pharmacological manipulation of intracellular thiol pools via the use of sulphur-containing molecules could potentially offer a new approach in the design of neuro-protective drug candidates for the treatment of retinal degenerative diseases such as glaucoma (Issels et al. 1988; Jokay et al. 1998; Fox et al. 2004). Also, GSH is clearly an important component necessary to maintain homeostatic oxidant/antioxidant conditions within a cell. It therefore follows that in diseases where oxidative stress is a possible cause, as in glaucoma the maintenance of optimum levels of GSH may provide a means of treatment.

1.7 Putative sulphur-containing/H_2S releasing compounds as neuroprotectants

The following sulphur containing compounds will be briefly reviewed: NAC, sulbutiamine, sodium hydrogen sulphide (NaHS) and dithiolethiones (ACS1 and ACS67).

1.7.1 NAC

The pharmacological agent acetylcysteine, also known as *N*-acetylcysteine or *N*-acetyl-*L*-cysteine (i.e. NAC), is used as a mucolytic agent (Klusha et al. 1976) and as a nephroprotective agent (Tepel et al. 2000). It is also used in cases concerning overdose of paracetamol (acetaminophen), where it is thought to augument GSH reserves (Perry & Shannon 1998). Other uses include sulphate repletion in conditions, such as autism, where cysteine and related sulphur amino acids may be depleted (Geier et al. 2006).

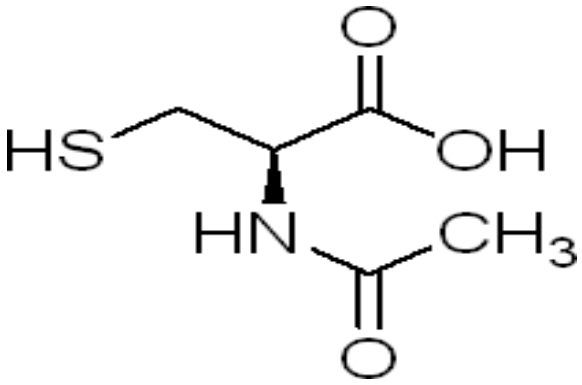


Figure 1.12: Chemical structure of NAC. Molecular formula is $C_5H_9NO_3S$, Mol. mass 163.19

As discussed above reduced GSH plays a central physiological role in maintaining the body's homeostasis and in protecting cells against oxidants, toxicants, DNA-damaging agents and carcinogens of either exogenous or endogenous origin. Unfortunately, the large GSH molecule is not transported efficiently into cells. Furthermore, L-cysteine, which is the rate-limiting amino acid in the intracellular synthesis of this tripeptide (γ-glutamyl-L-cysteinyl glycine) can be toxic to humans at high levels (Meister 1989). NAC is readily deacetylated in cells to yield L-cysteine thereby promoting intracellular GSH synthesis (De Flora et al. 1995). Besides being a GSH precursor, NAC is, *per se*, responsible for protective effects in the extracellular environment, mainly due to its nucleophilic and antioxidant properties, which influence the toxicokinetics of xenobiotics (De Flora et al. 1995). The ability of NAC to modulate the apoptotic process is also well established. One study addressed this problem by exposing normal human monocytes to cigarette smoke, a treatment which induced the expression of stress proteins, mitochondrial depolarization and apoptosis was attenuated by NAC (Banzet et al. 1999).

1.7.2 Sulbutiamine

Sulbutiamine (brand name: Arcalion) is a synthetic derivative of thiamine (vitamin B_1). It is a dimer consisting of two modified sulfurated thiamine molecules. It is lipophilic, crossing the blood-brain barrier more readily than thiamine, and able to increase the level of thiamine and thiamine phosphate esters in the brain (Bettendorff et al. 1990). Sulbutiamine is very lipophylic and, as a consequence, has better pharmacokinetic characteristics than many other thiamine derivatives (Volvert et al. 2008). Sulbutiamine is the only approved compound used to treat

asthenia (Van Reeth 1999). It is thought to selectively target the areas that are involved in the condition (Van Reeth 1999). In addition to its use as a treatment for chronic fatigue, sulbutiamine also appears to improve memory and erectile dysfunction (Micheau 1985; Dmitriev et al. 2005). Within therapeutic dosages, it has few reported adverse effects (Douzenis et al. 2006).

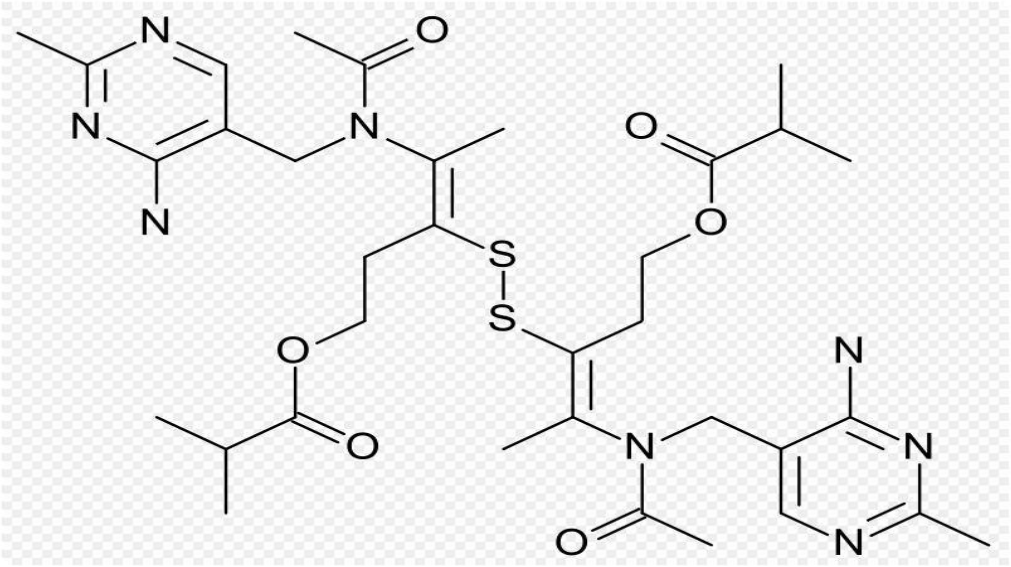


Figure 1.13: Chemical structure of sulbutiamine a synthetic sulfurated thiamine. Molecular formula of sulbutiamine is C32H46N8O6S2, Molecular weight is 702.89 g/mol

Several studies have shown that sulbutiamine improves memory through the potentiation of cholinergic, dopaminergic, and glutamatergic transmission. When sulbutiamine is administered to mice, they perform better on operant conditioning tests (Micheau et al. 1985) and object recognition tests (Bizot et al. 2005). Sulbutiamine also reduces the amnesiac effects of dizocilpine and improves memory in schizophrenics (Bizot et al. 2005). More recently,

sulbutiamine has been shown to improve everyday activities in patients suffering from early-stage and moderate Alzheimer's disease when used in conjunction with an acetylcholinesterase inhibitor. In a randomized double-blind study of Alzheimer's disease patients, the combination of sulbutiamine and donepezil improved episodic memory and daily life activities better than the combination of donepezil and a placebo (Ollat et al. 2007).

1.7.3 Anethole Dithiolethiones

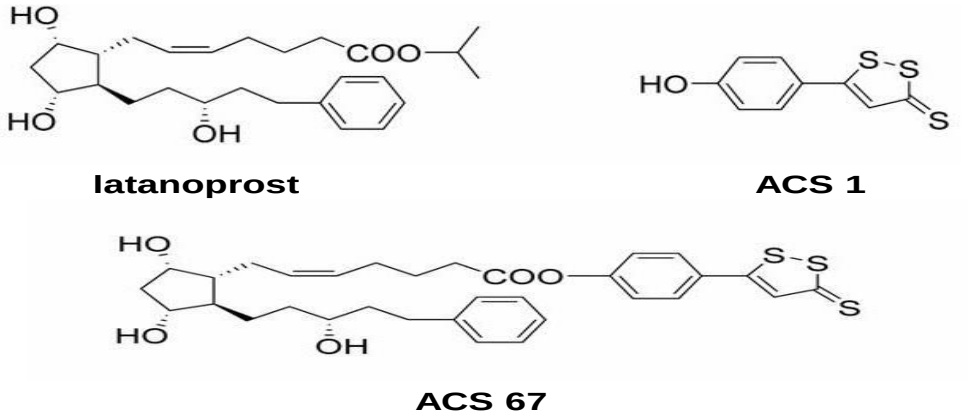


Figure 1.14: Chemical structure of derivatives of dithiolethione compounds ACS1 and ACS67.Also shown is the prostaglandin latanoprost. ACS1 or ADTOH (5-(4-hydroxyphenyl)-3H-1,2-dithiole-3-thione, Molecular weight:226.3 g/mol)

Anethole dithiolethione [5-(*p*-methoxyphenyl)3H-1,2-dithiole-3-thione; ADT] belongs to a group of cyclic, sulphur-containing compounds collectively known as the 1,2-dithiole-3-thiones,

some of which are presently being evaluated for their potential use in cancer chemoprevention (Christen 1995). ADT has been in clinical use for decades as a choleretic (stimulate bile) and sialogogue (stimulate saliva) without any major adverse reactions being noted. It is highly lipophilic and has been shown to increase cellular GSH levels both *in vivo* and *in vitro* and to protect against (oxidative) damage incurred by loss of GSH (Christen 1995; Davies et al. 1987; Mansuy et al. 1986; Sen et al. 1996), at least outside the nervous system. ADT also induces a rise in the GSH content of cultured astroglial cells (Drukarch et al. 1997).

It has been suggested that ADT and ADT-substituted compounds have the potential of releasing H_2S (Li et al. 2007) and, as a result, several ADT-hybrid molecules have been synthesized by a group in Italy (CTG Pharma) to produce potentially added benefits. Substances to which ADT were linked include aspirin, latanoprost, sildenafil, timolol and diclofenac, so providing them with the added capacity to release H_2S in certain situations. For example, ACS14 is aspirin containing a dithiolethione moiety and, once in the gut, H_2S is released where is thought to affect the redox state and protect the tissue from the negative effects of aspirin. Significantly, ACS14 still maintains the thromboxane-suppressing activity of aspirin. The precise mechanism by which ACS14 is able to protect the gastric mucosa from aspirin remains to be elucidated. ADT derivatives of latanoprost (ACS67) (Figure 1.14), diclofenac (ACS15), valproic acid (ACS2), aspirin (ACS14) and sildenafil (ACS6) have all been studied, both in cell cultures and in some cases in several animal models. Results suggest that, while these drugs maintain the therapeutic capacities of their parent molecules, they have several advantages, such as sparing the gastric mucosa (ACS14, ACS15), inhibiting histone deacetylase much more than valproic acid (ACS2), blocking superoxide formation and down regulating NADPH oxidase (ACS6) and enhancing

reduction in IOP (ACS67) (Sparatore et al. 2009; Li et al. 2007; Perrino et al. 2008; Muzaffar et al. 2008).

Mechanisms by which the addition of the ADT moiety exerts added pharmacological effects to parent molecules seem to be associated with a release of H_2S that either directly or indirectly increases GSH. It is speculated that H_2S is released at different rates from the three sulphur atoms of the trithione moiety, thus producing a phasic but sustained release for each of the ACS compounds (Guistarini et al. 2010). Dithiolethione-containing compounds actions on GSH seem to be one cause for these substances acting as antioxidants (Khanna et al. 1998). Indeed, it has been observed that acute and/or subchronic administrations of dithiolthiones (oltipraz and ADT) increase the activity of Phase II enzymatic detoxification systems and/or tissue GSH content (Buetler et al. 1995; Ansher et al. 1983).

1.7.4 NaHS

NaHS is quite soluble in organic compounds and is often used for the synthesis of organic and inorganic sulphur compounds. It is a toxic compound also known as sodium bisulfide, sodium hydrogen sulphide and sodium sulphydrate. It smells of H_2S due to hydrolysis caused by atmospheric moisture.

However, experimental studies have shown its multiple physiological roles in the CNS and vascular system. For example, systemic administration of NaHS (which donates H_2S) dramatically reversed the progression of movement dysfunction, loss of tyrosine-hydroxylase positive neurons in the substantia nigra and the elevated malondialdehyde level in injured striatum in a 6-OHDA-induced rat Parkinson's disease model. NaHS also specifically inhibited 6-OHDA evoked NADPH oxidase activation and oxygen consumption. Similarly, administration

of NaHS also prevented the development of Parkinson's disease induced by rotenone. Moreover NaHS treatment inhibited microglial activation in the substantia nigra and accumulation of pro-inflammatory factors (e.g. TNF-alpha and nitric oxide) in the striatum via NF-kappaB pathway (Hu et al. 2010).

In other studies, injection of either Escherichia coli lipopolysaccharide or NaHS in mice provokes neutrophil infiltration and edema in the lungs, coupled with elevated plasma tumour necrosis factor-α (TNF-α) concentration (Li et al. 2005) and (Bhatia et al. 2006), suggestive of a pro-inflammatory action. H_2S is also believed to act as a pro-inflammatory mediator in animal models of pancreatitis (Bhatia et al. 2004) and arthritis (Bhatia et al. 2005). These effects may be related to the vasodilator action of H_2S in the microvasculature.

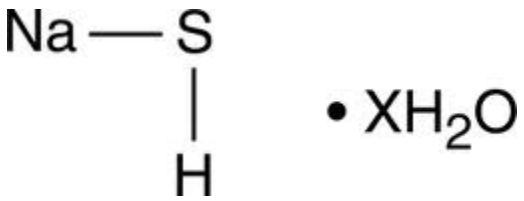


Figure 1.15: Chemical structure of sodium hydrogen sulphide hydrate (NaHS). Molecular formula NaHS x(H_2O). Molecular weight 56.063 g/mol.

1.8 Aims of thesis

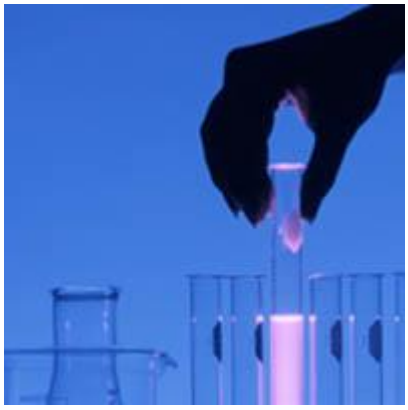
Experiments were conducted in order to understand the mode of actions of selected sulphur-containing compounds in relation to either cell death or neuro-protection. The thiol compounds used vary in structure and property, with some having the capacity to regulate the release of the gas H_2S in appropriate aqueous environments. The substances studied included NAC (an aminothiol compound), sulbutiamine (a thiolic thiamine derivative), various dithiolethionines (ACS1 and ACS67) and sodium hydrosulphide (NaSH).

The goal of the present work was to deduce whether any of the investigated substances might be candidate drugs for use as neuro-protective agents in attenuating different triggers for RGC death as might occur in glaucoma. Thiol compounds, as well as H_2S have a variety of putative actions that include influencing mitochondrial homeostasis in direct and indirect ways and can consequently enhance cell survival within a defined concentration range.

In Chapter 3, the neuroprotective effect of a well known over the counter aminothiol drug NAC and sulbutiamine were investigated. The aim was to determine the mechanisms by which NAC and sulbutiamine can act as neuroprotectants, including the role GSH formation. RGC-5 cells in culture were exposed to two different insults i.e. BSO plus glutamate (GB) and serum deprivation. NAC attenuated the negative effects of GB and serum deprivation but sulbutiamine was effective only against serum deprivation insult. The conclusion is that the mode of neuroprotection actions of NAC and sulbutiamine varies for different insults and does not solely involve the stimulation of GSH production.

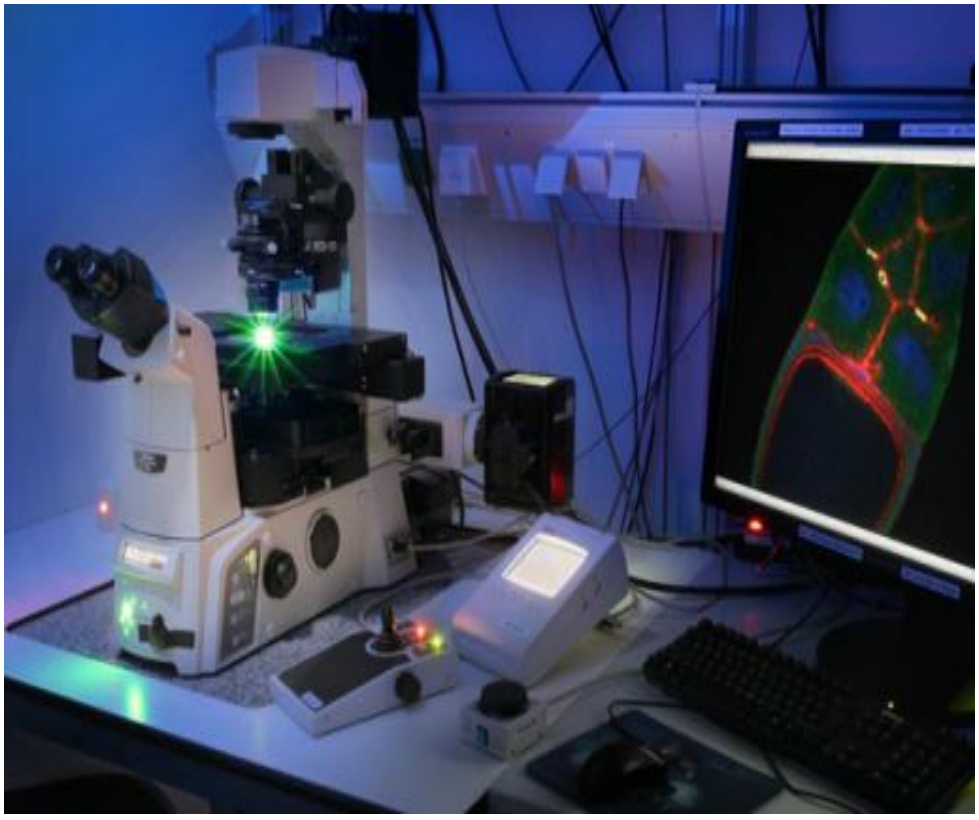
Chapter 4 is concerned with comparing the biochemical properties of the dithiolethione compound (ACS1) with NaHS, particularly in relation to GSH metabolism and cell death induced by GB. Dithiolethione compounds over a wide concentration range are thought to release H_2S at a rate that does not cause toxicity. However, when H_2S is released rapidly from substances such as NaHS, toxic levels of H_2S can occur. Also, the chemical property of NaHS means its thiol group is rapidly oxidized and causes a decrease in its efficacy to attenuate certain defined oxidative insult to cells given over a longer time period, thus limiting its neuroprotective properties. This was investigated by exposing RGC-5 cells in culture to an insult of GB and determining the influences of ACS1 and NaSH. The data generated are in general agreement with this view.

Chapter 5 is concerned with deducing whether the dithiolethione ACS67, a derivative of latanoprost is a neuro-protective agent. To achieve this goal, an insult of GB or ischaemia was delivered to RGC-5 cells in culture or the rat retina *in situ*, respectively. The general conclusion from the studies was that ACS67 is a very effective neuroprotectant because of its ability to regulate the release of H_2S, while latanoprost lacked this ability and indeed possessed effects toxic to cells. ACS67 maintains its capacity to lower IOP because of its latanoprost moiety. However unlike latanoprost, AC67 also has a neuroprotective effect because of its capacity to release H_2S. Its use for the treatment of glaucoma is worthy of further investigation.



Chapter 2

Materials and Methods



2.1. Materials

2.1.1. General reagents

Most of the chemicals used in this project were obtained from Sigma Chemical Company (Poole, UK) and were of the best quality available. The APOP*ercentage*™ apoptosis assay kit was from Biocolor Limited (Belfast, North Ireland) and cAMP ELISA kit was purchased from Cayman Chemical (Ann Arbor, Michigan, USA). Biotin-16-dUTP was obtained from Roche Diagnostics (Indianapolis, IN, USA). Terminal deoxynucleotidyl transferase (TdT) and Cell Titer Blue® (Resazurin reduction assay) (was obtained from Promega (Southampton, Hapshire, UK). Avidin-biotin complex kits were from Vector Laboratories (Peterborough, UK) and Deoxyribonuclease I (DNase I) was purchased from Boehringer Mannheim (Lewes, UK).

2.1.2. Cell Culture Reagents

Foetal bovine serum (that met E.U. regulations), DMEM, glutamine, 0.25% [w/v] trypsin solution, penicillin and streptomycin, fungazone (amphotericin B), gentamycin, MEM vitamins, MEM non-essential aminoacids and 24-wll plates were from GIBCO® Invitrogen (Paisley, UK). Sodium bicarbonate solution (7.5%) was from Sigma Chemical Company (Poole, UK). Tissue culture flasks; 25cm^2/ 75cm^2, (with ventilated cap), polypropylene (centrifuge) tubes and Cell Plus charged-coat 96-well plates were from Sarstedt (Leicester, UK).

2.1.3. Retinal Ganglion Cell line (RGC-5)

The transformed retinal ganglion cell line, RGC-5 (Krishnamoorthy et al. 2001) was a kind gift from Dr. N. Agarwal (Department of Pathology and Anatomy, UNT Health

Science Center, Fort Worth, Texas, USA). The cells used are transformed, neural like precursor cells known to exhibit a number of biochemical characteristics associated with retinal ganglion cells, which more recently have been found to originate from mice instead of from rat (Van Bergen et al 2009).

2.1.4. Animals

Procedures used in this thesis conformed to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and were approved by the Home Office in the United Kingdom. The adult female Wistar rats (weight 200–250g) were provided by the Physiology Department, University of Oxford. Rats housed in a 12-hour light-dark cycle were used for all experiments. Food and water were provided ad libitum and the animals were kept in a temperature and humidity-regulated animal house. Rats were sacrificed by cervical dislocation and subsequent decapitation. Rat pups were sacrificed by decapitation.

2.1.5. Anaesthetics

Sedation of the animals prior to intraocular injection, was with intramuscular injection of fentanyl/fluanisone (Hypnorm (0.3ml/kg); Janssen Pharmaceutica, Grove, UK) and diazepam (2.5mg/kg; Wockhardt UK Ltd., Wrexham, UK).

2.1.6. Pharmacological agents

NAC, NaHS and EGCG were purchased from Sigma Chemical Company (Poole, UK). ACS1 and ACS67 was a kind gift from Peiro Del Saldato CTG Pharma (Milan, Italy). Sulbutiamine was purchased from Shenzhen Sailing Chemical Co. (Schenzhen, P.R.China), and the purity was over 99% by HPLC.

2.1.7. Molecular biology

Throughout all the RT-PCR studies, deionised water (treated with 0.1% diethylpolycarbonate (DEPC) overnight and then autoclaved) was used. For gel electrophoresis, loading buffer (50% glycerol, 0.04M Tris-acetate, 1mM of EDTA, 1% bromophenol blue, 1% xylene cyanol) was added to the 10 µl sample and the products were separated on 1.2% agarose gel containing TBE buffer (88 mM Tris, 88 mM boric acid, 10 mM EDTA, pH 8), which came ready prepared (10x concentration) by Sigma Chemical Company (Poole, UK) and 1 µg/µL of ethidium bromide.

2.1.8. Antibodies

The primary rat monoclonal antibodies against choline acetyltransferase (ChAT) was purchased from Sigma Chemical Company (Poole, UK). The primary sheep antibody to neuronal nitric oxide synthase (nNOS) was a gift from Dr. Emson, University Cambridge, UK. The primary rat monoclonal antibodies against actin, and tubulin were purchased from Chemicon (Chandlers Ford, UK). The primary mouse antibody to neurofilament (NF-L) was also purchased from Chemicon (Chandlers Ford, UK). Primary mouse monoclonal antibody to Thy-1 was a gift from Dunn School of Pathology (Oxford University, Oxford, UK). The primary rabbit antibody to GABA was

purchased from Sigma Chemical Company (Poole, UK). The primary rabbit antibody to anti-glial fibrillary acidic protein (GFAP) was from Dako (Ely, UK). The primary mouse polyclonal antibody to vimentin and monoclonal antibody to calbindin were purchased from Sigma Chemical Company (Poole, UK). Other antibodies used in cell culture studies include rabbit anti-Nrf2 (Santa Cruz Biotechnology, USA) rabbit anti HO-1 (Assay Designs, USA), rabbit anti-NfkB (Santa Cruz Biotechnology, USA), mouse anti PKCα (Santa Cruz Biotechnology, USA), mouse anti-PARP (BD Biosciences Pharmingen, USA), mouse anti Bcl-2 (Santa Cruz Biotechnology, USA), mouse anti-XIAP (Abcam,UK) mouse anti-actin (Millipore, USA). The primary mouse monoclonal antibody against Poly (ADP-ribose) polymerase (PARP) was also purchased from Becton-Dickinson (Oxford, UK). The primary rat polyclonal antibody to cleaved PARP and primary rabbit monoclonal antibody to cleaved caspase-3 was purchased from Cell Signaling Technology (Danvers, MA USA). All secondary (IgG) antibodies were obtained were from Sigma Chemical Company (Poole, UK).

2.2. Methods

2.2.1. Cell Culture

2.2.1.1. RGC-5 cell line

RGC5-cells had been previously characterised as expressing certain ganglion cell markers in culture (Krishnamoorthy et al. 2001). This line was designated positive for certain characteristics of retinal ganglion cells including Thy-1 and Brn-3C expression. RGC-5 cells also express brain-derived neurotrophic factor, nerve growth factor neurotrophin-3, neurotrophin-4 (Agarwal et al. 2007). However, recently it was shown from the analysis of RGC-5 cell mitochondrial DNA that the cells originate from mice (Mus musculus) and not rats (Rattus norvegicus) (Van Bergen et al. 2009).

Cells were maintained in Dulbecco's modified Eagles's medium supplemented with 10% (v/v) heat inactivated fetal bovine serum, 4mM glutamine, 2.9 g/l glucose, 100 IU /ml penicillin/streptomycin in a humidified atmosphere of 95% air and 5% CO_2 at 37°C. Doubling time of these cells was approximately 20 hours under these conditions and they were generally passaged at a ratio of 1:8 to give a cell density of approximately 4-5 x 10^4 cells/ml. Cells were either passaged into sterile 75 cm^2 tissue flasks or plated onto sterile 6-, 24- or 96-well plates.

2.2.1.1.1 Passaging of RGC-5 cells

Cells were grown to confluence in 75 cm^2 culture flasks. Sterile PBS (10 ml) was added to the flasks and then poured off. Subsequently, 5 ml of 0.25% trypsin solution was added to the flasks, swirled and then placed in an incubator for 5 minutes. After that, 5 ml of 'RGC-5 culture medium' was added to the flasks and swirled. The cells were then scraped from the flasks using sterile cell scrapers and the contents were poured into a 10

ml test tube. Following a centrifugation (5 minutes at 100 x g) the supernatant was decanted from the test tube and the pellet resuspended in the 'RGC-5 culture medium' either for passaging into flasks or for plating, as described above.

2.2.2. Cell culture studies

Confluent cultures of RGC-5 cells from 75cm^2 filter-capped cell culture flasks were generally passaged at a ratio of approximately 1:8 to give a cell density of approximately 4-5 x 10^4 cells/ml. 100µl of these cells were plated onto CellPlus^{TM}-96 well plates or alternatively 500µl of cells plated onto sterilised borosilicate glass coverslips in 24-well plates. After approximately 5 hours to allow the cells to settle, 5 mM buthionine sulfoxime plus 5 mM glutamate (GB) was added to each well. In some instances ACS1, sulbutiamine, NaHS, ACS67, latanoprost (Sigma, Poole. UK) or acetylcysteine (NAC, from Sigma, Poole.UK) was added 30 minutes before adding GB. For serum deprivation experiments 10% FBS containing medium was exchanged with a medium which completely lacked serum. Sulbutiamine or NAC was added (preincubated) 30 minutes before the serum deprivation procedure (exchange of the medium) which is then consequently removed as a result of the procedure. After the serum deprivation procedure RGC-5 cells were reincubated with either drug and retained throughout the experiments for 48 hours before cell viability analysis was carried out. Cells cultured in 96-well plates were analysed 24 hours later for cell viability (RRA assay) or for apoptosis using an APOPercentage^{TM} kit (Biocolor, Belfast, Northern Ireland). Coverslips of cells in 24-well plates were processed at the same time for the localisation of reactive oxygen species (ROS) using the dye 2',7'-dihydroethidium and cell death using Hoechst 33258 (Sigma-Aldrich).

2.2.2.1 Viability Studies using Resazurin-Reduction Assay (RRA)

This was carried out using a kit bought from Promega (CellTitre-Blue® Assay). The assay is based on the ability of living cells to convert the redox dye, resazurin, into a flurescent product (excitation 530nm, emission 590nm), resorufin (Ahmed et al. 1994, Fields and Lancaster 1993). The CellTiter-Blue® Cell Viability Assay provides a homogeneous, fluorometric method for estimating the number of viable cells present in multiwell plates. It uses the indicator dye resazurin to measure the metabolic capacity of cells, an indicator of cell viability. Viable cells retain the ability to reduce resazurin into resorufin, which is highly fluorescent (Figure 2.1). Nonviable cells rapidly lose metabolic capacity, do not reduce the indicator dye, and thus do not generate a fluorescent signal.

Resazurin is dark blue in color and has little intrinsic fluorescence until it is reduced to resorufin, which is pink and highly fluorescent (579Ex/584Em). The visible light absorbance properties of CellTiter-Blue® Reagent undergo a blue shift. upon reduction of resazurin to resorufin. The absorbance maximum of resazurin is 605nm and that of resorufin is 573nm (Figure 2.2). Either fluorescence or absorbance may be used to record results; however, fluorescence is the preferred method because it is more sensitive and involves fewer data calculations.

A flow diagram summarizing the CellTiter-Blue® Assay protocol is shown in Figure 2.3. The homogeneous assay procedure involves addition of a single reagent (CellTiter-Blue® Reagent) directly to cells cultured in serum supplemented medium. After an incubation step, data are recorded using either a fluorometer or spectrophotometer. Cell washing, removal of medium and multiple pipetting steps are not required, making the assay ideal for adaptation to automated high-throughput screening for cell viability and

cytotoxicity. Under most experimental conditions, the fluorescent signal from the CellTiter-Blue® Reagent is proportional to the number of viable cells. There is a linear relationship between cell number and fluorescence. The linear range and lower limit of detection are dependent on the cell type and the ability to reduce resazurin.

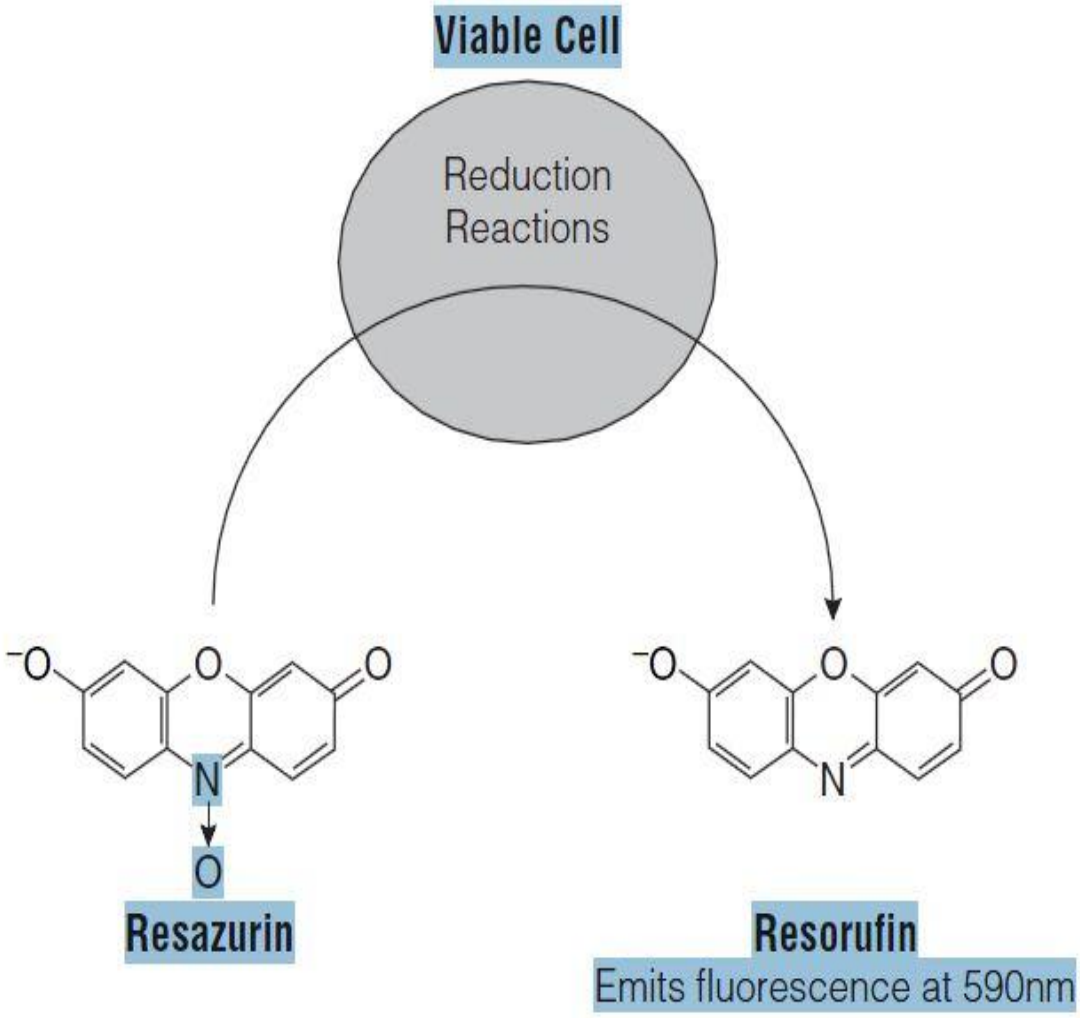


Figure 2.1: Conversion of resazurin to resorufin by metabolically active cells results in the generation of a fluorescent product. The fluorescence produced is proportional to the number of viable cells.

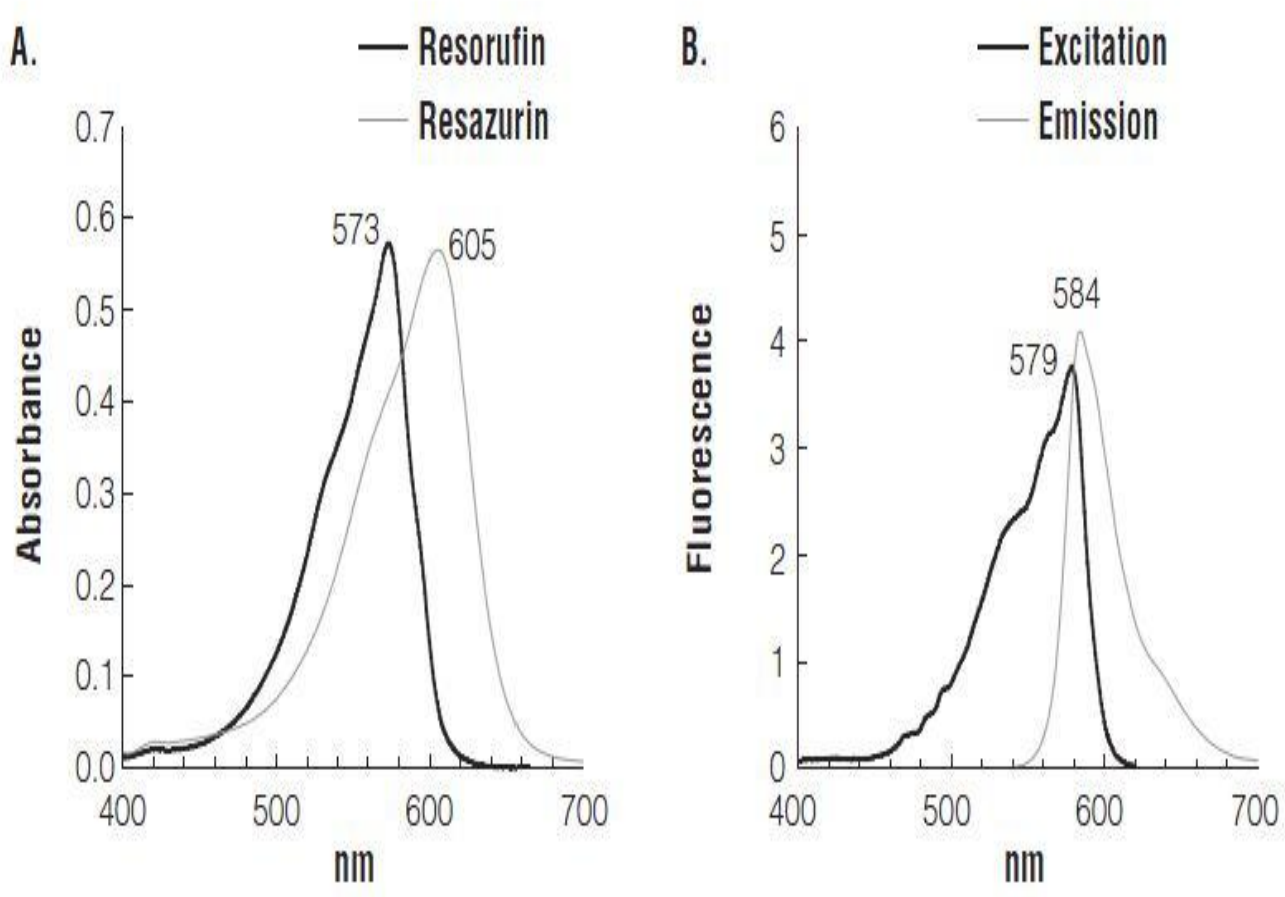


Figure 2.2: Spectral properties of resazurin and resorufin in RPMI medium + 10% fetal bovine serum. **Panel A**. Absorbance spectra for resazurin and resorufin. **Panel B.** Fluorescence excitation and emission spectra for resorufin.

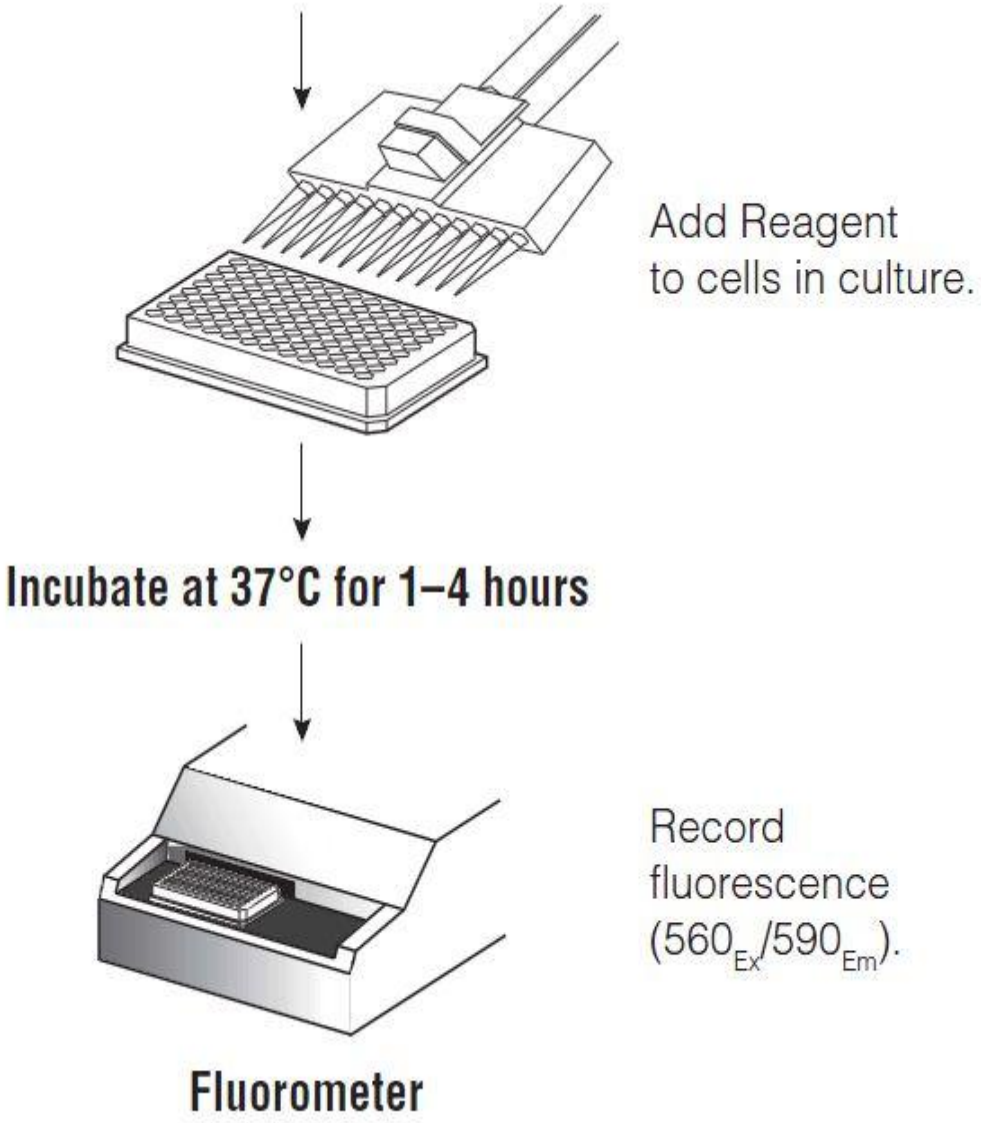


Figure 2.3: The CellTiter-Blue® Cell Viability Assay protocol. Multiwell plates (96- or 384-well) compatible with fluorescent plate readers are prepared with cells and the compounds to be tested using standard methods. CellTiter-Blue® Reagent is added directly to each well, the plates are incubated at 37°C to allow cells to convert resazurin to resorufin, and the fluorescent signal is measured.

2.2.2.2. Identification of apoptosis by use of APOPercentage™ kit.

The APOP*ercentage*™ apoptosis assay (Biocolor Ltd., Belfast, North Ireland) was used to quantify apoptosis. The assay was performed according to instruction provided by Biocolor Ltd. The APOP*ercentage*™ assay is a dye-uptake assay, which stains only the apoptotic cells with a pink/red dye, whereas the normal and necrotic cells that are present remain unlabelled. The assay uses a dye that stains pink/red the apoptotic cells undergoing the membrane "flip-flop" event when phosphatidylserine is translocated to the outer leaflet of the cell membrane. Exposure of phosphatidylserine to the exterior surface of the membrane has been linked to the onset of the execution phase of apoptosis. 100 µl per well fresh culture medium containing 5 µl APOP*ercentage*™ dye was added to each well followed by incubation for 30 minutes at 37°C. After removal of the culture medium cells were gently washed two times with 200 µl per well PBS and then immediately viewed under light microscope. Apoptotic cells (red stained cells) were counted using a cell counter under a Zeiss light microscope at 100 times magnification. Ten different fields per well were use to estimate the total number of apoptotic cells per treatment (means SD represent single experiments on different days, 3 experiments total; minimum 300 cells/plate counted).

2.2.2.3. Localising apoptotic cells with Hoechst and FDA/PI staining.

After treatment with 5 mM BSO plus glutamate for 24 hours, cells were initially fixed in 4% paraformaldehyde and incubated with Hoechst 33258 dye (2.5µg/ml dissolved in distilled water) for 30 minutes at room temperature. Cells with positively stained nuclei caused by Hoechst affinity for fragmented DNA were defined as cells in the process of apoptosis. Microscopic analysis for live and dead cells caused by BSO plus glutamate

was also characterized by staining cells with fluorescein diacetate (FDA) and propidium iodide (PI) staining. Cells incubated with 5 μM FDA for 2 min and 3 μM PI for 30 min at room temperature. And then washed once with PBS and examined under fluorescence microscopy.

PI positive cells or Hoechst positive cells were counted using a cell counter under a fluorescence microscope at 100 times magnification. The total number of positive cells in four different representative fields per well were quantified for each treatment group to estimate the percent of positive cells out of the total cell number. (Minimum 200 cells/well were counted and 4 wells were sampled for each treatment group).

2.2.2.4. Assay for H_2S quantification

RGC-5 cells H_2S-synthesizing activity was determined as described previously with modification (Chunyu et al., 2003). RGC-5 cells were grown in 40 mm wells in the presence of different concentration of ACS1 and NaHS for 6 h, 12h and 24 h. After treatment, medium was aspirated, and cells were washed twice and homogenized with potassium phosphate buffer (100 mM; pH 7.4). Each sample (50% weight/volume; 100 μL) contained L-cysteine (10 mM; 20 μL), pyridoxal 5′-phosphate (2 mM; 20 μL) and PBS (30 μL). The reaction was performed in parafilmed eppendorf tubes and initiated by transferring the tubes from ice to bath at 37°C. After incubation for 2 h, zinc acetate (1% weight/volume; 100 μL) was added to trap evolved H_2S followed by tricloroacetic acid (10% weight/volume; 100 μL) to precipitate protein and thus stopping the reaction. After centrifugation, N**,**N-dimethyl-p-phenylenediamine sulphate (20 mM; 50 μL) in HCl 7.2 M followed by $FeCl_3$ (30 mM; 50 μL) in HCl 1.2 M was then added to 50 μL of the supernatant, and optical density was measured at 670 nm.)

2.2.2.5. Assay for glutathione (GSH) and glutathione-S-transferase (GST)

The assay is based on glutathione-S-transferase reacting with GSH in the presence of monochlorobimane (mCB) to form the fluorescent product bimane-glutathione (Cook and Mitchell 1995). After maintaining cell cultures in 40mm wells in the presence of different substances for 8 hours approximately 1x 10^6 RGC-5 cells (collected from two 40mm wells) were collected and centrifuged at 700 x g for 10 minutes at 4ºC. The pelleted cells were then resuspended in cell lysis buffer (100µl) and after 10 minutes centrifuged at 10,000 x g for 10 minutes. 20µl supernatant samples were transferred to a microplate and to each sample were added 2µl 20 mM mCB and 4µl of GST 25U/ml. For GSH quantification, after incubation at 37º C for 15 minutes, fluorescence was measured in a plate reader where the excitation and emission spectra were 380 and 460nm, respectively. GST activity is measured by measuring the rate of change of fluorescence intensity changes over different times. GST plays an important role in detoxification of xenobiotics catalyzing the transfer of the thiol group of glutathione to electrophilic xenobiotics. It utilizes glutathione to scavenge potentially toxic compounds - including those produced as a result of oxidative stress - and is part of the defense mechanism against the mutagenic, carcinogenic and toxic effects of such compounds. The Fluorometric GST Assay Kit provides a simple, fluorescence-based *in vitro* assay for detecting GST activity using a fluorescence plate reader. The assay utilizes monochlorobimane (mCB), a dye that reacts with GSH. The free form of mCB is almost nonfluorescent, whereas the dye fluoresces blue (ex/em = 380/461 nm) when reacting with glutathione. GST catalyzes the mCB-glutathione reaction and the fluorescence levels are proportional to the amount of GST present in the reaction. Thus, the GST level in samples can be easily detected using a fluorometer or a 96-well

fluorometric plate reader. The assay can detect GST activity in crude cell lysate or purified protein fraction, and can also be used to quantitate GST-tagged fusion proteins.

A. Cell Sample Preparation:

1. Cells were collected by centrifugation. To collect adherent cells, a rubber policeman for scraping the cells was used.
2. The cells were then homogenized or sonicated in Sample Buffer.
3. Cells were then centrifuged 10,000 x g for 15 minutes at 4°C.
4. The supernatant was then collected and used for the assay. The remaining sample was stored at –80°C and is stable for at least 1 month.

B. GST Assay Protocol:

1. Samples were prepared in a total of 100 µl volume with GST Sample Buffer, including a negative control with 100 µl of GST Sample Buffer alone and a positive control with 2 µl of GST and 98 µl of Sample Buffer.
2. 10 µl of Glutathione was then added to each well containing the sample and control above.
3. The Substrate Mix was prepared by adding 2 µl mCB Solution + 98 µl of GST Assay Buffer for each sample and standard assayed. This was mixed well and 100 µl of the Substrate Mix was transferred into each sample and control well.

4. The plate was carefully shaken to start the reaction.

5. Fluorescence intensities of samples were read at Ex/Em 380/460 nm at various time points within 1 hour using a fluorescence plate reader. At least 5 time points were collected for kinetic studies. For general comparison, sample intensities were taken between 0.5-1 hours to compare the fluorescence level of treated vs. control samples.

6. GST Activity can be expressed as RFU/min/mg protein. Alternatively the standard calibration curve could be used to determine the GST activity in the samples.

2.2.2.6. Catalase Quantification Using The Amplex Red Catalase Assay Kit (A22180)

The Amplex Red Catalase Assay Kit (A22180) is a very sensitive, fluorescence-based assay used for measuring of catalase activity (Molecular Probes, 2004). In the first step of the assay, catalase reacts with H_2O_2, producing water and oxygen (O_2) (Mueller et al. 1997). In the second step, an agent called the Amplex Red Reagent (ARR) reacts with any unreacted H_2O_2 in the presence of horseradish peroxidase (HRP) and this generates the highly fluorescent oxidation product resorufin (Zhou et al. 1997; Mohanty et al. 1997). This means that the higher catalase activity in the test, the weaker the resorufin signal gets. Resorufin has a very strong absorption capacity and because of this either a fluorometer or a spectrophotometer can be used for the assay (Molecular Probes 2004).

Briefly after RGC-5 cells were incubated with different treatments for 24 hours or 48 hours for serum deprivation experiments, the microplate was emptied by placing Kleenex on top of it then turning it upside-down. Amplex Red Catalase Assay procedure was then carried out according to the Amplex Red Catalase Assay Kit protocol (Molecular Probes 2004). All additions in the catalase assay were pipetted

horizontally into the microplate wells. The 37°C incubation time chosen was 35 minutes, and the excitation and emission detection levels chosen for the fluorescence measuring were set to 530 nm and 590 nm, respectively.

The fluorescence intensity was measured at 8 time points, each separated by 15 minutes. The first measurement was done 15 mins after placing of the microplate in the Fluoroskan Ascent (Thermo Fisher Scientific, MA, USA).

The result values from the Fluoroskan were statistically analyzed in SigmaPlot12 where graphs were drawn on basis of the result figures. The figures used for the graphs were mean values, that is, an average was calculated for the 4 replicates of each different drug concentration as well as controls. The analyses made were a one-way ANOVA. The significance threshold was set to $p<0.05$.

2.2.2.7. Assessment of Reactive Oxygen Species (ROS) production

2.2.2.7.1. Dihydroethidium (DHE) ROS staining

Cells were assessed for the production of reactive oxygen species using the dye DHE according previous methods (Carter et al. 1994; Vanden Hoek et al. 1997). DHE is a reduced form of ethidium bromide, which is non-fluorescent and can passively cross the membrane of live cells (King et al. 2004; Bass et al. 1983). DHE can be oxidized in the cell by superoxide anions or hydrogen peroxide to ethidium bromide, which binds to DNA and, when excited, emits red fluorescence that is proportional to the intracellular superoxide anion or H_2O_2 levels (Vanden Hoek et al. 1997). After different treatments, cells on cover-slips were stained with DHE (10 mg/ml) by incubating for 30 min at 37℃ in culture medium in a humidified chamber and then fixed with 4% paraformaldehyde for 20 min. After washing in PBS, cover-slips were mounted in 1%

glycerol and red fluorescence was detected using a Zeiss epifluorescence microscope.

2.2.2.7.2. Quantification of ROS.

Keston and Brandt originally described a method employing 2',7'-dichlorofluorescin diacetate (DCFH-DA) to measure hydrogen peroxide in aqueous solution (Brandt and Keston 1965). DCFH-DA is freely permeable across cell membranes and is incorporated into hydrophobic lipid regions of the cell (Bass et al. 1983). Esterase cleaves the acetate group of DCFH-DA and traps the nonfluorescent 2',7'-dichlorofluorescin (DCFH) in the cell. Hydrogen peroxide and the hydroxyl radicals produced by the cell oxidize DCFH to 2',7'-dichlorofluorescein (DCF) which is fluorescent (530 nm). The green fluorescence measured is thus proportional to the H_2O_2 produced (Carter et al. 1994). To quantify intracellular ROS, RGC-5 cells were exposed to 5 mM 2',7'- dichlorofluorescin diacetate (DCFH-DA) for 1 h according to previous methods (LeBel et al. 1992) and the fluorescence intensity was determined using Fluoroskan Ascent (Thermo Fisher Scientific, MA, USA) at an excitation wavelength of 485 nm and an emission wavelength of 530 nm. The scavenging activity of DCFH fluorescence (%) was calculated using the equation (Fluorescence control - Fluorescence sample) x 100 / (Fluorescence control - Fluorescence normal).

Briefly RGC-5 cells (5×10^3) were seeded in 96 well plates and cultured for 24 h before a 1-h pretreatment with different concentrations of sulbutiamine, NAC, NaHS or ACS compounds and subsequent exposure to GB 5 mM for 24 h. After the treatment, cells were incubated with DCFH diacetate for 15 min in the dark. Then the fluorescence of liberated DCF was quantified, using a fluorescence plate reader (Fluoroskan Ascent, Thermo Scientific,Waltham, MA, USA) (Ex/Em =485/535 nm).

2.2.2.7.3. Antioxidant capacity assay

The antioxidant capacities of NAC, NaHS, sulbutiamine and ACS compounds were measured using 5- (and-6)-chloromethyl-2', 7'-dichlorodihydrofluorescein diacetate, acetyl ester (CM-H_2DCFDA; Molecular Probes, Eugene, OR, USA) according to previous methods (Shimazawa et al. 2009; Nakajima et al. 2009, Jung et al 2010). CM-H_2DCFDA (inactive for ROS) is converted to DCFH (active for ROS) by being taken into the cell and acted upon by an intracellular enzyme (esterase). Hydrogen peroxide (H_2O_2), superoxide anion (O_2•) and hydroxyl radical (HO•) oxidizes intracellular DCFH (non-fluorescent) to DCF (fluorescent) which is detected by the spectrofluorometer (Adom and Liu 2005).

Briefly RGC-5 cells were seeded at a density of 2×10^3 cells per well into 96-well plates, and then incubated in a humidified atmosphere of 95% air and 5% CO_2 at 37°C. Twenty-four hours later, the cell-culture medium was replaced, before any treatment with drugs or their vehicle (DMEM containing 1% FBS). After pretreatment with selected compounds at different concentrations or its vehicle for 1 h, the radical probe, 5-(and-6)-chloromethyl-2', 7'-dichlorodihydrofluorescein diacetate, acetyl ester (CM-H_2DCFDA) (Molecular Probes, Eugene, OR, USA) at 10 μM was added and incubated for 20 min at 37°C (Adom and Liu 2005). Then, the cell culture medium was replaced to remove the surplus probe. To generate the ROS H_2O_2 at 1 mM (H_2O_2) or KO_2 at 1 mM ($O_2^{\cdot-}$) as the radical probe was added to the loading-medium. Fluorescence was measured, after the ROS-generating compounds had been present for various time-periods, using a fluorescence plate reader (Fluoroskan Ascent, Thermo Scientific,Waltham, MA, USA) (Ex/Em =485/535 nm). In addition, to detect the $HO^{\cdot}$

formed in the Fenton reaction, 2-[6-(4'-amino) phenoxy-3H-xanthen-3-on-9-yl] benzoic acid (APF) (Enzo Life Sciences (UK) LTD.) (Setsukinai 2003) was used. Briefly, cells were loaded with APF by incubation for 20 min at 37°C in Hanks/Hepes buffer solution containing APF (10 μM). To perform the Fenton reaction, H_2O_2 was added to the Hanks/Hepes buffer solution of APF, and then iron (II) perchlorate hexahydrate was added. Fluorescence was measured at excitation/emission wavelengths of 490/515 nm. Total fluorescence intensity was measured at 20 minutes after treatment with ROS-generating compounds.

2.2.2.8. Immunocytochemistry and Immunohistochemistry

2.2.2.8.1. Immunocytochemistry

For immunocytochemistry, RGC-5 cells were grown on 13 mm borosilicate glass cover-slips, which were coated with 10μg/ml poly-L-lysine in PBS. Cover-slips containing cell cultures were fixed in 4% paraformaldehyde in 0.1 M sodium phosphate buffer (pH 7.4) for 30 minutes at room temperature. After rinsing with PBS plus Triton X-100 (0.1%, v/v; PBS-T) for 5-10 minutes, the fixed cultures were incubated at room temperature for 4 hours with the primary antibodies listed above, followed by a further 5-10 minute wash in PBS-T. Cover-slips containing cultures were then exposed to secondary antibodies labelled with (Fluorescein isothiocyanate) FITC for 60 minutes followed by 5-10 minute wash in PBS-T. Cover-slips were then mounted to glass slides using 1% glycerol. Fluorescent antigens were visualized using a Zeiss fluorescence microscope.

For Nrf2 staining cells were grown on poly-L lysine coated slides, Polysine™ (Menzel Gmbh & Co KG, (Braunschweig, Germany). Cultures were incubated with and without drug treatment for 24 h. After treatment, medium was aspirated, and cells were washed twice with PBS and fixed with 4 % (v/v) for 25 min, washed in PBST and then blocked with BSA (0.5% w/v in PBST) for 30 min. The cells were then incubated with the antibody rabbit polyclonal anti-Nrf2 (ab31163, 1:200) (Abcam,Cambridge,UK) overnight at +4°C. They were subsequently exposed to appropriate secondary antibodies conjugated to FITC (1:300; Sigma) and then washed with PBS. Concurrent nuclear staining was then performed by incubating 4',6-diamidine-2'-phenylindole dihydrochloride (DAPI) at 0.5 µg/ml in PBS for 5 min. Cells were washed extensively with PBS before being mounted with buffered glycerol containing phenylenediamine to reduce fluorescence fading. Images were obtained under 40 x magnification with a fluorescence microscope (Nikon, Melville, NY). Images were then processed with Image- J software (NIH, NY, USA).

2.2.2.8.2 Immunohistochemistry

The eyes of anaesthetized rats were enucleated and their retinas removed and fixed in 2% paraformaldehyde / 0.1 M sodium phosphate buffer (pH 7.4) for 45 minutes. The fixed retinas were then cryopreserved for roughly 4 hours in 30% sucrose / 0.1 M sodium phosphate buffer (pH 7.4). Individual retinas were then frozen onto a microtome chuck using dry ice/isopentane slurry at 20℃. A microtome was then used to cut 10µm thick sections, which were recovered onto gelatin-coated glass slides. Sections were first washed in PBS-T (5 mM sodium phosphate buffer, pH 7.4, containing 150 mM

NaCl, 0.25 mM KCL and 0.2% [v/v] Triton X-100) for 10 minutes and incubated at 4℃ overnight with mouse anti-choline acetyltransferase (ChAT) monoclonal antibody (clone 1E6; 1:250) in PBS-T or sheep anti–neuronal nitric oxide synthase (nNOS) antibody (1:2000) in PBS-T. The sections were again rinsed in PBS-T for 10 minutes and incubated at room temperature for 50 minutes with goat-raised secondary IgG antibody (1:100 in PBS-T) conjugated with FITC. After a final washing for 10 minutes in PBS-T, sections were mounted using PBS-glycerol (5 mM sodium phosphate buffer, pH 7.4, containing 150 mM NaCl, 0.25 mM KCL and 75% [v/v] glycerol) and then viewed with Zeiss epifluorescence microscope. Photographs were taken at 250x magnification using a digital camera (Nikon Coolpix 995) mounted to the eyepiece of the microscope.

2.2.3. Animal Studies

Animal procedures conformed to the ARVO Statement for the Use of Animals in Ophthalmology and Vision Research and approved by the Home Office in the United Kingdom. Rats were housed in a 12-hours light-dark cycle and provided food and water ad libitum. Adult animals were anaesthetised with an intramuscular injection of Hypnorm (Janssen, Grove, UK; 0.3 ml/kg), and diazepam (CP Pharmaceuticals Ltd., Wrexham, UK; 2.5 mg/kg) and their electroretinograms (ERGs) were recorded from both eyes. Chemicals and reagents were from Sigma Chemical Company (Poole, UK).

2.2.3.1. Induction of retinal ischaemia

Ischaemia was induced by inserting a 30-gauge needle, connected to a sterile saline reservoir at a certain height, into the anterior chamber of one eye to produce an intraocular pressure of 120 mmHg. Ischaemia was confirmed by immediate whitening of the fundus and was for 50 min. Normal body temperature was maintained and monitored both during ischemia and in the recovery period before transfer to animal house conditions. In some instances 5µl of ACS67 (4 nmoles) or vehicle (50% DMSO) was injected into the vitreous humour directly after ischaemia and these animals killed after seven days of reperfusion. Retinas and optic nerves were dissected and analysed for levels of defined mRNAs and proteins. In some instances retinas were fixed and processed for immunohistochemistry.

2.2.3.2. Electroretinography (ERG)

2.2.3.2.1. Basic principles of ERG

Electroretinography (ERG) allows for the measurement of voltage changes experienced by the eye following an external light stimulus, typically by a flash of light, by recording the difference potentials between an electrode placed on the corneal surface and a reference electrode placed elsewhere on the head. Flash ERG was performed in order to determine functional alterations to rat retinal tissue, where the voltage potentials represent the summed electrical response of the retina, with each component (e.g., a- and b-wave) comprising the distinct response of the particular groups or types of cells. Following a bright light stimulus the ERG produces a complex waveform that consists of major and minor components.

There are three prominent phases. The initial a-wave represents a negative polarity

relative to the corneal surface, followed by a corneal positive upward sloping b-wave. In the rod-mediated ERG a second much slower corneal-positive potential, called the c-wave is seen. Most of the ERG signals are generated not directly from the neurons of the retina, but rather by non-neuronal elements; especially the pigment epithelium and Müller cells. Only the leading edge of the a-wave can be attributed to light evoked activity of both rod and cone photoreceptors, whereas the b-wave is generated by Müller cells of the retina and inner retinal neurons, such as bipolar cells, while the c-wave is predominantly generated by the pigment epithelium. The potentials generated in the Müller and pigment epithelial cells arise from light evoked changes in K^+ levels that occur as a result of neuronal or photoreceptor cell activity. Therefore, the ERG can be used to analyse neuronal cell activity and gives a measure of the health of the retina.

2.2.3.2.2. Methodology of ERG

Adult Wister rats were dark adapted for a period for 1-2 hour prior to recordings. Throughout readings of ERG was prepared under dim red light and the rats were kept warm during and after the procedure. The rats were anaesthetized with an intramuscular injection of Hypnorm (Janssen, Grove, UK; 0.3ml/kg), and diazepam (CP Pharmaceuticals Ltd., Wrexham, UK; 2.5mg/kg). After anaesthesia was achieved, the pupils were dilated with 1% tropicamide (Smith and Nephew Pharmaceuticals Ltd. Romford, UK) and 2.5% phenylephrine (Smith and Nephew Pharmaceuticals Ltd. Romford, UK). During readings a platinum electrode was placed in contact with the central cornea, with a 'loop' reference electrode surrounding the sclera of the eye. A ground electrode was placed on the tail of the rat. The cornea was kept moist with hypromellose eye drops (Martindale pharmaceuticals, Romford, Essex), this was in

order to maintain the baseline recording and to prevent exposure keratopathy. The stimulus of the frequency 0.5Hz was presented from a white strobe light source placed approximately 50 cm in front of the rat. Five to ten consecutive responses, sampled at 10 kHz were amplified and averaged using model 1902 Signal Conditioner/1401 Laboratory Interface (CED, Cambridge, UK). This was connected to a computer that generated the data using a software programme called 'Signal Averager' (CED, Cambridge, UK). The b-wave amplitude was measured from the trough of the a-wave to the peak of the b-wave, and the a-wave was measured as the difference in amplitude between the recording at onset and the trough of the negative deflection. Baseline recordings were taken 1 to 4 days before injection with preferred drugs. Readings were then taken 5 days following injection/pressure induced ischemia. ERGs from both eyes were recorded 2-3 days before and 5 days after ischemia. Data were analyzed by the Student's unpaired *t*-test and a *p* value less than 0.05 was considered significant.

2.2.3.3. RT-PCR procedure for animal studies:

The levels of cyclophilin, Thy-1, NF-L, PARP, GFAP, caspase-3 and caspase-8 mRNAs were determined in the retina from rats seven days after ischaemia using semi-quantitative RT-PCR as described previously (Nash and Osborne 1999; Chidlow et al.,2005). Briefly, total RNA was isolated, and first strand cDNA synthesis performed on 2 μg DNase-treated RNA. The individual cDNA species were amplified in a reaction containing an aliquot of cDNA, PCR buffer, 5mM $MgCl_2$ for NF-L, caspase-3 and caspase-8 and 4mM $MgCl_2$ for all other primers, dNTPS, the relevant primer pairs and DNA polymerase. Reactions were initiated by incubating at 94°C for 10 minutes and PCRs (94ºC, 15 s; 52ºC, 55ºC or 56ºC, 30 s; 72ºC, 30 s) performed for a suitable

number of cycles, followed by a final extension at 72ºC for 3 minutes. Inter-experimental variation was avoided by performing all amplifications in a single run. PCR reaction products were separated on 1.2% agarose gels using ethidium bromide for visualization. The relative abundance of each PCR product was determined by quantitative analysis of digital photographs of gels using Labworks software (UVP Products, CA, USA). The sequence and annealing temperatures of the primers used (Gibco Life Technologies, Paisley, Scotland) are shown in Table 2.1.

Prior to semi-quantitative amplification of the experimental samples, the amount of cDNA in all of the samples was equalized. In addition, the optimal conditions (e.g. Mg^{2+} concentration, annealing temperature) for each set of primers were determined. Subsequently, cycle-dependent reactions were performed for each mRNA species in order to determine the linear range of detection by ethidium bromide. Once the linear range was established, PCRs were performed at the lowest cycle number that gave a reliably detectable product. To minimize variability, duplicate runs were performed for each mRNA amplified and the data were averaged.

For assessment of the levels of the various mRNAs in the retina, all values were normalized to that of the housekeeping gene cyclophilin. Cyclophilin thus acted as an internal standard to correct for any variations in RNA isolation and/or cDNA synthesis. The primers were selected from GenBank (www.ncbi.nlm.com) and designed using the primer design software Primer Express (Applied Biosystems). Most of the primers were supplied by Invitrogen (Paisley, UK).

2.2.3.3.1 Basic principles of RT-PCR

The purpose of polymerase chain reaction (PCR) is to synthesise, *in vitro*, a large number of copies of a gene. There are three steps in each PCR cycle which are repeated for a set number of cycles. PCR is performed using an automated thermal cycler that can rapidly heat and cool the tubes containing the reaction mixture. Briefly, the three major steps in PCR are as follows (Figure 2.5):

1) **Denaturation** at 94ºC: during denaturation, the double strand unwinds and is separated into two single strands of DNA. All enzymatic reactions stop at this high temperature. Subsequent rapid cooling of the reaction mix ensures the separated (denatured) strands do not re-anneal.

2) **Hybridisation** at 50-60ºC: the molecules are in perpetual motion and weak hydrogen bonds are constantly being formed and broken between different pieces of DNA. Stable bonds are formed between the base pairs on the oligonucleotide primers and complementary base pairs on the single stranded DNA templates. The large excess of the primers ensures that the two DNA strands of interest always bind to the primers rather than to each other.

3) **Extension** at 72ºC: DNA polymerase attaches to the small pieces of double-stranded DNA (template and primer) and adds deoxynucleotide triphosphate (dNTPs) to the primer in a 5' to 3'direction. The primers flank the target for amplification and face inwards. DNA synthesis therefore proceeds across the region between the primers. 72ºC is the optimal working temperature for the heat-stable DNA polymerase. Primers that have bound to DNA strands at positions with no exact nucleotide base pair match are

not extended because of weaker hydrogen bonding, which is unstable at higher temperatures.

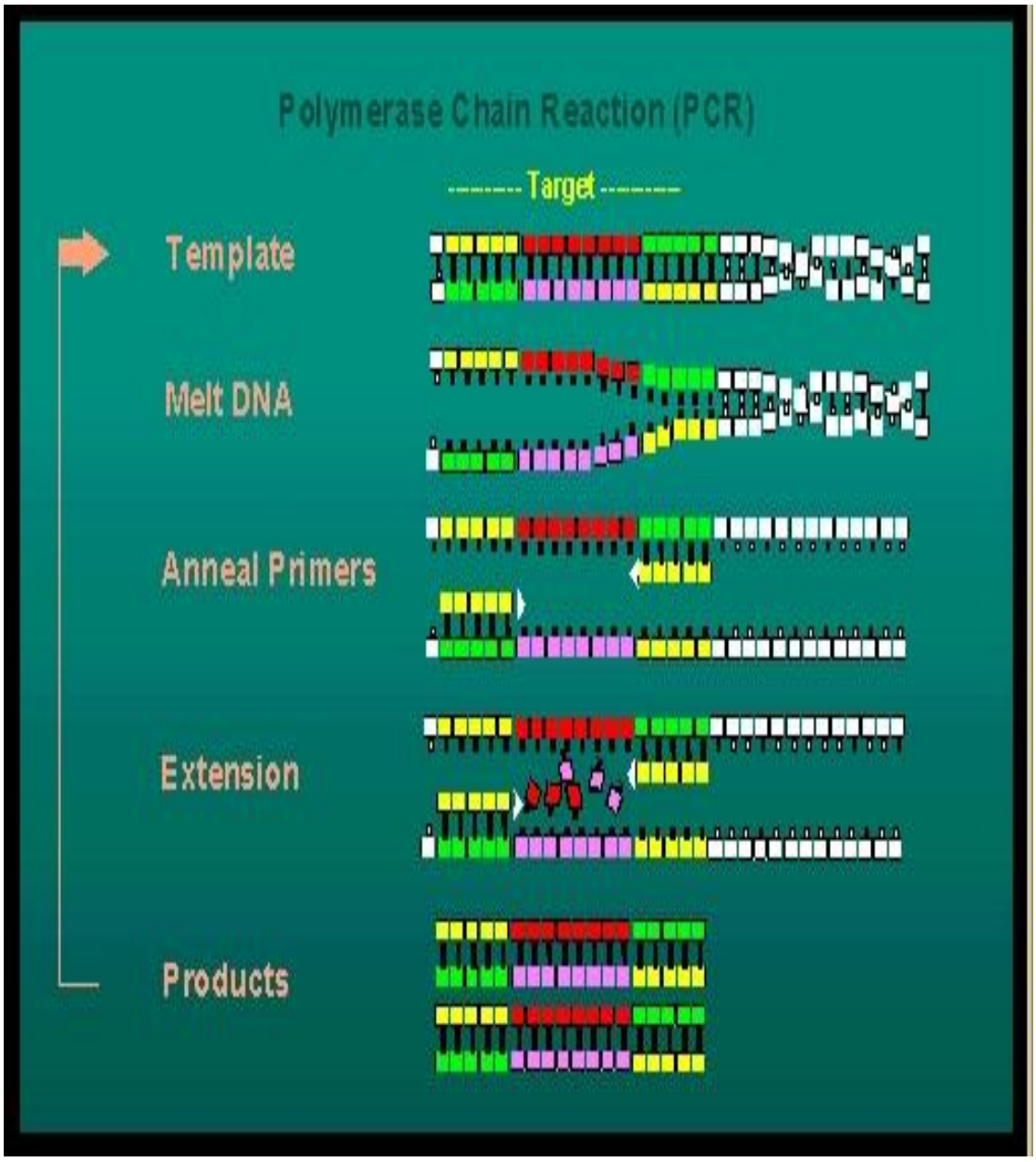


Figure 2.5: Schematic drawing of the PCR cycle. (1) Denaturing (Melting) at 94-96 °C. (2) Annealing at ~65 °C (3) Extension at 72 °C. Only one cycle is shown here. (Adapted from Chris Lee Go for your PCR, Optics Express 2009)

2.2.3.3.2 Methodology of PCR

1) Preparation of tissue and RNA isolation

Using methodology outlined by Chidlow et al (2005) and Nash and Osborne (1999), rats were killed and the globes were enucleated. The cornea was excised and the lens and vitreous removed. The sensory retina was then gently extracted from the eye cup and homogenized in 400 µl of ice cold TriReagent^{TM} with a sonicator (Burkard Scientific Ltd, Uxbridge, UK) for 13 seconds, and stored at -70°C until used. Once thawed, the homogenates were vortexed and centrifuged at 14000 rpm for 10 minutes to remove insoluble materials. The supernatant was then transferred to a new tube. After 5 minutes at room temperature to ensure complete dissociation of nucleoprotein complex, 80 µl of chloroform was added, the mixture was vortexed for 15-30 seconds and allowed to stand at room temperature for a further 10 minutes and then another 10 minutes at 4°C (on ice) before centrifugation at 14000 rpm for 15 minutes at 4°C. This produced separation of the sample into three phases: a top colourless aqueous phase containing RNA, an inter-phase containing DNA and a bottom organic phase containing protein. 150 µl of the RNA containing phase was carefully removed and transferred to a new 1.5ml tube. After 200 µl of isopropanol was mixed with the RNA-containing phase, the samples were vortexed and then placed on ice for 60 minutes, to help precipitate the RNA, followed by centrifugation at 14000 rpm for 15 minutes. The supernatant was then decanted off and the RNA pellet washed with 1ml of 75% ethanol (to resolubilise various salts), vortexed and centrifuged at 14000 rpm for 5 minutes at 4°C. After decanting the supernatant and removing excess liquid the pellet was allowed to dry at

room temperature for at least 15 minutes before resuspending in 50µl of diethylpyrocarbonate (DEPC) treated water and the samples were placed in an ice bath. The quality and quantity of RNA was determined using spectrophotometry, where 10 µl of solution was added to 990 µl of DEPC-treated water and the ratio of the optical density (OD) at 260 and 280nm wavelengths (OD_{260}/OD_{280}) was calculated. OD_{260} correlates with the amount of nucleic acid in the sample and the OD_{280} correlates with the protein content. Only samples with OD_{260}/OD_{280} ratios greater than 1.6 were considered appropriate for further analysis, and the volume of original sample containing 2 µg of RNA was determined using the following sequence of calculations:

1 OD_{260} unit = 40 µg of total RNA/ml

OD_{260} (measured) x 40 = [RNA] in original sample (µg/ml)

[RNA] in diluted sample (µg/ml) x 100 = [RNA] in original sample (µg/ml)

[RNA] in original sample (µg/ml)/1000 = [RNA] in original sample (µg/ µl)

Volume of sample containing 2 µg RNA = 2/[RNA] in original sample (µg/ µl)

The RNA sample was made up to a total volume of 7 µl using DEPC-treated water.

2) DNase treatment

Residual DNA was digested by incubating at 37°C for 10 minutes 2 µg of total RNA and the appropriate volume of DEPC-treated water with 0.5 µl RNasin (2U/ µl), 1.5 µl of DNase (0.05U/ µl), 1.0 µl reaction buffer (Sigma Chemical Co. Poole, UK) to give a total reaction mixture of 10 µl. 1.0 µl of stop solution (Sigma Chemical Co. Poole, UK) was then added to each tube and the mixture incubated for 10 minutes at 70°C in order to denature the DNase.

3) Reverse transcription

The synthesis of the first-strand of cDNA was performed in a 17 µl reaction mix at 42°C for 60 minutes. Each sample contained 11 µl aliquote from the DNase-treated RNA sample, 0.6 µl RNasin (24U), 1.0 µl oligo[dT]$_{30}$ (5ng), which attaches to the poly-A-tail of the mRNA in order to form the cDNA product, 0.4 µl dNTPs (0.6mM each dNTP) and 1 µl M-MLV reverse transcriptase (200U) and 3 µl reverse transcriptase buffer pH 8.3 (Sigma Chemical Co. Poole, UK). Following this incubation 133 µl of DEPC-treated water was added to each tube to give 150 µl of the final cDNA product.

4) PCR amplification of cDNA

Individual cDNA (2 µl of sample from the RT stage) were amplified in a reaction mixture containing 1 µl GeneAmp PCR buffer, 1.6 µl $MgCl_2$ (4mM), 0.8 µl dNTPs (200 µM each dNTP), 0.4 µl of both the sense and antisense primers (4ng/ µl each primer), 0.1 µl of 1U AmpliTaq Gold^{TM} and 3.7 µl of DEPC-treated water. PCR reactions were initiated by incubating tubes at 94°C for 10 minutes (a pre-PCR thermal requirement to active the AmpliTaq Gold^{TM}). Subsequently, a suitable number of cycles were used in order to amplify the samples within the linear phase while avoiding satuation. Each cycle was comprised of denaturation at 94°C for 12 seconds, hybridisation (annealing temperature) for 30 seconds, and extension at 72°C for 30 seconds, using a Hybaid PCR Sprint thermal cycler (Ashford, Middlesex, UK). The reaction was completed by a final extension at 72°C for 3 minutes. The appropriate number of cycles to be used for each

set of primers had been previously determined in the laboratory. Moreover, all of the PCR products yielded single bands corresponding to the expected size in base pairs and had previously been sequenced to ensure their validity. The number of cycles are shown in Table 2.2

5) Visualisation and analysis of the PCR product

PCR samples (10 µl) plus loading buffer (2 µl) were separated on a 1.2% agarose minigel. A fresh minigel was prepared each time by boiling 0.6g of agarose in 45ml DEPC-treated water and 5ml Tris-borate-ethylene-diamino-tetra-acetic acid (TBE) buffer. Ethidium bromide (5µl added to gel and running buffer) was used for DNA visualization under ultraviolet fluorescence. Digital images of the gel were taken and band densitometry performed using Labworks software (Ultra-Violet Products Ltd., Upland, CA). Sequences and annealing temperatures of each primer are shown in Table 2.1.

Primers	Sequences	Annealing temp (°C)
Caspase-3	GTTAACACGAGTGAGGATGTG ACTGGCTGCCCTCAGTTCCTGTGC	52
Caspase-8	ACTGGCTGCCCTCAAGTTCCTGTGC TCCCTCACCATTTCCTCTGGGCTGC	60
GAPDH	CATCAAGAAGGTGGTGAAGCAGG CCACCACCCTGTTGCTGTAGCCA	56
GFAP	ATTCCGCGCCTCTCCCTGTCTC GCTTCATCCGCCTCCTGTCTGT	55
NF-L	ATGCTCAGATCTCCGTGGAGATG GCTTCGCAGCTCATTCTCCAGTT	52
Opsin	CAGTGTTCATGTGGGATTGACT ATGATTGGGTTGTAGATGGAGG	52
Thy-1	CGCTTTATCAAGGTCCTTACTC GCGTTTTGAGATATTTGAAGGT	52

Table 2.1: Table showing the sequences of primers used throughout the animal study. Their product size (base pairs) and their accession number are located in the GenBank database, (*www.ncbi.nlm.nih.gov/Genbank/).*

Primer	Number of cycles	Annealing Temp(°C)
Caspase-3	26-28	52
Caspase-8	32-35	60
GAPDH	19-21	52
GFAP	26-29	55
NF-L	25-27	52
Opsin	17-19	52
Thy-1	24-25	52

Table 2.2: Cycle numbers for each set of primers and the optimum annealing temperature for each set of primers used in the PCR cycle.

. 2.2.4 Quantitative (Real Time) PCR studies for cell culture

Real time PCR (qPCR) was used to quantify the mRNA levels of Nrf-2, Bcl-2, HO-1, PARP, xCT, XIAP, nfkB and p-AKT in RGC-5 cells. Cells were harvested 24 hours after GB treatment and 48 hours for serum deprivation experiments. The protocol for RNA isolation were carried out using Trizol and Qiagen RNAeasy Columns. The Tmº for each primer is shown in Table 2.3.

1. **Total RNA Isolation Using Trizol and Qiagen RNAeasy Columns**

This protocol works well for preparing 20 to 40 µg of very clean, salt free, RNA. The RNA prepared from this protocol is ready for cDNA synthesis or further applications.

A. Materials for qPCR

1. RNase-free cell scraper
2. RNase-free 1.5 or 2.0 mL microfuge tubes
3. Microfuge
4. RNase-free pipette tips
5. Qiagen RNAeasy Mini Kit (Qiagen Catalogue No: 74204)
6. Qiagen RNase-Free DNase Set (Qiagen Catalogue No: 79254)
7. RNase free or DEPC-treated H_2O
8. Trizol (Invitrogen)

B. Procedure

1. Cells are harvested with a cell-scraper and centrifuged at 600 x g. The cell pellet is then resuspended in a small volume of PBS and 5-10 equivalent volumes of RNAlater is added. Samples then can be stored at 4ºC for up to 1 month.

2. 50 µl of Trizol is then added and mixed by vortexing and incubated for 5 minutes at room temperature with occasional vortex. 0.2ml of chloroform is then added to the Trizol and further vortexed for 15 seconds. This solution is then incubated for 1 minute at room temperature and then vortexed for further 15 seconds.

3. The solution is then centrifuged at 15000 x g for 10 minutes to separate phases. 300 µl is then removed from the top layer and added to 300 µl of Qiagen RLT buffer in a new tube. The rest of the top layer is then removed and freezed at -20 to serve as a backup of low initial yield.

4. 500 µl of 96-100% ethanol is then added to the sample and vortexed. Half of the sample (aprox. 700 µl) is then applied to a Qiagen spin column and placed in a 2 ml microfuge tube. This is then spun for 15 seconds at about 10000 rpm. The flow through is discarded and the procedure repeated with the second half of the sample.

5. DNase I incubation mix is then prepared (10 µl DNase I + 70 µl Buffer RDD) 350 µl Buffer RW1 is first added to the column, spun for 15 seconds at 10,000 rpm and the flow through discarded. 80 µl of the prepared DNase I incubation mix is the mixed directly onto the column membrane an incubated for 15 minutes. The column is then washed with 350 µl Buffer RW1, spun for 15 seconds at 10,000 rpm and the flow through discarded. This is repeated once more and then washed with 500 µl of Buffer RPE. The column is then spun for 15 seconds at 10,000 rpm and the flow through discarded. This is again repeated but for a period of 2 minutes.

6. The column is then placed in a new collection tube and centrifuged at full speed for 1 minute. The column is then placed in a new 1.5 ml collection tube and 30-50 µl of RNase-free water is then added directly to the spin column membrane. This is then incubated for 1-2 minutes on the bench and further centrifuged for 1 minute at 10,000 rpm to elute the RNA.

7. The RNA yield can then be quantified at this stage.

2. Turning mRNA into cDNA for qPCR analysis

For this process the reverse transcriptase-Superscript III is used.

1. The following reaction/sample initially prepared: 1µl of Oligo dT (50µM) + 1µl of dNTPs (10mM each) + 11µl of 10pg -5µg total RNA each.

2. The mixture is then heated to 65ºC for 5 minutes and cooled on ice for 1 minute. The mastermix for each reaction /sample is then prepared as follows:

   4 µl of 5x first strand buffer + 1µl 0.1M DTT+ 1µl RNaseOUT, +1µ Superscript III

3. The mastermix is then mixed by mixing up and down using a pipet and heated to 50ºC for 60 minutes which is then followed by inactivation at 70ºC for 15 minutes. The cDNAs can then be used immediately or stored at -20ºC.

3. Preparing cDNAs for qPCR analysis

The SybrGreen Mastermix protocol is employed for this process. Briefly the following reaction mixture is prepared:

10µl of SybrGreen mastermix + 0.5µl of forward primer (10µM) +0.5µl of reverse primer (10µM) + 8µl molecular biology grade water (RNase free water)

19 µl of the mastermix is then pipette into each well and 1 µl of sample cDNA is added into each well.

4. Determining melting temperature for primers:

1. Each primer is run with a single reaction to determine the melting temperature as recorded by the qPCR program.

2. The temperature for measuring fluorescence of each primer is adjusted by subtracting 2.5ºC from the melting temperature.

3. From these data, the melting temperature of primers is determined to run qPCR analysis at the same time on the same plate.

Primers	Sequences	Melting Temp (°C)
ARP	Fwd: CGACCTGGAAGTCCAACTAC Rev: ATCTGCTGCATCTGCTTG	76.1
xCT	Fwd: CCCAAGGGCATACTCCAGAC Rev: CTCCGCACTGATGGTGGTAAA	79.9
XIAP	Fwd: CCCAAATTCAACAAACTC Rev: GACTTGACTCATCCTCCG	75.8
p-Akt	Fwd: TGGAGGACAACGACTACGG Rev: ACACGATGTTGGCAAAGAA	84.4
PKCα	Fwd: CCACGGCGTCTCAGGACGTGG Rev: TTGCTCCTGGGGTCGTCAGTGT	82.2
Nrf-2	Fwd: TTCTTTCAGCAGCATCCTCTCCAC Rev: ACAGCCTTCAATAGTCCCGTCCAG	79.9
HO-1	Fwd: CAAGCCGAGAATGCTGAGTTCATG Rev: GCAAGGGATGATTTCCTGCCAG	81.7
NfkB	Fwd: ATTCCGGGCAGTGACGCGAC Rev: GCCCACGGCGCGCTAAAGTA	83.8
Bcl-2	Fwd: TGCACCTGACGCCCTTCAC Rev: AGACAGCCAGGAGAAATCAAACAG	82.2

Table 2.3: Table showing the sequences and melting temperature of primers used throughout the cell culture study.

2.2.5. Electrophoresis/SDS-PAGE

2.2.5. Basic principles and methodology of electrophoresis/SDS-PAGE

Electrophresis is the migration of charged molecules in solution in response to an electric field (Figure 2.4). The rate of this migration depends on the strength of the field, size and shape of the molecules, viscosity and the temperature of the medium in which the molecules are moving. Typically, the samples are run in a support matrix such as agarose or polyacrylamide gel. The matrix inhibits convective mixing caused by heating and provides a visual record of the electrophoretic run.

Both the agarose and polyacrylamide matrices, being porous gels, provide a means of separating molecules by size. A porous gel can act as a sieve by retarding or in certain cases completely obstructing the movement of large macromolecules, while allowing the movement of smaller molecules.

Sodium dodecyl sulphate (SDS) is an anionic detergent, which denatures proteins by wrapping around the polypeptide backbone by binding to proteins in a mass ratio of 1.4:1. This thereby confers a negative charge to the polypeptide, which is directly proportional to its length. The denatured polypeptides become rods surrounded by a negatively charged cloud with equal charge densities per unit length. The denaturing process of SDS-PAGE separation allows migration of proteins not by intrinsic electrical charge but by molecular weight.

1. **Preparations of samples for electrophoresis**

RGC-5 cells were grown in 6 well plates and were harvested from the well using sterile

cell scrapers. The cells were centrifuged (5 minutes at 100 x g) and sonicated for 30 seconds in 100 µl homogenization buffer/protease inhibitors solution (20 mM Tris HCl, pH 7.4, containing 2 mM EDTA, 0.5 mM EGTA, 1% SDS, 0.1 mM phenyl-methylsulphonyl fluoride, 50 µg/ml aprotinin, 50 µg/ml leupeptin, and 50 µg/ml pepstatin A), using an ultrasonic tissue disrupter (Burkard Scientific Ltd., Uxbridge, UK). Subsequently, 100 µl of sample buffer (62.5 mM Tris-HCl, pH 7.4, containing 4% SDS, 10% glycerol, 10% β-mercaptoethanol and 0.002% bromophenol blue to colour the sample) was added. The samples were boiled for 3 minutes and an aliquot was analyzed for total protein content using a bicinchoninic acid protein kit (Sigma-Aldrich, St. Louis, MO).

For animal tissues, after enucleation from adult Wistar rats, retinas and optic nerves (6-8 mm long) were extracted and placed into 0.4 ml of Tri Reagent®. Tissues were sonicated and centrifuged, with the resulting supernatant placed into a new sterile tube. Chloroform was then added to the sample, and the sample was left at room temperature and on ice for equal measures of time. The samples were then centrifuged at 4ºC at for 10 minutes, separating the solution into three layers; an aqueous phase of RNA, an interphase containing DNA and an organic phase that contained proteins. RNA was carefully removed for RT-PCR analysis and after the removal of RNA, 100% ETOH was added to the tubes and mixed by inversion. The tubes were then centrifuged, allowing the DNA to form into a pellet (which was then discarded) and the supernatant removed to new tubes, to which isopropanol was added and kept at room temperature for 20 minutes. The samples were then centrifuged at 4ºC for 15 minutes to form a protein pellet. The supernatant was then discarded and the pellet was washed 3 times with a 0.3 M guanidine solution (containing 95% ETOH), during each wash the samples

were stored in the wash solution for 20 minutes at room temperature and centrifuged (9600 rpm) for 5 minutes at 4ºC. The pellets then had a further wash with 100% ETOH, which was aspirated off and the samples were left to dry before being placed into a homogenization buffer/protease inhibitors solution (20 mM Tris HCl, pH 7.4, containing 2 mM EDTA, 0.5 mM EGTA, 1% SDS, 0.1 mM phenyl-methylsulphonyl fluoride, 50 μg/ml aprotinin, 50 μg/ml leupeptin, and 50 μg/ml pepstatin A) until completely dissolved. Once dissolved an equal volume of sample buffer (62.5 mM Tris-HCl; pH 7.4, containing 10% glycerol, 10% β-mercaptoethanol, 4% SDS and 0.002% bromophenol blue) was added to the samples, which were then heated to boiling point for 3 minutes. The samples were then run on SDS-PAGE prior to immunoblotting.

2. SDS-PAGE

Electrophoresis was performed according to the method described in previous studies (Laemmli 1970) and was performed on 1.5 mm thick, 10% polyacrylamide gels containing 1% SDS in 360 mM Tris-HCl. The gel was polymerized with the addition of ammonium persulphate at a concentration of 0.1% (w/v), with 0.005% (v/v) N,N,N',N',- tertramethyl-ethylenediamine (TEMED). Samples (15 μl) were loaded into each well, of a 4.5% polyacrylamide stacking gel (containing 4% acrylamide, 0.1% SDS, 0.045% ammonium persulphate, 0.15% TEMED in 0.5 M Tris-HCl, pH 6.8) that was set above the resolving gel. The gels were run at a constant 30 mA for 2-3 hours.

2.2.6. Western Blotting

2.2.6.1 Methodology

The electrophoretic transfer of the proteins from the gel onto nitrocellulose was performed according to the method described by Towbin et al. (Towbin et al. 1979). The transfer was run overnight at 30 mA, with electrodes that were 10 cm apart. Following transfer of the proteins, the nitrocellulose was washed in Tris buffered saline (TBS; 10 mM Tris-HCl; Ph 7.4, containing 140 mM of NaCl) and then 10-15 minutes in TBS containing 0.1% Tween-20 (TBS-T).

Selected antibodies specific for immunoprobing were diluted accordingly in a volume of 2 ml of TBS-T. The appropriate nitrocellulose blots were then incubated with the antibody for a period of 3 hours at room temperature and were rotated continuously. Primary antibodies for immunostaining were diluted as follows: actin, 1:2000; GFAP, 1:2000; NF-L, 1:2000; tubulin, 1:2000; cleaved caspase-3, 1:500; cleaved PARP,. Other antibodies used for cell culture studies include rabbit anti-Nrf2 1:500; rabbit anti HO-1 1:500; rabbit anti-NfkB 1:500; mouse anti PKCα 1:500; mouse anti-PARP 1: 500; mouse anti Bcl-2 1:500; mouse anti-XIAP 1:1000; mouse anti-actin 1:2000.
The blots were then given a series of washes with TBS-T, high salt Tris buffer (HST; 10 mM Tris-HCl; pH 7.4, that contained 1 M NaCl and 0.5% Tween-20) and with TBS-T, for equal times of 10 minutes. The blots were then incubated for 90 minutes with a conjugated secondary antibody, specific to the primary antibody that was prepared in HST with a dilution of 1:2000 to 1:1000. After incubation with the secondary antibody the blots were washed with TBS-T, HST and a final wash with TBS-T for equal times of 10 minutes prior to developing.

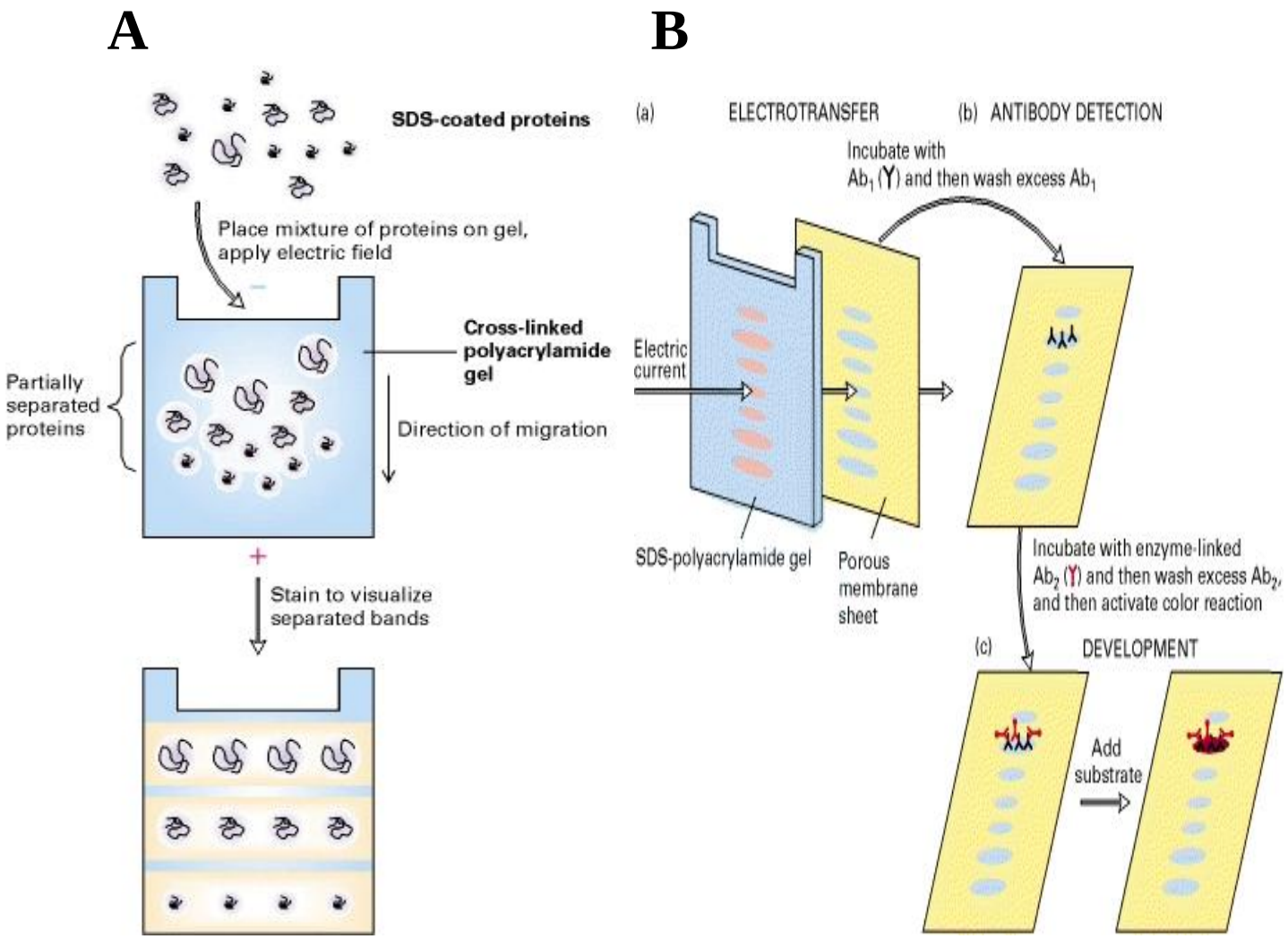


Figure 2.4: The detection of protein using Western blotting. SDS coated protein (A) is subjected to electrophoresis on SDS polyacrylamide gel and electroblotting transfers the separated proteins from the gel to the nitrocellulose membrane. Membrane with protein bands (B) is incubated with primary and secondary antibody. The visualization of the antibody bound proteins is performed by enzymatic reaction (A,B). (Adapted from http://www.ncbi.nlm.nih.gov/books/NBK21589/)

The nitrocellulose blots were developed in a 0.016% (w/v) solution of 3-amino-9-ethylcarbazole (AEC) in 50 mM sodium acetate (pH 5) that contained a 0.05% (w/v) Tween-20 and 0.03% H_2O_2. To stop the colour from developing further, 0.05% sodium azide was added to the solution. The blots were then scanned into the computer and analysed using Labworks software (Ultra-Violet Products Ltd., Upland, CA) and optical density readings were taken of each of the bands. The ratio of the densitometric reading of the protein of interest was expressed as a percentage ratio of the control protein (actin). To ensure consistency of the readings the nitrocellulose blots were divided to allow for staining of actin, allowing for actin to act as an internal standard.

2.2.7. Lipid Peroxidation Assay

1. Preparation of rat brain homogenate

Adult Wistar rats were killed by cervical dislocation and subsequent decapitation. The homogenate from a single animal was used for each set of lipid peroxidation experiments and was used immediately after decapitation as storage/freezing of the brain was shown to affect the lipid peroxidation control value. The cerebral tissue (the whole brain) was then removed from the rats and was homogenized in 10 volumes of ice-cold 0.9% saline (pH 7.0), by a motor-driven poly-tetrafluoroethylene-glass homogenizer. The homogenate was then centrifuged at 1000 x g for 10 minutes at 4℃, and 0.5 ml of the resulting low-speed supernatant was used for assaying lipid peroxidation. The cerebral homogenate from a single animal was used for each set of experiments.

2. Incubation with drug and initiation of lipid peroxidation

0.4 ml of 0.9% saline (pH 7.0) containing 100 µl of a defined concentration of drug was added to each of the 0.5 ml rat brain/rat retinal/bovine retinal samples followed by a pre-incubation period of 10 minutes at 37°C. Lipid peroxidation was then initiated via the addition of 100 µl of SNP (at 10µM concentrations). Buffer was substituted in the place of SNP with control samples. The process was terminated after 45 minutes of incubation at 37°C by placing tubes on ice. The degree of lipid peroxidation in each sample was determined by measurement of thiobarbituric acid species (TBARS).

3. Measurement of Thiobarbituric Acid Reactive Species (TBARS)

TBARS are the coloured products resulting from the reaction of thiobarbituric acid with compounds formed during lipid peroxidation such as malondialdehyde. TBARS were determined as described by Ohkawa et al. (Ohkawa et al. 1979), with some modifications (Melena and Osborne 2003), by measuring colour products resulting from the reaction of thiobarbituric acid with compounds formed during lipid peroxidation such as malondialdehyde.

The colour reaction was developed by the sequential addition of 0.2 ml of 8.1% wt/vol sodium dodecyl sulphate (1.5ml of 20% v/v acetic acid (pH 3.5) and 1.5 ml of 0.8% w/v thiobarbituric acid. This mixture was incubated for 30 minutes in a boiling water bath. After cooling with tap water, the mixture was then transferred into centrifuge tubes (Sarstedt, Leicester, UK). 1.5 ml of n-butanol:pyridine (15:1 v/v) was added and vortexed several times, the reaction mixture was then centrifuged at 2000 x g for 10 minutes at 4°C. The mixture was then left at room temperature for 10 minutes before

measurements began. The organic layer (1 ml) was carefully removed from the top of the tube and placed into a clean cuvette. Absorbance of the organic layer was measured at a fixed wavelength of 532 nm, with the amount of TBARS being determined from using a standard curve of the malondialdehyde derivative 1,1,3,3-tetraethoxypropane (at 1–30 nanomoles). All measurements were standardised to a cuvette filled with 1 ml of distilled water.

2.2.8. Measurement of protein-bicinchoninic protein assay kit assay

Protein concentration was determined using a bicinchoninic protein assay kit (Sigma) using BSA (bovine serum albumin) as a standard. This was to ensure the protein content of the samples were comparable to one another. Briefly, 50 µl total volume of sample/BSA with distilled water was added to 0.95ml of bicinchoninic acid and 4% copper sulphate solution (1:50 dilution of copper sulphate in bicinchoninic acid). The solution was then incubated in a water bath for 30 minutes at 37℃, which was then removed and allowed to cool. Measurements were then taken at a fixed wavelength of 562 nm.

2.2.9. Caspase-3 activity assay

For caspase-3 activity assay the colometric Caspase 3 Assay Kit (Product Code: CASP-3-C) from Sigma was used according to manufaturer's protocol. The caspase 3 colorimetric assay is based on the hydrolysis of the peptide substrate acetyl-Asp-Glu-Val-Asp p-nitroanilide (Ac-DEVD-pNA) by caspase 3, resulting in the release of the p-nitroaniline (pNA) moiety. P Nitroaniline has a high absorbance at 405 nm (εmM =

10.5). The concentration of the pNA released from the substrate is calculated from the absorbance values at 405 nm or from a calibration curve prepared with defined pNA solutions.

2.2.10. Statistical analysis

Student's t-test and one-way ANOVA were used to compare two or more independent means, respectively. The number determined involving each experimental condition was kept approximately equal to prevent the influence of unequal variances. Data is expressed as mean ± standard error of the mean (SEM) for the indicated number of experiments. Statistical significance was determined by ANOVA was by using SPSS for Windows, version 12.0 (SPSS Inc, Chicago, IL, USA). Student's independent (unpaired) t-test was used to compare two independent means, such as the two left eyes of two different animals. Student's paired t-test was used to compare two correlated means, such as the left and the right eye of the same animal. In the case of Student's t-test statistical evaluations were performed using Sigma Plot 10. For western blot and PCR bands the data was taken from the integrated optical densitometric (IOD) reading of the protein/DNA band of interest and was expressed as a percentage of the control protein/gene product. In all experiments a p-value of <0.05 was generally considered statistically significant (Field 2003).

Chapter 3

Influence of sulbutiamine and NAC on RGC-5 cell death induced by GB or serum deprivation



Chapter Abstract

The protective effects of different sulphur-containing drugs, sulbutiamine and NAC, on RGC-5 survival were investigated in relation to two different insults - serum deprivation and GB exposure. The neuroprotective mechanisms of each drug were investigated, including their effects on GSH stimulation. RGC-5 cells grown in culture were exposed to an insult of GB 5 mM for 24h or serum deprivation for 48h. NAC or sulbutiamine at different concentrations, or vehicle, was added to the cultures over 24h or 48h. ROS generation was measured using a dye dihydroethidium. Cell death was measured by use of resazurin-assays (RRA) and evidence for apoptosis was obtained with an *APOP*ercentage™ procedure. GSH, catalase and GST were also determined using different fluorescent procedure. The effects of NAC and sulbutiamine on the oxidative stress related transcription factor Nrf-2 levels and its dependent phase II enzyme HO-1 were determined using western blot analysis and quantitative-PCR.

NAC and sulbutiamine dose-dependently attenuated serum deprivation-induced RGC-5 death by a caspase-3 dependent apoptosis. However NAC, but not sulbutiamine, attenuated GB-induced cell death by a non-caspase-3 dependent mechanism. NAC and sulbutiamine both independently stimulated the production of GSH and GST but scavenged different types of ROS with different efficacy. Moreover only sulbutiamine stimulated catalase and significantly increased Nrf-2 and HO-1 levels.

NAC and sulbutiamine significantly blunts oxidative-induced death to RGC-5 cells induced by serum deprivation insult but only NAC attenuate the effects of GB. Evidence is also provided to show that NAC and sulbutiamine increases intracellular GSH, GST levels and scavenge different types of ROS with different efficacy. The neuroprotective action of different sulphur compounds NAC and sulbutiamine is therefore different and is not solely caused by an action on GSH.

3.1. Introduction

3.1.1. Aim

Compare how NAC and sulbutamine influence the survival of RGC-5 cells in cultures subjected to two different insults, one being serum withdrawal and the other GB.

3.1.2. Brief overview

In a neurodegenerative disease like glaucoma, oxidative stress caused by ROS overproduction is thought to play an important role in causing retinal ganglion cell death (Whitmore et al. 2005; Quigley 1999; Weinreb and Khaw 2004). ROS over production also appears to play an important role in the pathogenesis of other progressive neurodegenerative disorders such as Alzheimer's disease, Parkinson's disease, amyotrophic lateral sclerosis, multiple sclerosis and acute syndromes of neurodegeneration, such as ischaemic and hemorrhagic stroke (Rothstein 1996; Sen 1998; Butterfield et al. 2001; Finkel and Holbrook 2000; Gilgun-Sherki et al. 2001, Gilgun-Sherki et al.2002).

One cause for an elevation of intracellular ROS is thought to stem from an elevation of extracellular glutamate (Maher and Henneken 2005). Excess extracellular glutamate can affect the influx of cystine into cells so reducing the rate of intracellular GSH formation (Maher and Henneken 2005) and in the process undermining a major cellular defense mechanism to maintain homeostatic levels of ROS. Mitochondria are a major target of ROS attack and at the same time is a source for ROS production such as the superoxide anion (Brand et al. 2004). Intracellular

ROS levels in healthy cells therefore exist in a balanced redox state and over production as a consequence of an underlying pathogenic state leads to breakdown of homeostasis. Importantly, excess intracellular ROS can lead to a depletion of sulphur containing thiols (Maher and Henneken 2005). In addition cellular components such as catalase, superoxide dismutase, the thiol redox systems GSH/GSSG (glutathione/oxidized glutathione) and thioredoxin function to minimize the harmful effects ROS might have on the cell (Powers and Jackson 2008).

The molecular mechanism of how endogenous cellular antioxidants are generated to combat any oxidative stress that results in neuronal death remains a matter of speculation (Ikonimidou et al. 2001). However, a number of signaling proteins have been implicated to be involved in the process. These include, for example, the transcription factor Nrf-2 which induces antioxidant enzymes and involves activation through the antioxidant-responsive element (ARE) (Lim et al. 2007). In this case, electrophilic agents including ROS induce a set of genes encoding Phase-II enzymes, including HO-1, NADPH, quinine oxidoreductase-1 and γ-glutamyl cysteine ligase (γ-GCL). These enzymes provide efficient cytoprotection, in part, by regulating the intracellular redox state (Lim et al. 2007). For example, Sun et al (2010) showed that elevation of HO-1 throughout the retina caused by cobalt protoporphyrin attenuated the negative effects of ischemia-reperfussion injury to the retina.

The enzyme catalase is another important enzyme that decomposes hydrogen peroxide (H_2O_2) to water and molecular oxygen and functions as a natural antioxidant by protecting cells against oxidative damage to proteins, lipids and nucleic acids (Greenwald 1990; Putnam et al. 2000). This enzyme has also been studied especially in its response to ROS generation, gene expression and apoptosis (Hyoudou et al. 2007; Islam et al. 1997). However, its exact physiological function and regulation is not yet fully understood (Doronicheva et al. 2007). Catalase has not been specifically determined in RGCs or photoreceptors, but catalase levels are highest in the RPE where it appears to suppress the generation of lipid peroxides (Miceli et al. 1994).

The processes by which sulphur containing compounds overcome the negative effects of excess intracellular ROS remains poorly understood. Sulbutiamine and NAC are both well known sulphur containing antioxidants and used for decades as adjuvants in the treatment of certain human diseases. NAC is a well known amino thiol molecule with clinical uses ranging from mucolytic therapy for patients with inspissated respiratory secretions and dry eyes to standard treatment of acetaminophen overdoses (Perry & Shannon 1998) and more recently for the prevention of contrast-induced nephropathy (Walker et al. 2003; Tepel et al. 2000). NAC partly acts as a free radical scavenger as well as a GSH precursor to increase intracellular antioxidant capacity and hence protect cells against ROS (Tredger 2000; Zafarullah 2003). However, it is also thought to work through other mechanisms. It has also been studied especially in its response to ROS generation, gene expression and apoptosis and its efficacy may be secondary to detoxification of ROS, local vasoactive properties and/or interference with gene expression

(Malorni et al. 1993; Heussler et al. 1999; Talley et al. 1995; Cossarizza et al. 1995). Furthermore, cysteine (a structural component of NAC), methionine and homocysteine, act as important substrates for the metabolic processes in transufuration pathway. The enzymes CBS and CSE in the transulfuration pathway, are essential in the production of H_2S (Levonen et al. 2000) a potent gaseous neuromodulator. Importantly it has been shown that homocysteine is critically involved in the pathogenesis of certain neurodegenerative disorders (Ho et al. 2001). This is believed partly to be due to its role in the generation of ROS including H_2O_2 and O^{2-} (Heinecke et al. 1987, Levonen et al. 2000). It has also been shown that methionine induced ROS production was effectively blocked by NAC in brain endothelial cells (Tyagi et al. 2009). Also when a H_2S donor was combined with substances like NAC, it synergistically increased their antioxidant effect to scavenge ROS and protect vascular endothelium from methionine induced cytotoxicity (Tyagi et al. 2009).

NAC has also been shown to inhibit cortical neuronal injury independent of an effect on meningeal inflammation (Auer et al. 2000). NAC thus has potential benefits as a neuroprotectant in acute brain injury caused by for example bacterial meningitis. It has also been reported that NAC modulates the gene expression of the vasoconstrictor endothelin-1 (ET-1) to enhance cerebral blood flow in acute brain injury as well as being able to reduce ROS (Sury et al. 2006). Conflicting data however show that NAC only poorly penetrates the blood-brain barrier (McLellan et al. 1995), and has been reported not to prevent the loss of cortical GSH in the infant rat model of pneumococcal meningitis (Christen et al. 2001). Various clinical studies show NAC

to have a high safety profile and can be used for the treatment of various neurological conditions such as depression (Berk et al. 2008) Furthermore, in a controlled trial of patients with symptoms of Alzheimer's disease and having had treatment with NAC for 3-6 months showed improvement on nearly every outcome of measure, although significant difference were obtained only for a subset of cognition tasks (Adair 2001).

Sulbutiamine also has beneficial effects in the treatment of Alzheimer's disease as it enhances cholinergic and glutamatergic transmission, mainly in hippocampus and prefrontal cortex (Ollat et al. 2007). A multicentric, randomized and double-blind trial which evaluated the effects of the association of sulbutiamine to an anticholinesterasic drug in cognitive functions in patients with Alzheimer's disease at an early stage (episodic memory, working memory, executive functions, attention), concluded that it can be an effective adjuvant to treatment in early stage and moderate Alzheimer's disease by anticholinesterasic drugs (Ollat et al. 2007). Interestingly it has been previously reported that thiamine blood levels have been found to be lower in glaucoma patients in comparison to controls (Asregadoo et al. 1979). Also, Suzuki et al. (1997) have suggested a link between thiamine deficiency and optic neuropathy (Suzuki et al. 1997). Intravenous administration of thiamine in a patient with optic and peripheral neuropathy produced rapid improvement in visual acuity with absence of further episodes of weakness/ataxia (Sedel et al. 2008). The challenge with using thiamine however is its poor lipophilicity. A synthetic derivative of thiamine, sulbutiamine was hence synthesized in order to overcome the poor oral bioavailability of thiamine. Sulbutiamine as opposed to thiamine is therefore able to readily cross the blood-brain barrier (BBB) (Bettendorff et al.1990). Sulbutiamine has also been shown to

elicit several neuro-modulating functions including memory improvement in mice and enhancing cognitive functions (Micheau 1985).

Sulbutiamine is clinically approved to treat asthenia (Shah 2003) and is the most bio-available anti-asthenic compound as it efficiently crosses the blood-brain barrier to act on certain regions of the brain which is thought to be affected by asthenia (Van Reeth 1999). In addition to these beneficial effects, it has been also used in the treatment of erectile dysfunction (Dmitriev et al. 2005). At therapeutic dosages, sulbutiamine has few reported adverse effects, though it may interfere with the therapeutic outcome of bipolar disorder (Douzenis et al. 2006). However the exact mechanism of sulbutiamine is still not clearly understood and its influence in oxidative stress conditions is not known. However thiamine deficiency is known to affect cellular metabolism via the oxidoreductase pathway by decreasing NADPH levels which eventually leads to GSH depletion (Bruce et al. 2003). Thiamine has also been shown to stimulate GSH activity and attenuate oxidative insults in certain defined conditions (Portari et al. 2008, Shangari et al. 2007).

The potential use of NAC and sulbutiamine as neuroprotectants in attenuating the gradual loss of ganglion cells in a disease such as glaucoma remains to be investigated. In the present study the effectiveness of sulbutiamine and NAC at attenuating the detrimental consequence of insults to RGC-5 cells in culture was therefore investigated. The main aim of the present study was to deduce whether these two selected sulfurated compounds are able to reduce cellular oxidative stress and improve neuronal survival *in vitro***.**

3.1.3 Summary of results

The results presented in this section are summarised as follows:

a) Serum deprivation and GB reduced the amount of viable RGC-5 cells in culture in a time-dependent manner evidenced by reduced mitochondrial dehydrogenase activity (RRA assay), cellular membrane alteration, DNA breakdown, GSH depletion and the generation of ROS. GB caused a greater reduction in cell viability, DNA breakdown, GSH depletion and ROS generation.
b) NAC attenuated the negative effects of GB more effectively than the influence of serum deprivation on RGC-5 cells. In contrast, sulbutiamine significantly attenuated the effect of serum deprivation but not that caused by GB.
c) GB induced cell death of RGC-5 cells without any change in caspase-3 levels. The negative effect of GB was attenuated by necrostatin-1, an inhibitor of necroptopsis.
d) Serum deprivation induced cell death of RGC-5 cells is blunted by sulbutiamine. The death process is caspase-dependent, involving caspase-3 and is unaffected by necrostatin-1.

3.2 Results

3.2.1 Cell viability results and apoptosis detection show sulbutiamine is more effective than NAC in attenuating serum deprivation-induced cell death however is ineffective in GB-induced cell death

By use of RRA, it was shown that 48 hours serum deprivation reduced the viability of RGC-5 cells by 40% (Figure 3.1A) and 24 hours of 5mM GB resulted in an approximate 45% decrease in cell viability (Figure 3.1B).

The effect of 5mM GB was significantly nullified by inclusion of NAC (1mM and 5mM) (Figure 3.1D). Moreover, NAC at 5mM, but not at 1mM nullified the detrimental effect of 48 hours serum deprivation (Figure 3.1C). Sulbutiamine (Sulb) at both 50µM and 100µM significantly increased cell viability by approximately 18% and 28% respectively relative to serum deprivation (Figure 3.1C) but did not influence cultures exposed to 5mM GB (Figure 3.1D).

Cytological evidence demonstrated the negative effects of exposure of RGC-5 cells to 5mM GB for 24h or serum deprivation for 48h. Cell death was revealed by FDA/PI staining where live cells appear green and dead cells appear red (Figure 3.2E-H and Q-T). Furthermore the death process was by apoptosis which was revealed by use of the Apopercentage^{TM} dye (Figure 3.2A-D and M-P) and Hoechst staining (Figure 3.2I-L and U-X). The negative effect on cell survival induced by 5mM GB in terms of cell death (F), apoptosis (B and G) was unaffected by the inclusion of 100µM sulbutiamine (C,G,K) while NAC (5mM) significantly blunted the negative effects of GB (D,H,L). Moreover, the detrimental effect of serum deprivation for 48h in terms of

cell death (R), apoptosis (N and V) was more efficaciously blunted by the inclusion of 100μM sulbutiamine (O,S,W) than 5mM NAC (P,T,X).

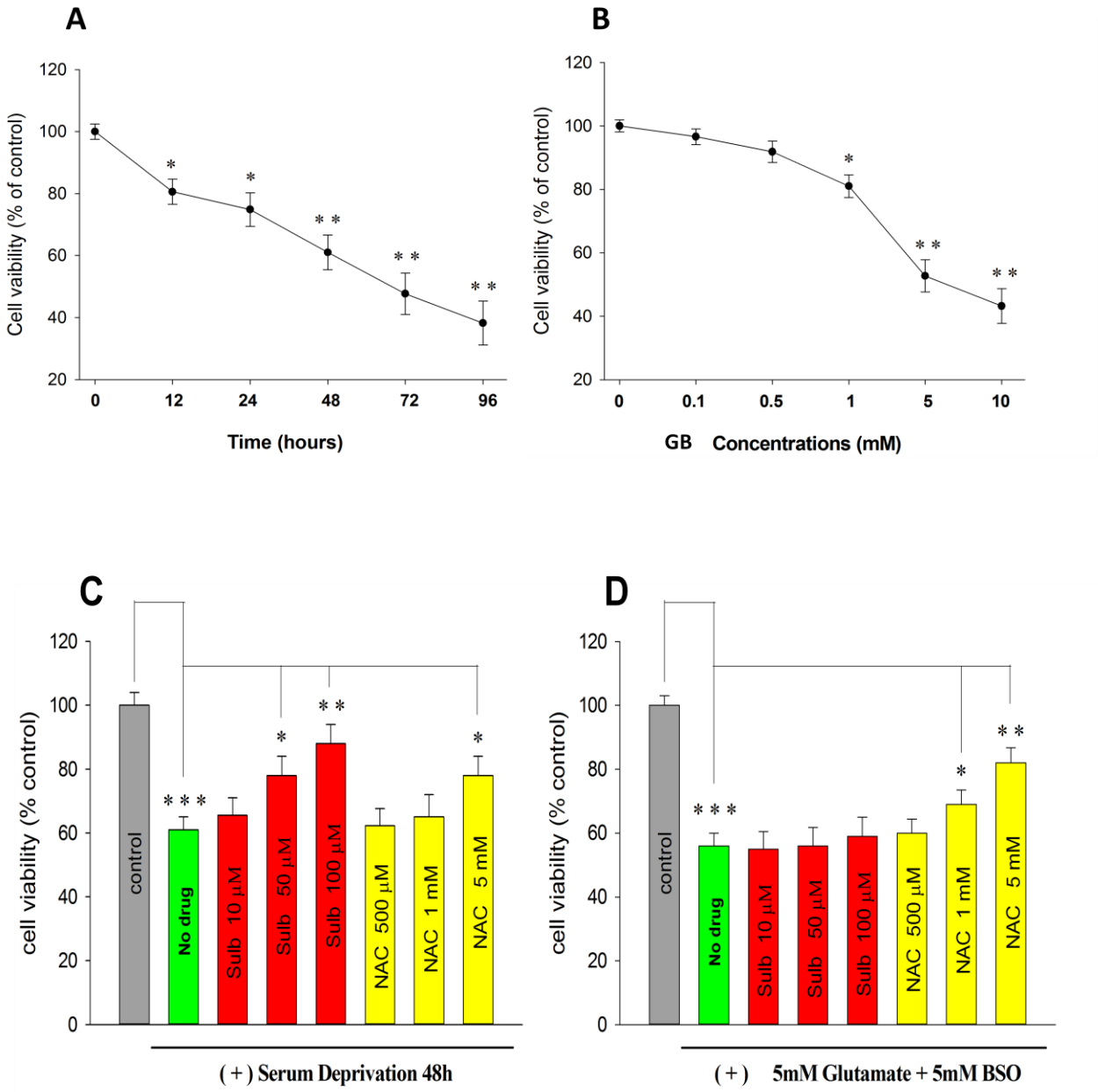
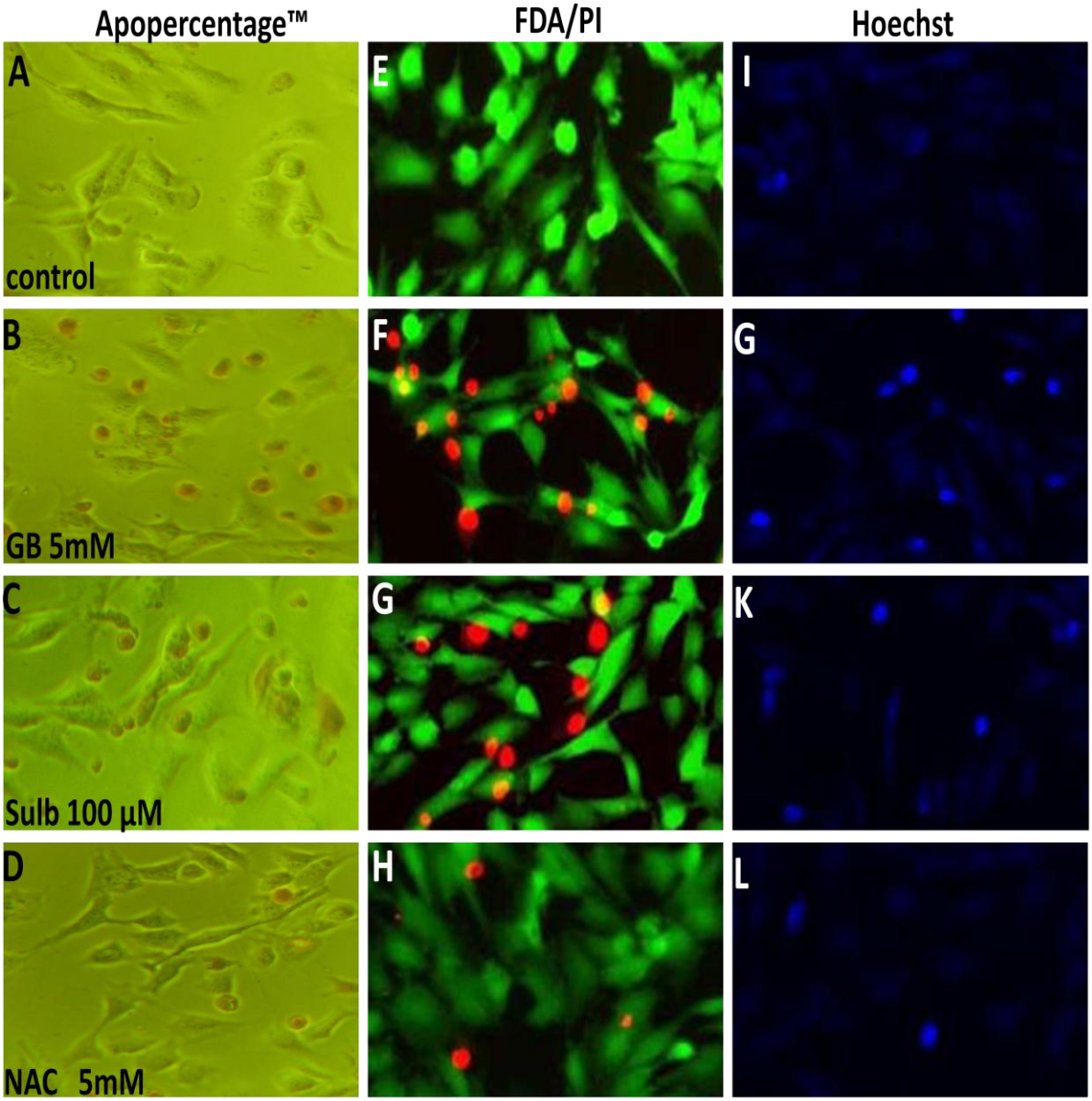


Figure 3.1: Cell viability study was determined by use of Resazurin Reduction Assay (RRA). (A) Serum deprivation caused a time-dependent reduction in cell viability and reduced the cell viability by 40% after 48 hours. (B) GB caused a dose-dependent reduction in cell viability and resulted in an approximate 45% decrease in cell viability at concentration of 5mM. (C) The negative effect of serum deprivation (48h) was attenuated by sulbutiamine (50-100µM) and NAC 5mM. (D) The detrimental effect of 5mM GB (24h) was blunted by NAC (1 and 5mM) but not by sulbutiamine (10-100µM). Data are expressed as mean ± SEM (n=5), *p < 0.05, **p < 0.01 and ***p < 0.001 vs control, Bonferroni test.


Apopercentage™
FDA/PI
Hoechst
A
control
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GB 5mM
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Sulb 100 µM
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NAC 5mM
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L

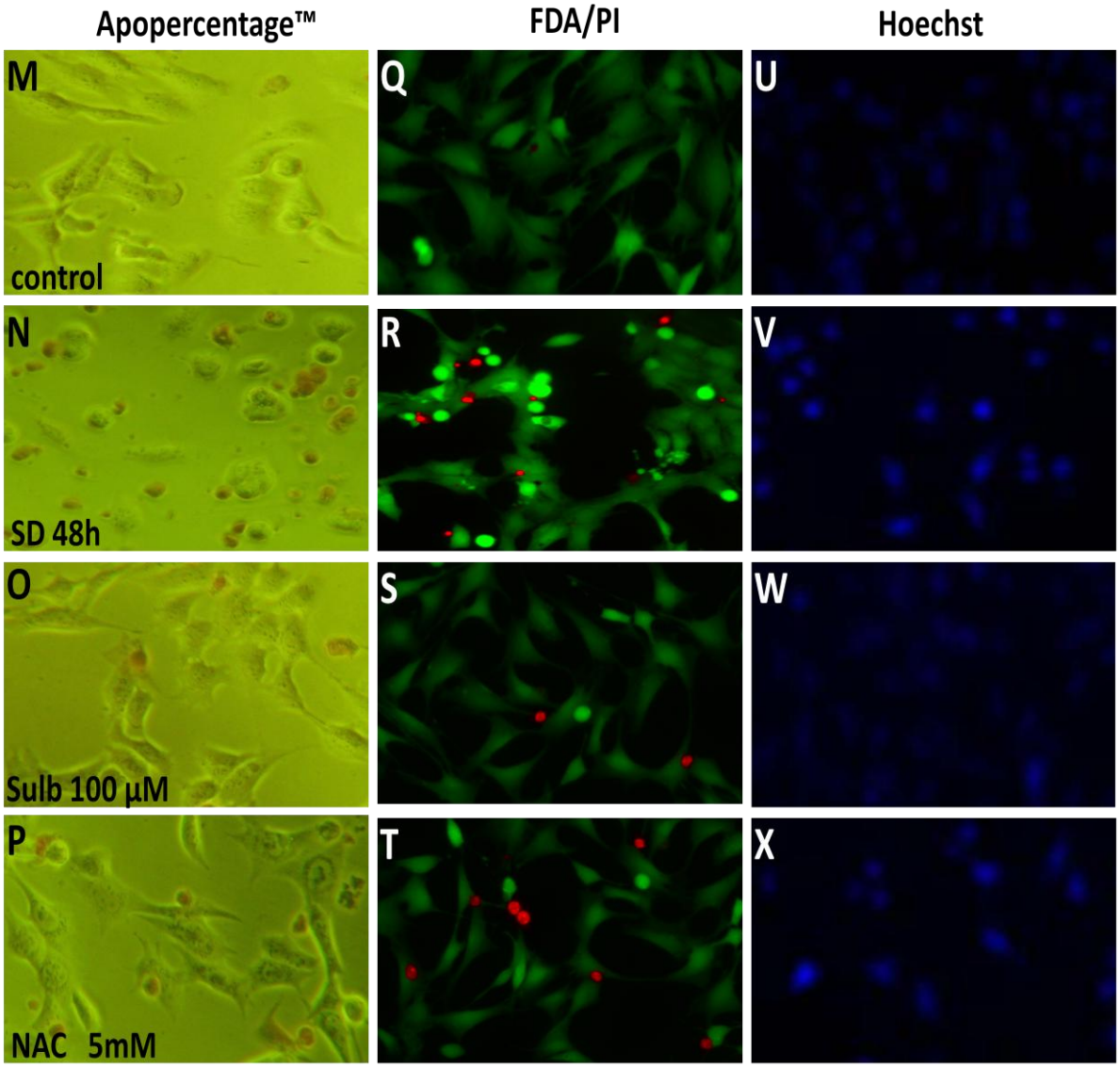


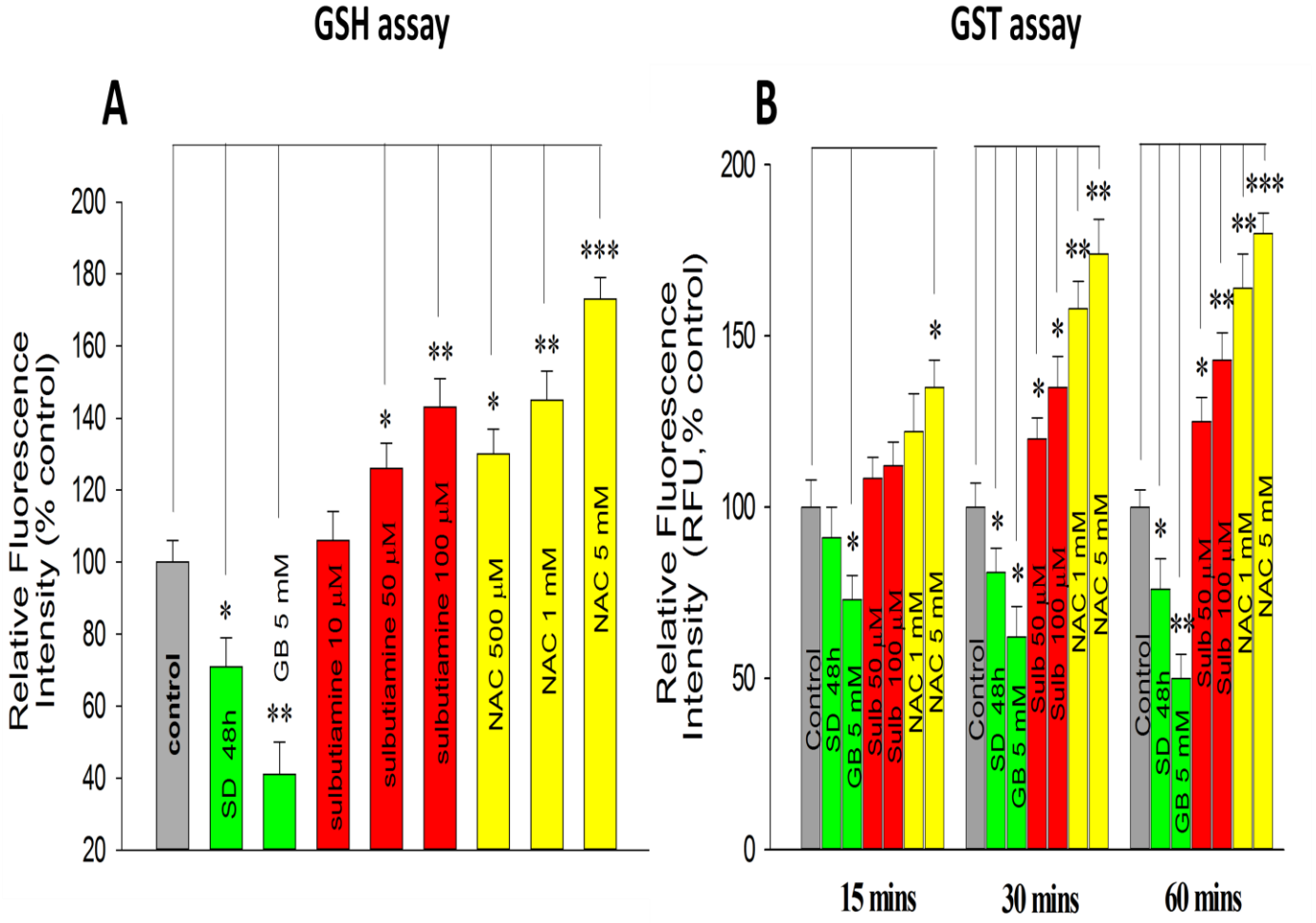
Figure 3.2: Apoptotic cell death was detected by use of Apopercentage^{TM} dye (red) (A-D and M-P), FDA/PI staining (red= dead cells) (E-H and Q-T) and Hoechst staining (bright blue=damaged DNA) (I-L and U-X). The negative effect on cell survival induced by 5mM of GB in terms of cell death (F), apoptosis (B and G) was unaffected by the inclusion of 100µM of sulbutiamine (C,G,K) while NAC (5mM) significantly blunted the negative effects of GB (D,H,L). Moreover, the detrimental effect of serum deprivation for 48h in terms of cell death (R), apoptosis (N and V) was more efficaciously blunted by the inclusion of 100µM sulbutiamine (O,S,W) than 5mM NAC (P,T,X). These experiments were repeated in three separate experiments.

3.2.2 The effect of sulbutiamine and NAC on the antioxidant defense process

To further evaluate the beneficial effects of sulbutiamine and NAC, their influence on key intracellular antioxidant components were investigated. The relative fluorescent intensity which corresponds to reduced glutathione levels in RGC-5 cells was measured after 24h exposure to 5mM GB and 48h exposure to serum deprivation. Both 5mM GB for 24h and serum deprivation for 48h resulted in significant depletion of GSH levels compared to control (Figure 3.3A). The GSH levels treated with defined concentrations of sulbutiamine (50μM and 100μM) and NAC (500μM, 1mM and 5mM) over 24 hours were significantly increased (Figure 3.3A).

Figure 3.3B shows the levels of GST following exposure to sulbutiamine (50 and 100μM), NAC (1 and 5mM) and 5mM GB for 24 h or serum deprivation for 48h. Post-incubation sulbutiamine (50 and 100μM) at 30 and 60 mins significantly stimulated the formation of GST. NAC 1mM also stimulated GST at 30 and 60 minutes. NAC at 5mM stimulated GST not only at 30 and 60 minutes but also in as little as 15 minutes (Figure 3.3B). Cells incubated with either serum deprivation for 48h or GB 5mM for 24h also significantly reduced GST levels at 30 and 60 minutes.

The relative levels of catalase were measured over 1 hour after exposure to sulbutiamine (100μM), NAC (5mM) and 5mM GB for 24h or serum deprivation for 48h. Catalase levels significantly decreased over 1h period subjected to serum deprivation for 48 hours or GB 5mM for 24 hours (Figure 3.3C). NAC 5mM caused no significant change however sulbutiamine 100μM significantly increased catalase levels at 60 minutes (Figure 3.3C).


GSH assay
GST assay
A
B
Relative Fluorescence Intensity (% control)
Relative Fluorescence Intensity (RFU,% control)
control
SD 48h
GB 5 mM
sulbutiamine 10 µM
sulbutiamine 50 µM
sulbutiamine 100 µM
NAC 500 µM
NAC 1 mM
NAC 5 mM
Control
Sulb 50 µM
Sulb 100 µM
15 mins
30 mins
60 mins

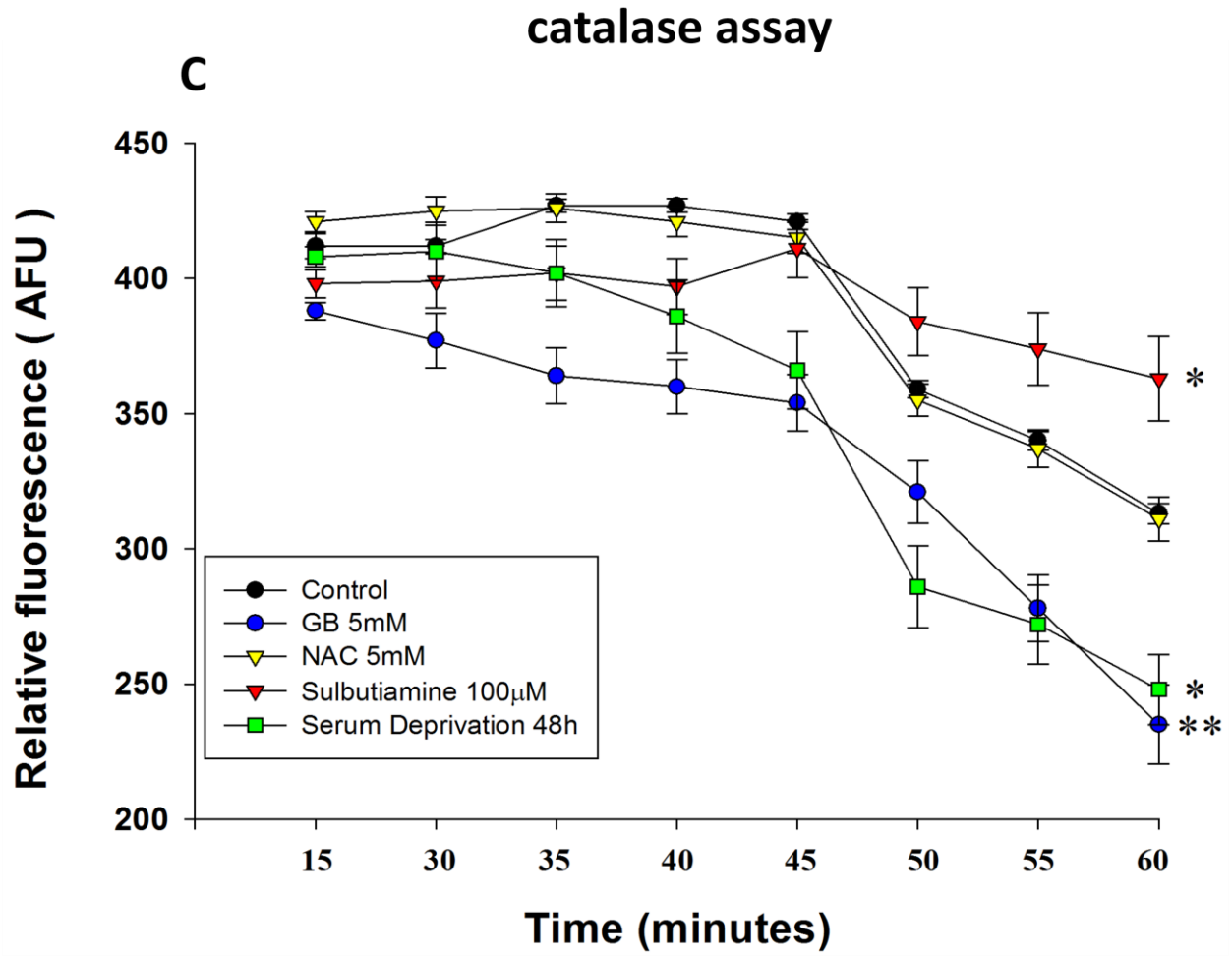
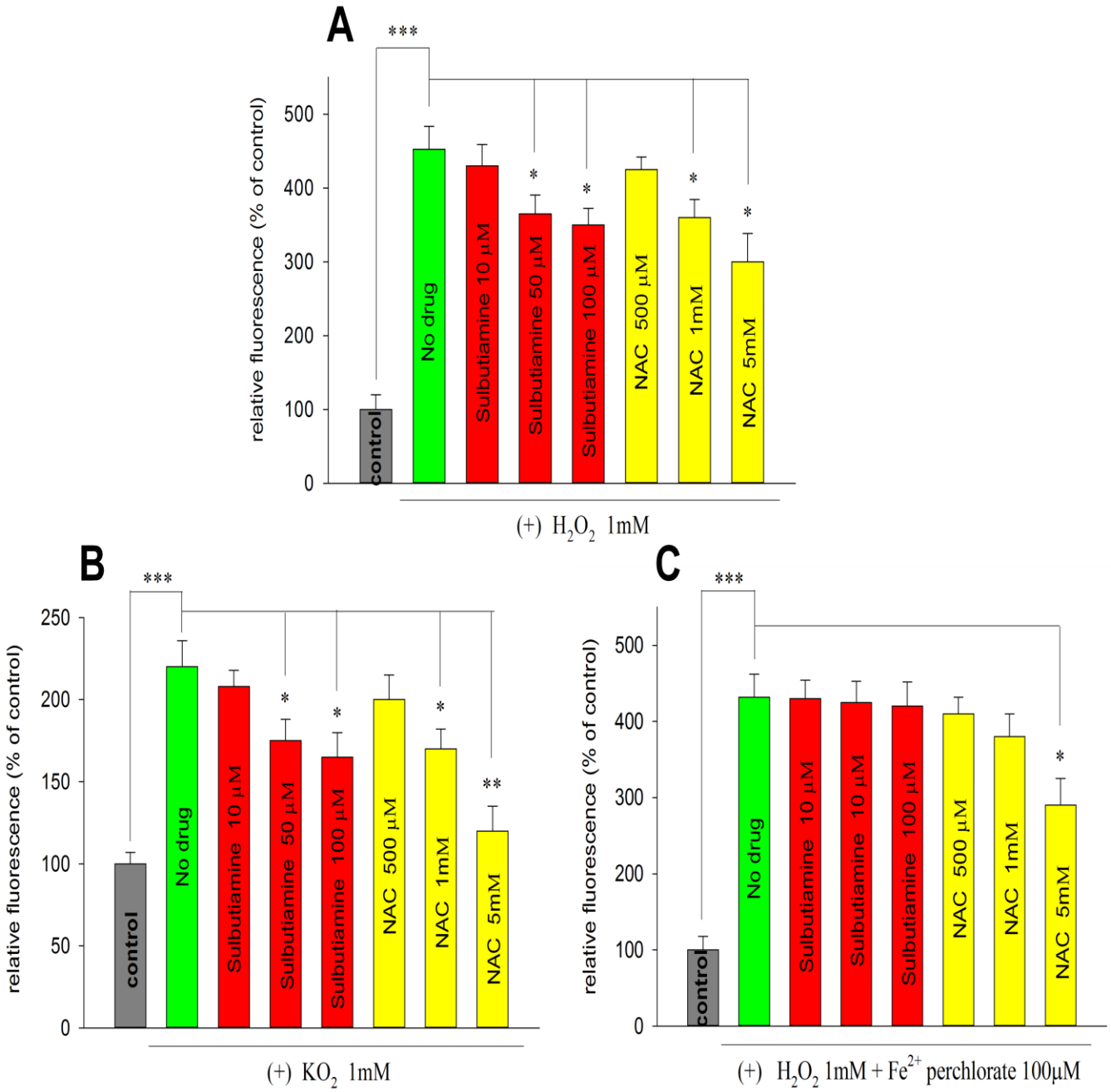


Figure 3.3: The effect of sulbutiamine and NAC on the antioxidant defence process. (A) Relative GSH fluorescence intensity compared to control in cultures treated with either sulbutiamine or NAC for 24h. Incubation with GB 5mM over 24h or serum deprivation 48h decreased GSH significantly. Cells incubated with different concentrations of sulbutiamine (50 and 100µM) and NAC (500µm- 5mM) showed a dose dependent increase in reduced glutathione (GSH) levels. (B) GST levels compared to control in cultures treated with different substances. Post incubation, GST levels were measured over 60 minutes at three different time intervals (15, 30 and 60 mins). Sulbutamine (50 and 100µM) was effective in increasing GST levels at 30 and 60 minutes. NAC 1mM stimulated GST at 30 and 60 minutes whereas 5mM stimulated GST from 15 to 60 minutes. Both GB and serum deprivation significantly decreased GST levels at 30 and 60 minutes. (C) The influence of different substances on catalase levels measured over 60 minutes. Following serum deprivation for 48 hours or GB 5mM exposure over 24 hours, catalase levels decreased significantly at 60 minutes. Sulbutiamine (100µM) significantly increased catalse above control levels. NAC 5mM caused no significant change in catalase levels relative to control at 60 minutes. Data are expressed as mean ± SEM (n=5), *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$ vs control, Bonferroni test.

3.2.3 NAC is more effective than sulbutiamine in scavenging different types of ROS

The antioxidant capacity was subsequently determined and it was shown that NAC was more effective than sulbutiamine at scavenging different types of ROS. ROS activity (relative fluorescence units: RFU) was significantly increased When 1mM H_2O_2 or KO_2 were added to cells. Both sulbutiamine (50 and 100µM) and NAC (1 and 5mM) significantly scavenged H_2O_2 (Figure 3.4A) and KO_2 induced $O_2\cdot^-$ radicals (Figure 3.4B). Hydroxyl radicals (HO^-), was produced by adding 1mM H_2O_2 plus 100µM Ferrous (II) perchlorate. It was seen that only NAC at 5mM was able to scavenge H_2O_2 plus Ferrous (II) perchlorate induced generation of HO^- (Figure 3.4C).

Moreover, microscopic analysis of cell cultures processed for the localization of ROS (Figure 3.4D) and quantification of ROS (Figure 3.4E) provided confirmatory data to support the view that NAC is more effective than sulbutiamine at counteracting ROS production induced by 5mM GB for 24h and serum deprivation for 48h.


A
relative fluorescence (% of control)
0
100
200
300
400
500
***
*
control
No drug
Sulbutiamine 10 µM
Sulbutiamine 50 µM
Sulbutiamine 100 µM
NAC 500 µM
NAC 1mM
NAC 5mM
(+) H2O2 1mM
B
relative fluorescence (% of control)
0
50
100
150
200
250
***
*
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control
No drug
Sulbutiamine 10 µM
Sulbutiamine 50 µM
Sulbutiamine 100 µM
NAC 500 µM
NAC 1mM
NAC 5mM
(+) KO2 1mM
C
relative fluorescence (% of control)
0
100
200
300
400
500
***
*
control
No drug
Sulbutiamine 10 µM
Sulbutiamine 10 µM
Sulbutiamine 100 µM
NAC 500 µM
NAC 1mM
NAC 5mM
(+) H2O2 1mM + Fe2+ perchlorate 100µM

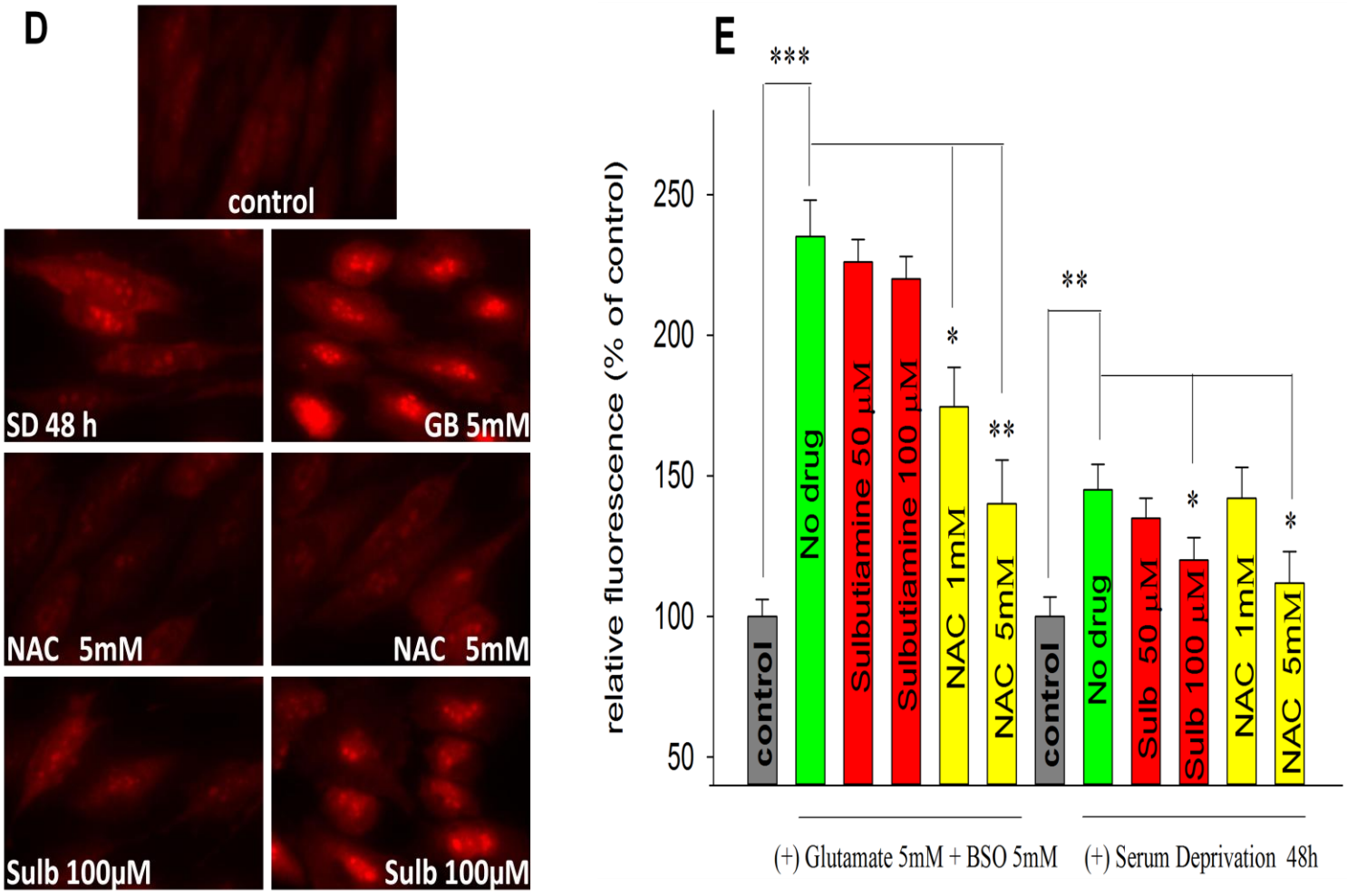
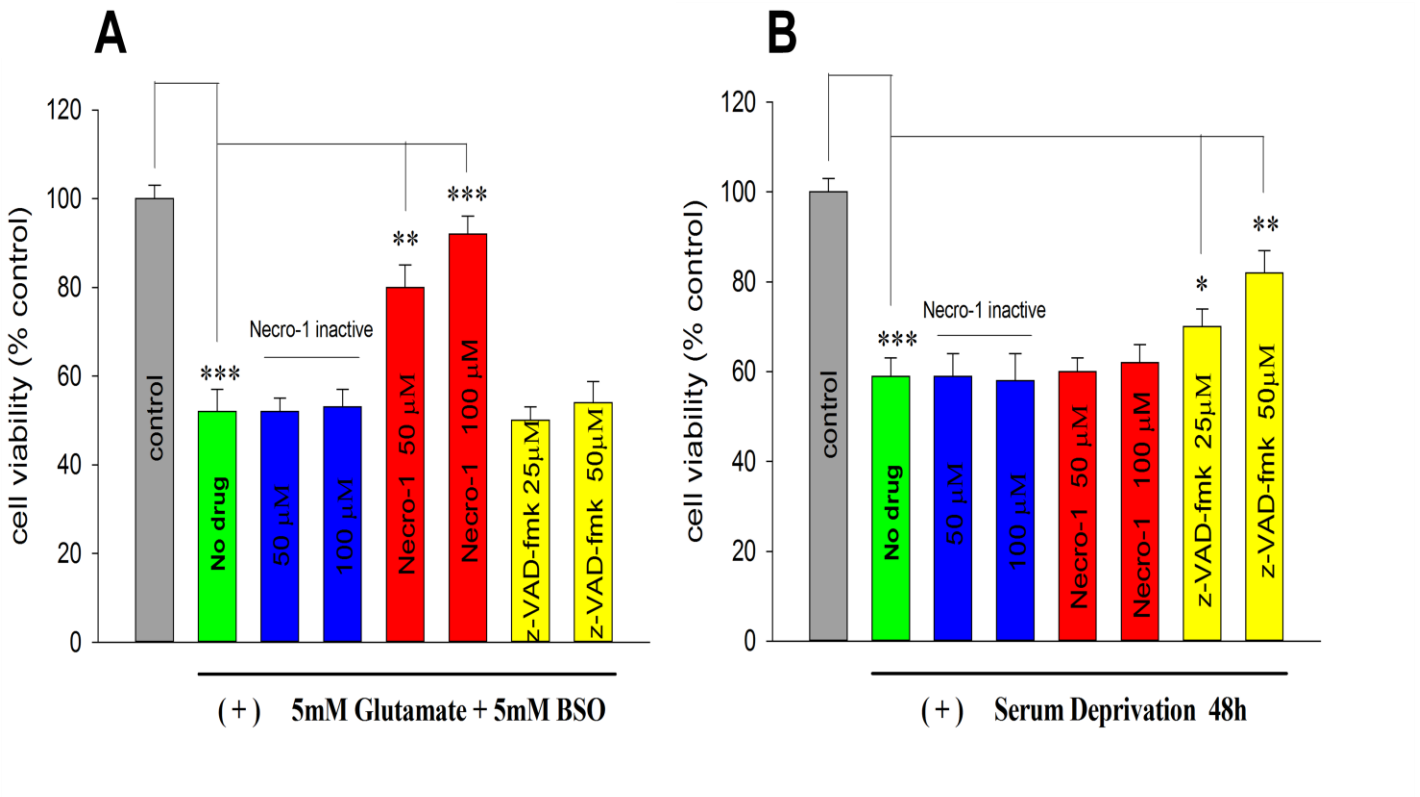


Figure 3.4 Radical scavenging capacities of sulbutiamine and NAC on various radical species (H_2O_2, $O_2\cdot^-$ and HO^-). (A) Sulbutiamine (50 and 100µM) and NAC (1 and 5mM) significantly scavenged 1mM H_2O_2-induced ROS. (B) Sulbutiamine (50 and 100 µM) and NAC (1 and 5mM) significantly scavenged 1mM KO_2-induced ROS. (C) Only NAC at 5mM significantly scavenged 1mM H_2O_2 plus 100µM ferrous perchlorate (II)-induced ROS. (D) Microscopic analysis of cell cultures processed for the localization of ROS showed that 5mM of NAC significantly scavenged ROS produced by both GB (5mM) and serum deprivation (48h) and 100µM sulbutiamine slightly scavenged ROS produced by serum deprivation (48h) but not GB (5mM). (E) Quantification of ROS also showed that NAC at 5mM significantly reduced ROS induced by 5mM of GB and 48h of serum deprivation. Sulbutiamine only at 100µM significantly reduced ROS induced by 48h of serum deprivation. Data are expressed as mean ± SEM (n=5), *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$ vs control, Bonferroni test.

3.2.4 The effect of Necrostatin-1 (necroptosis inhibitor) and z-VAD-fmk (pan-caspase inhibitor) on GB and serum deprivation treatment

Cell viability analysis (RRA) demonstrated the neuroprotective effect of Necrostatin-1 and z-VAD-fmk on RGC-5 cells exposed to either GB 5mM over 24h (Figure 3.5A) or serum deprivation for 48h (Figure 3.5B). Necrostatin-1 (50 and 100µM) significantly blunted the effect of 5mM GB over 24h but not the effect of serum deprivation for 48h. Necrostatin-1 inactive was used as negative control. In contrast, z-VAD-fmk (25 and 50µM) attenuated the detrimental effect of serum deprivation for 48h but not 5mM GB over 24h. Moreover, this was confirmed by microscopic analysis using the APOP*ercentage*™ method (Figure 3.5C). Consistent with these findings is the observation that caspase-3 activity was elevated by serum deprivation over 48h but not by 5mM GB for 24h. Staurosporine was used for positive control (Figure 3.5D).



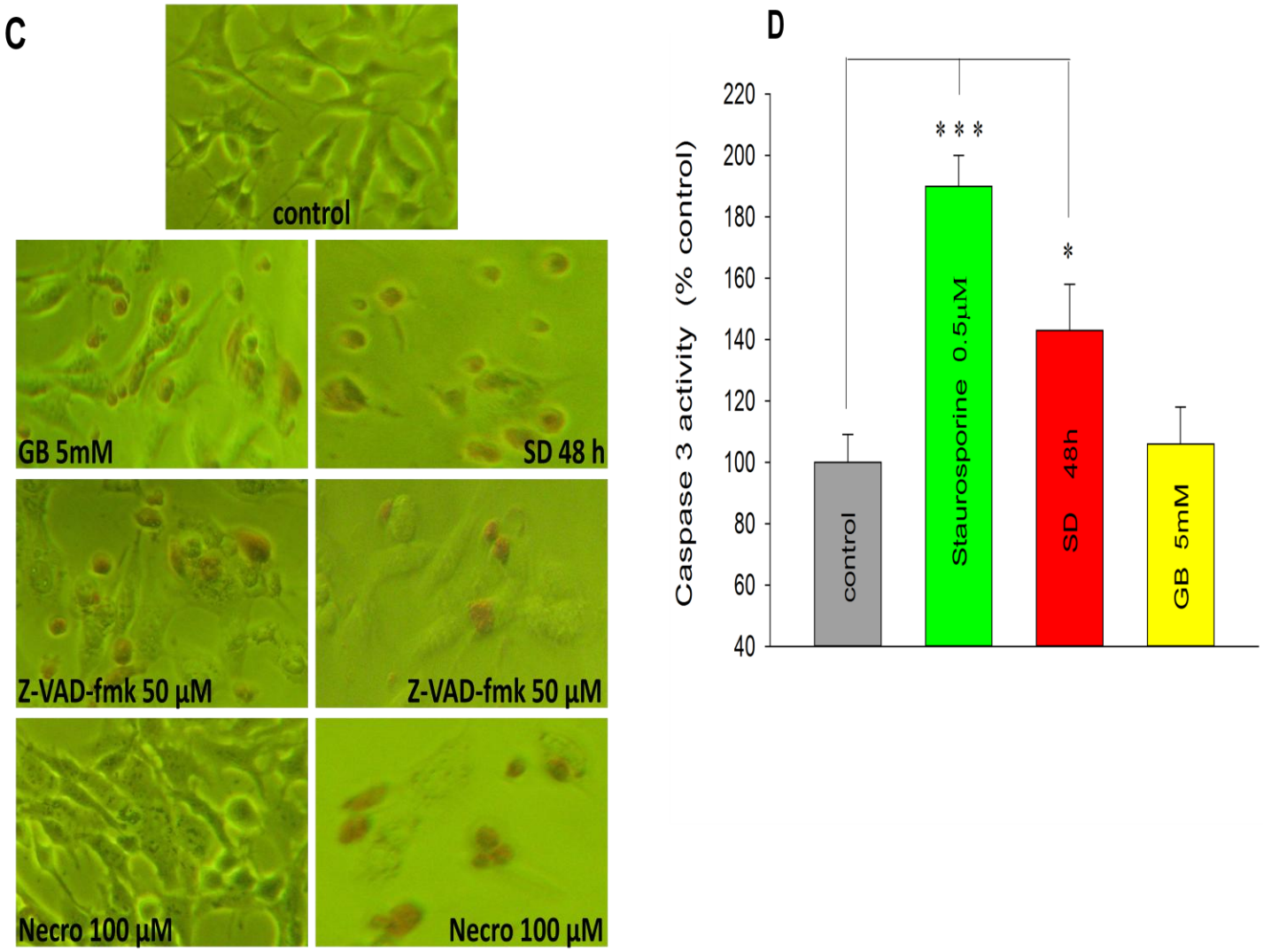
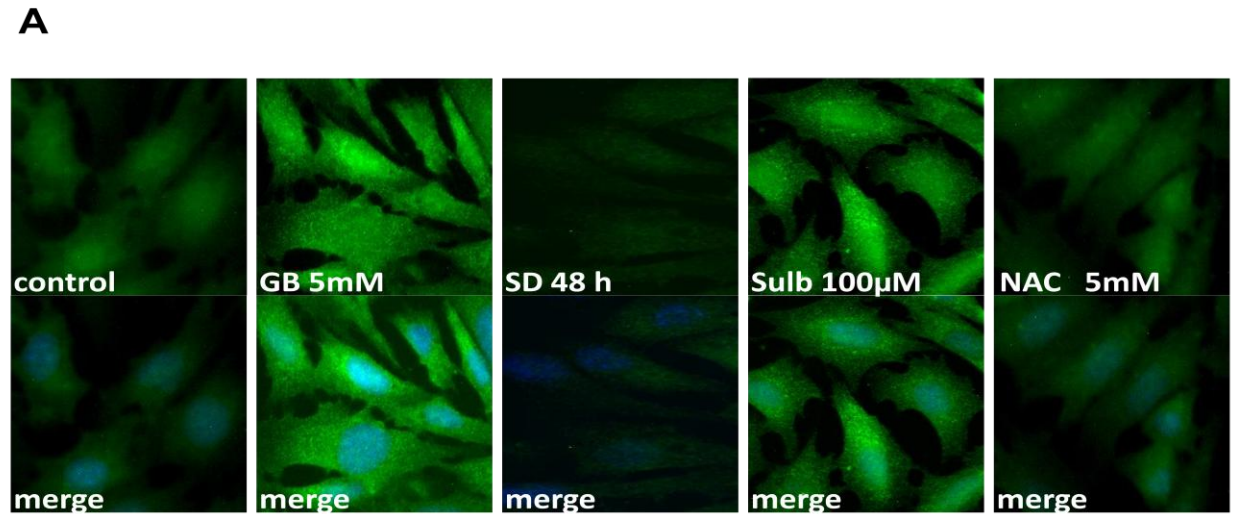


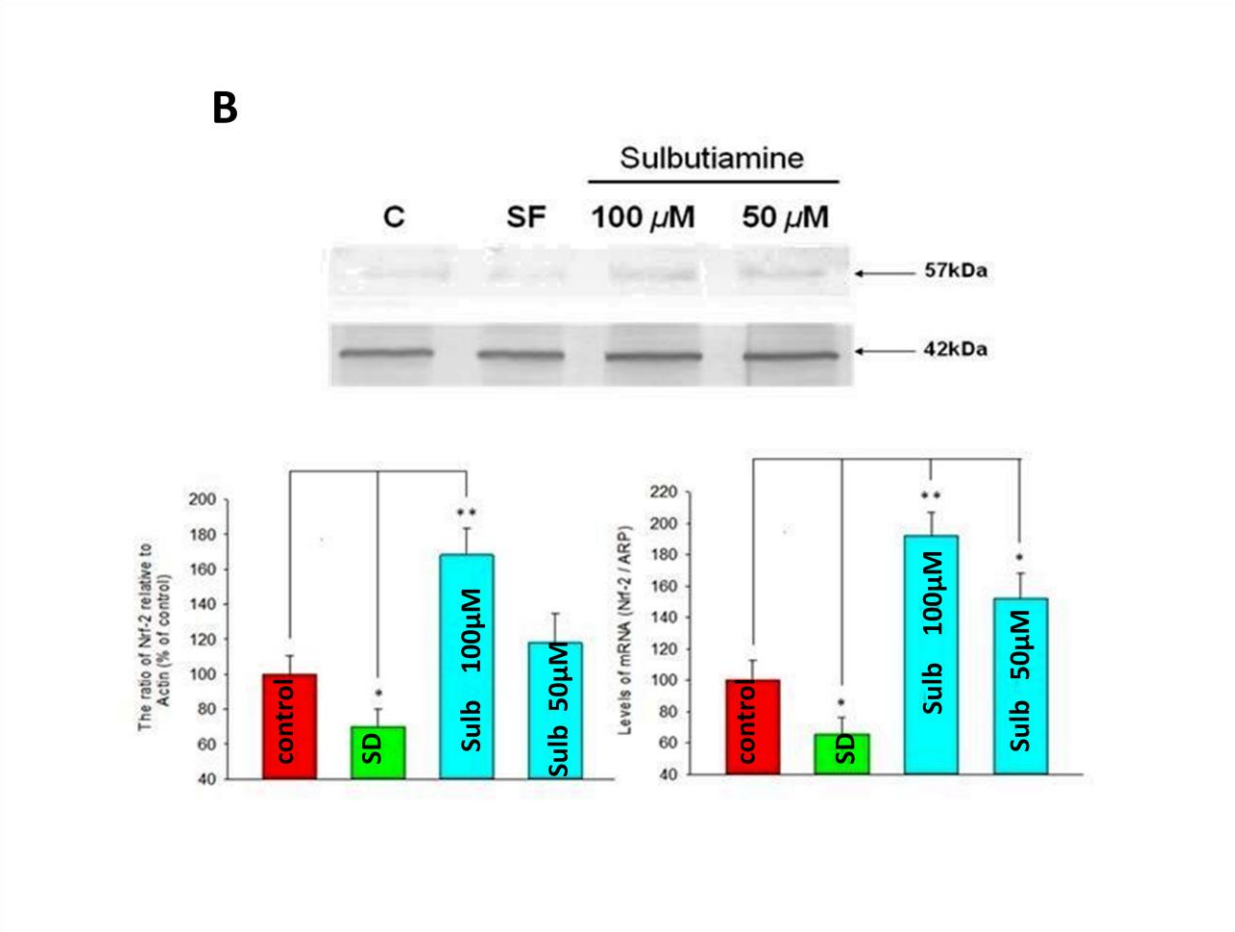
Figure 3.5: The effect of pan-caspase inhibitor (z-VAD-fmk) and necroptosis inhibitor (Necrostatin-1) on 5mM of GB and 48h of serum deprivation treatment. (A) Necrostatin-1 (50 and 100µM) but not z-VAD-fmk attenuated the negative effects of GB (5mM). Necrostatin-1 inactive used as negative control. (B) z-VAD-fmk (25 and 50µM) but not Necrostatin-1 blunted the detrimental effects of serum deprivation (48h). Necrostatin-1 inactive used as negative control. (C) Microscopic analysis determined by use of Apopercentage™ kit also showed that Necrostatin-1 at 100µM nullified the negative effects of GB (5mM) and z-VAD-fmk at 50µM significantly reduced the apoptotic cells induced by serum deprivation (48h). (D) The caspase-3 activity showed that 48h of serum deprivation and 0.5µM of staurosporine caused a significant up-regulation of caspase-3 activity while 5mM of GB was ineffective. Data are expressed as mean ± SEM (n=5), *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$ vs control, Bonferroni test.

3.2.5 The effect of GB, serum deprivation, sulbutiamine and NAC on protein and mRNA levels of Nrf2 and HO-1

RGC-5 cells were exposed to GB (5mM), sulbutiamine (100μM), NAC (5mM) for 24h or serum deprivation for 48h and processed for the localization of Nrf2 immunoreactivity. Nrf2 immunoreactivity was increased by 5mM of GB or 100μM sulbutiamine and decreased by 48h of serum deprivation. There was no significant difference between NAC (5mM) and control (Figure 3.6A). Western blot and qPCR studies provided support for the idea that GB (5mM) or sulbutiamine (50 and 100μM) increased (Figures 3.6B and C) and serum deprivation (48h) decreased both Nrf2 protein and mRNA contents (Figure 3.6B). In contrast, NAC (1 and 5mM) did not significantly affect Nrf2 at mRNA and protein levels (Figure 3.6C).

HO-1 immunoreactivity was increased by 5mM of GB or 100μM sulbutiamine and decreased by 48h of serum deprivation. There was no significant difference between NAC (5mM) and control (Figure 3.7A). Western blot and qPCR studies provided support for the idea that GB (5mM) or sulbutiamine (50 and 100μM) increased (Figures 3.7B and C) and serum deprivation (48h) decreased (Figures 3.7B) both Nrf2 protein and mRNA contents. In contrast, NAC (1 and 5mM) did not significantly affect Nrf2 on mRNA and protein levels (Figures 3.7C).


A
control
GB 5mM
SD 48 h
Sulb 100µM
NAC 5mM
merge
merge
merge
merge
merge


B
Sulbutiamine
C
SF
100 µM
50 µM
57kDa
42kDa
The ratio of Nrf-2 relative to Actin (% of control)
200
180
160
140
120
100
80
60
40
control
SD
Sulb 100µM
Sulb 50µM
Levels of mRNA (Nrf-2 / ARP)
220
200
180
160
140
120
100
80
60
40
control
SD
Sulb 100µM
Sulb 50µM

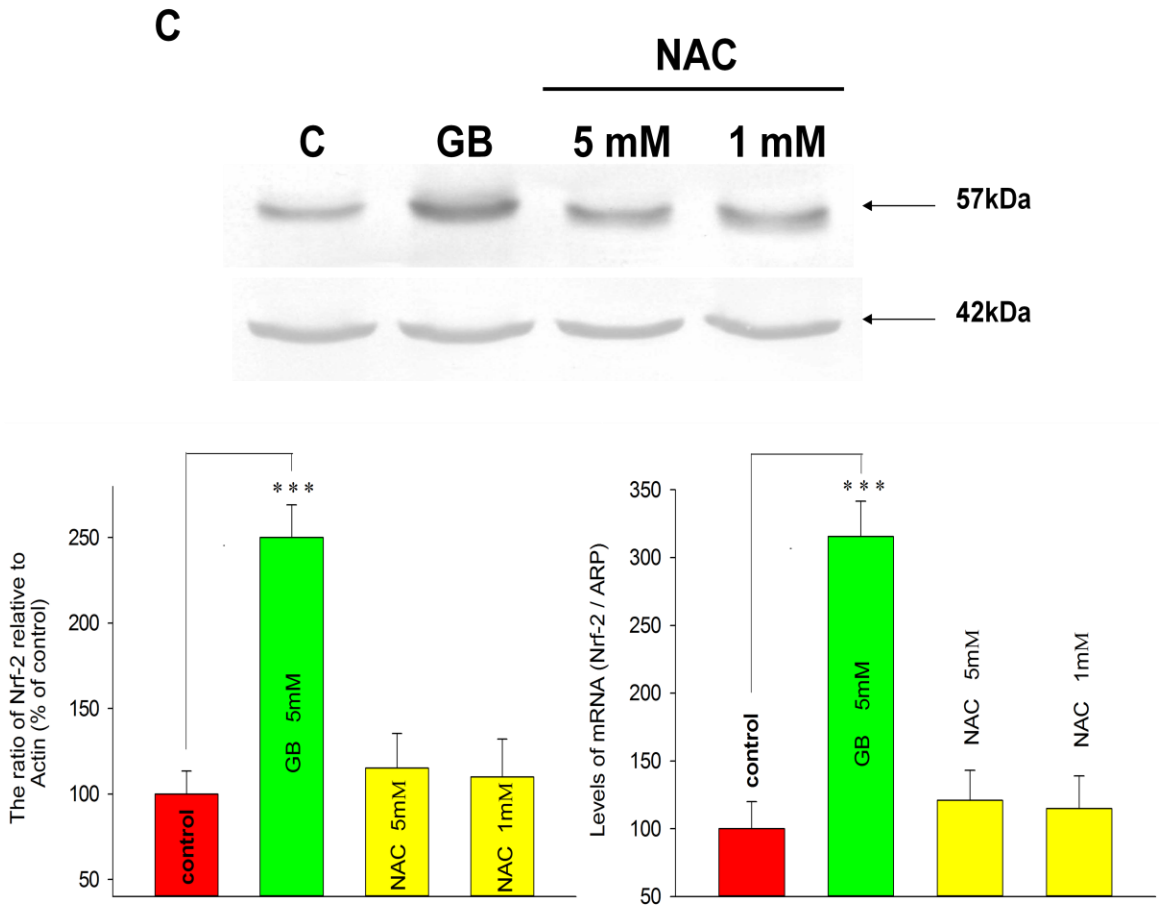
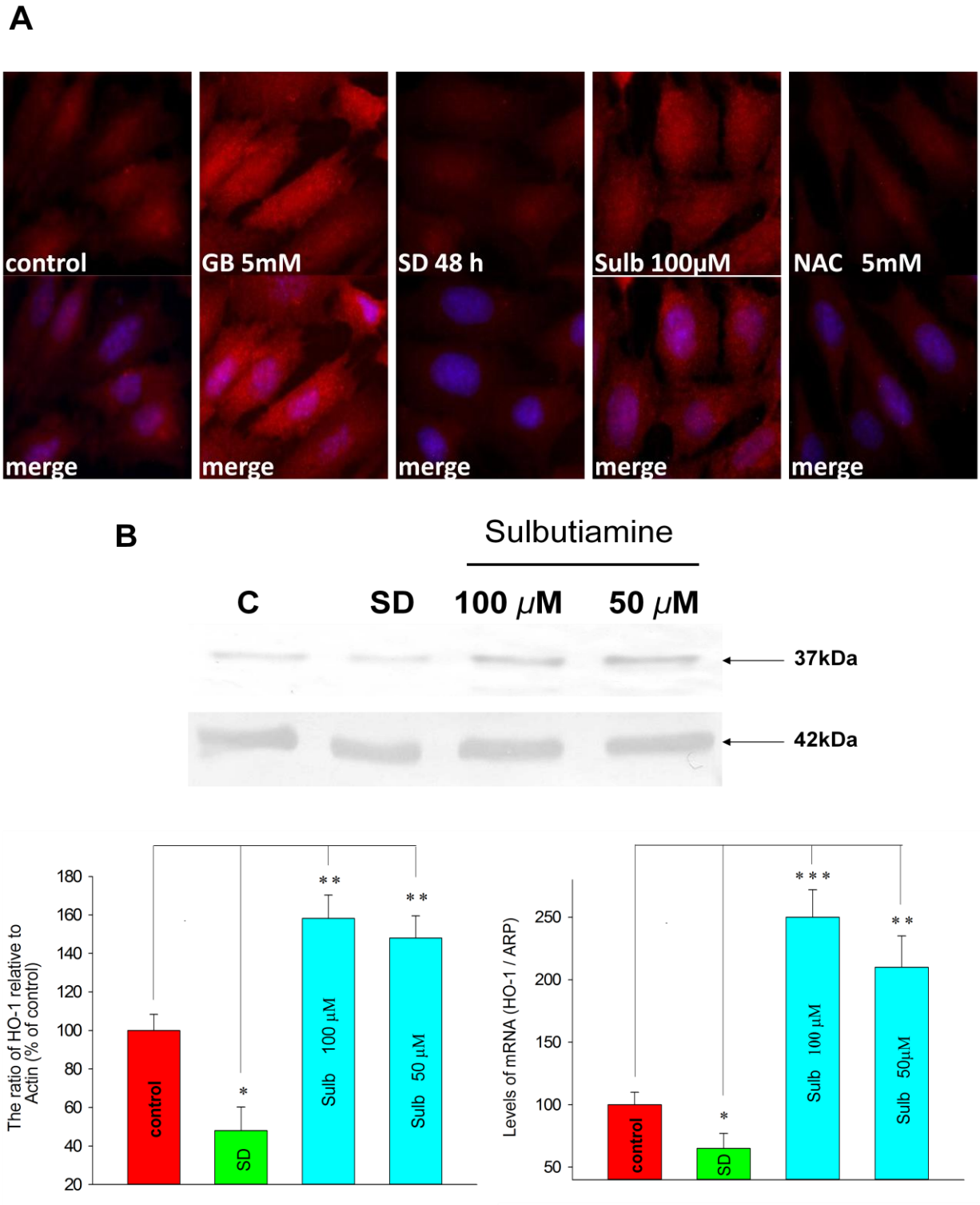


Figure 3.6: The changes of Nrf2 at protein and mRNA levels exposed to different substances. (A) Immunocytochemistry staining showed that Nrf2 immunoreactivity was obviously increased by 5mM of GB or 100μM sulbutiamine and decreased by 48h of serum deprivation. There was no significant difference between NAC (5mM) and control. (B) The bands intensity corresponding to the levels of Nrf-2 (57kDa) relative to β-actin (42kDa) and the mRNA levels relative to acid ribosomal phosphoprotein (ARP). It can be seen that serum deprivation decreased protein and mRNA levels of Nrf-2 whereas sulbutiamine at 50 and 100μM increased the levels. (C) Western blot and qPCR results showed that 5mM of GB significantly increased protein and mRNA levels of Nrf-2 whereas NAC at 1 and 5mM had no effect on the levels. These experiments were repeated in four separate experiments. Data are expressed as mean ± SEM, *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$ vs control, Bonferroni test.


A
control
GB 5mM
SD 48 h
Sulb 100μM
NAC 5mM
merge
merge
merge
merge
merge
B
Sulbutiamine
C
SD
100 μM
50 μM
37kDa
42kDa
The ratio of HO-1 relative to Actin (% of control)
180
160
140
120
100
80
60
40
20
control
SD
Sulb 100 μM
Sulb 50 μM
Levels of mRNA (HO-1 / ARP)
250
200
150
100
50
control
SD
Sulb 100 μM
Sulb 50μM

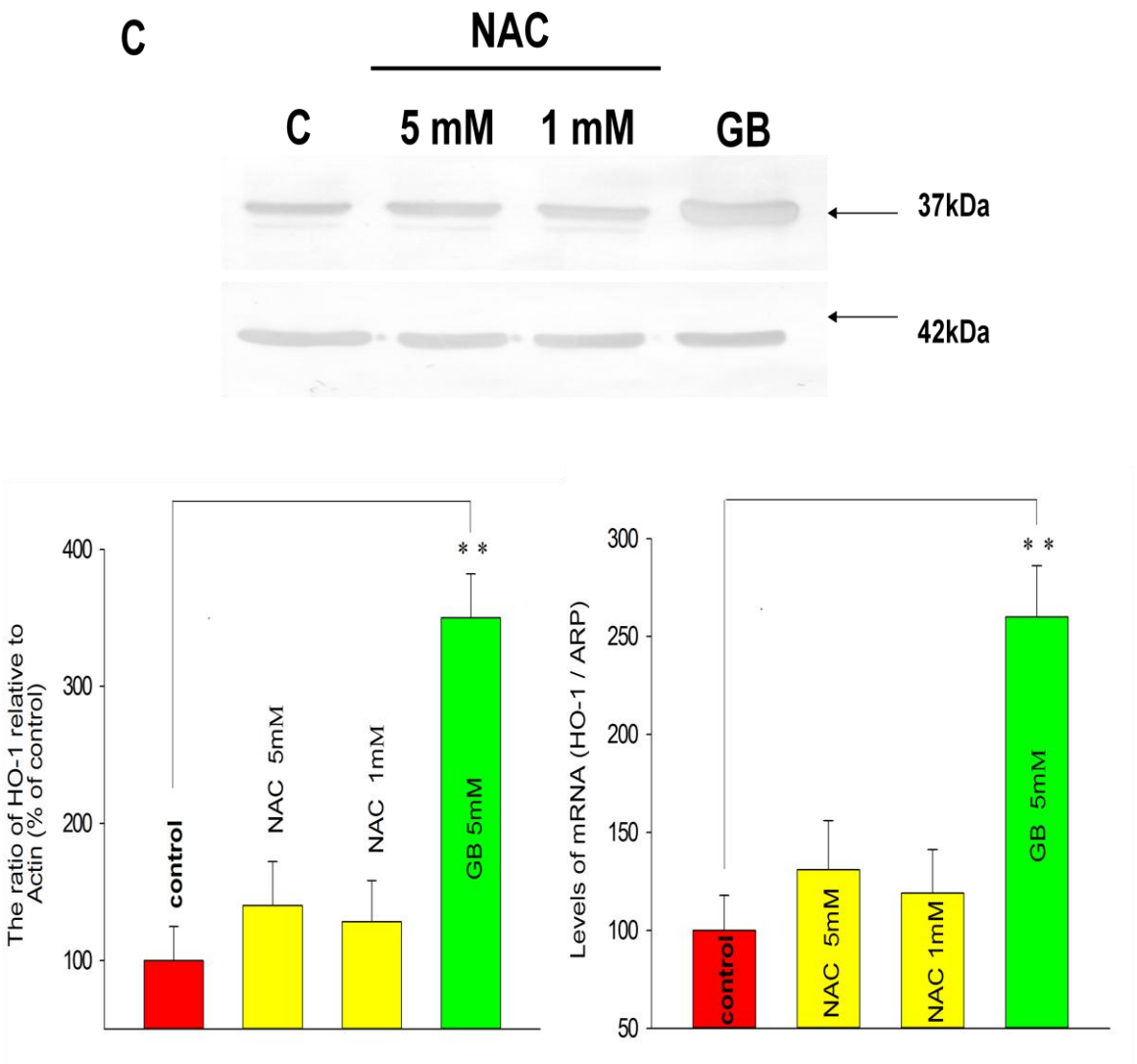


Figure 3.7 The changes of HO-1 at protein and mRNA levels exposed to different substances. (A) Immunocytochemistry staining showed that HO-1 immunoreactivity was obviously increased by 5mM of GB or 100μM sulbutiamine and decreased by 48h of serum deprivation. There was no significant difference between NAC (5mM) and control. (B) The bands intensity corresponding to the levels of HO-1 (37kDa) relative to β-actin (42kDa) and the mRNA levels relative to acid ribosomal phosphoprotein (ARP). It can be seen that serum deprivation decreased protein and mRNA levels of HO-1 whereas sulbutiamine at 50 and 100μM increased the levels. (C) Western blot and qPCR results showed that 5mM of GB significantly increased protein and mRNA levels of HO-1 whereas NAC at 1 and 5mM had no effect on the levels. These experiments were repeated in four separate experiments. Data are expressed as mean ± SEM, *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$ vs control, Bonferroni test.

3.3 Discussion

Neuronal damage in glaucoma, including Alzheimer's disease has been linked to increased free radical production and to a low concentration of endogenous antioxidant defense (Izzoti et al. 2006; Markesbery 1997). NAC and sulbutiamine are relatively safe sulphur compounds which are both currently used as adjuvant therapeutic agents for several clinical conditions (Perry & Shannon 1998; Walker et al. 2003; Tepel et al. 2000; Ollat et al. 2007; Shah 2003). Both are also known to have cytoprotective properties such as ROS scavenging and anti-apoptotic properties in several different cell lines (Malorni et al.1993; Heussler et al. 1999; Talley et al. 1995; Cossarizza et al. 1995; Shin et al. 2004; Wang et al. 2000). However, both *in vivo* and *in vitro* studies to support their potential use in the treatment of neurodegenerative diseases such as glaucoma are still lacking. Additionally the molecular mechanism underlying their potential neuroprotective effects still remain unclear.

To further understand which mechanism of NAC and sulbutiamine could potentially mediate neuroprotection at the cellular level, this study examined the effect of these compounds on two insults to RGC-5 cells. Thus the efficacy of NAC and sulbutiamine as ROS scavengers in oxidative stress conditions, their effects on cellular antioxidants, stress proteins and several anti-apoptotic proteins were investigated. NAC attenuated the detrimental effects of GB and although significant its protective effect is less dramatic in serum deprivation conditions. Sulbutiamine however significantly attenuated the effects of serum deprivation but not the effects of GB

(Figures 3.1 and 3.2). Both sulfur compounds prevented cell death by modulating certain cellular defense mechanisms differently.

NAC and sulbutiamine both had positive effects on the antioxidants GSH and GST levels but only sulbutiamine showed significant stimulation of the antioxidant enzyme catalase (Figure 3.3). NAC is thought to enhance the biosynthesis of GSH under conditions when the demand for GSH is increased (De Vries et al. 1993; Ferrari et al. 1991; Cacciatore 2010), such as in some pathological conditions where excessive oxidative stress is implicated. GSH homeostasis is regulated by de novo synthesis as well as GSH redox state. It is possible that the differences observed between these two drugs in the stimulation of Nrf2 is due to the differential effects of each drug in maintaining GSH levels since cellular GSH status has also been found to be important in modulating apoptosis in other cell types (Beaver and Waring 1995; Martinez et al. 1995; Sandstrom et al. 1994; Watson et al. 1996). Furthermore Nrf2 activity also regulates the sensitivity of death receptor signals by means of regulating intracellular GSH levels (Morito et al. 2003). The importance of GSH depletion in promoting apoptosis has been amply demonstrated in that apoptosis is prevented by replenishing intracellular GSH concentrations in several models of apoptosis (Beaver and Waring 1995; Bojes et al. 1997; Ho et al. 1997; Sandstrom et al. 1994; Watson et al.1996).

Nrf2 is the primary transcription factor protecting cells from oxidative stress by regulating cytoprotective genes, including the antioxidant GSH pathway. The expression of the transcription factor Nrf2 induces antioxidant enzymes by transcriptional activation through the antioxidant-responsive element (ARE) (Lim et al. 2007). In this case, electrophilic agents including ROS induce a set of genes encoding Phase II enzymes, such as HO-1, NADPH quinine oxidoreductase 1, and γ-glutamyl cysteine ligase (γ-GCL). These enzymes provide efficient cytoprotection, in part by regulating the intracellular redox state (Lim et al. 2007). Sun et al. (2010) showed that pharmacological induction of HO-1 by cobalt protoporphyrin resulted in HO-1 expression in the retina. Furthermore, HO-1 over-expression alleviated retinal apoptosis, preserved RGCs, and attenuated reduction of inner retinal thickness after ischemia-reperfussion injury (Sun et al 2010). In the current study, sulbutiamine significantly caused a stimulation of Nrf-2 and the Phase II-dependent enzymes HO-1, while NAC had no significant effect (Figures 3.6 and 3.7). Interestingly Forresti et al. (1997) reported that co-incubation with NAC significantly attenuated HO-1 induction by the NO donor SNP and SNAP, and a reduction in HO-1 activity was also observed when bovine endothelial cells were pre-incubated with NAC for 16 hours prior to exposure to NO donors. Since HO-1 induction involves Nrf-2 activation, NAC may be inhibiting HO-1 via a negative feedback mechanism on Nrf-2. The different effect of these sulphur compounds on the endogenous cellular antioxidant architecture thus potentially varies. The differences between these compounds in Nrf-2 stimulation may partly explain the observed variation in their degree of neuroprotection. Sulbutiamine stimulated Nrf-2 but did not significantly attenuate the effects of GB, and insult which decreases GSH significantly compared to serum deprivation. Since NAC is more efficacious at high concentrations than sulbutiamine in stimulating GSH, this could explain why these substances do not equally attenuate the

detrimental effects caused by GB. Also neuroprotection via Nrf-2 induction may be more specific or important in a particular cell death mechanism, stage or cell type. It is interesting to note that GB caused a significant increase in both Nrf-2 and HO-1 levels but still resulted in significant cell death. Hence there may be a threshold level where Nrf-2 induction can be beneficial. Furthermore, NAC at 5mM is more efficacious than sulbutiamine in scavenging ROS which may play a more important role in GB induced cell death. NAC stimulated the enzyme GST, which is thought to be induced by the Nrf-2 pathway. However it is also possible that NAC may be acting on the ARE directly independent of Nrf-2 stimulation or by indirectly modulating GST and possibly other antioxidant enzymes.

Studies on the effects of the two sulphur compounds on different types of ROS showed NAC at high concentrations scavenged OH^-, H_2O_2 and $O_2^{\cdot-}$. Sulbutiamine at effective concentrations was not as efficacious in scavenging OH^-. H_2O_2 can give rise to the formation of OH^- which is the most reactive species among the three radicals, a process markedly accelerated in the presence of ferric ions via the Fenton reaction (Gutteridge et al. 1979). H_2O_2 can be removed when GSH is oxidized to GSSG via the action of glutathione peroxidase (Meister and Anderson 1983). In the present study the effective concentration of NAC (5mM) caused greater stimulation of GSH than sulbutiamine (100µM) (Figure 3.3). High GSH levels can be readily oxidized to GSSG in the presence of ROS such as OH^- and therefore could be one reason why NAC was more effective in scavenging OH^- since it effectively stimulated GSH at high concentrations. Futhermore, it cannot be excluded whether sulbutiamine acts on low concentrations of OH^-. Although this method has been used previously to cause stimulation of different radical species (Shimazawa et al. 2009), its

sensitivity in detecting different types of ROS however is a factor that also needs to be considered.

In this study, NAC showed no effect on catalase stimulation at 60 minutes whereas sulbutiamine has a significantly stimulatory effect. GB and serum deprivation both caused a drastic decrease in catalase levels compared to control (Figure 3.3). The differences between NAC and sulbutiamine in stimulating catalase could be related to differences in chemical properties. Cetinkaya et al. (2005) evaluated the protective effects of NAC against acetic acid induced colitis in a rat model which showed no influence of NAC on catalase activity. However in another study which examined the effects of NAC on the age associated oxidative stress related parameters in different rat brain regions, showed that supplementation with NAC caused decreased lipid peroxidation, enhanced GSH levels, along with enhanced SOD and catalase activity (Kanwar and Nehru 2007). Currently there are no specific studies on the effects of sulbutiamine on catalase although a study carried out in thiamine deficient cell-free rat-liver extracts showed a decrease of catalase concentration and activity (Hauschildt 1975). Furthermore a study which looked at the biochemical activities and protein expression of catalase in isolated rats hearts submitted to thiamine deprivation, it was shown that catalase activity increased 2.1 times (Gioda et al. 2010).

Interestingly a study which evaluated the antioxidative effect of several types of antioxidants on catalase activity at a physiological H_2O_2 concentration showed that certain antioxidants can

partially suppress catalase activity (Doronicheva et al. 2007). The study demonstrated that the catalase activity increased depending on the structure of antioxidative flavonoids and their derivatives. A combination of many factors such as the position of functional groups on the flavonoid structure and their interaction with catalase, including that of the ferriprotoporphyrin group, also contribute to this effect. The authors also did not find a clear relationship between the apparent IC50 values for scavenging H_2O_2 and the enhancing effects on catalase activity (Doronicheva et al. 2007). The complex physicochemical relationships between drug and enzyme activities thus may also apply to thiol antioxidants such as NAC and sulbutiamine.

In the present study, necroptosis, which has characteristics of apoptosis, necrosis and also autophagy (Degterev et al. 2005; Hitomi et al. 2008) was inhibited by Necrostatin-1 in GB exposed RGC-5 cells (Figure 3.5A). Necrostatin-1 targets a serine threonine kinase receptor that interacts with protein kinase-1 (RIP1) that is specifically involved in necroptosis (Degterev et al. 2005; Galluzi et al. 2009). Cell death inhibition by Necrostatin-1 is thought to differentiate between apoptotic cell death and necroptosis (Degterev et al. 2005; Hitomi et al. 2008). The death mechanism involving serum deprivation for 48h appears to be more like apoptosis as evidenced by increase of caspase-3 activity. Moreover, caspase-3 activity was not elevated in GB exposure (Figure 3.5D). A similar study also did not show any evidence of caspase-3 activation in RGC-5 cells exposed to GB (Maher and Hanneken 2005). It is possible that sulbutiamine protects the culture exposed to serum deprivation by decreasing caspase-3 levels and NAC protects GB induced cell death by inhibiting the necroptotic pathway. Shin et al. (2004) showed

that thiamine has a cytoprotective effect on cultured neonatal rat cardiomyocytes under hypoxic insult, and also protects the cardiomyocytes against hypoxia-induced apoptosis. Their findings also showed that caspase-3 activation, PARP cleavage and DNA fragmentation were all inhibited by thiamine (Shin et al. 2004). Furthermore, it has been shown that the hemoglobin oxidation by-product, hemin-induced necroptotic cell death in cortical astrocytes can be attenuated by NAC (Laird et al. 2008).

In conclusion, GSH depletion may be a pivotal mechanism of damage caused by several insults such as GB. GB exposure inhibits GSH synthesis to cause a type of cell death, however serum deprivation induced oxidative stress and cellular damage to RGC-5 cells may have a lesser dependence on GSH and ROS and induces a different mechanism of cell death. Both NAC and sulbutiamine helped to prevent the exhaustion of GSH and to preserve the thiol antioxidant pool and also to decrease intracellular ROS accumulation and cell death. They also showed significant but variable effects on the modulation of crucial antioxidant enzymes, stress proteins which may contribute to their differences in cyptoprotective effects. It appears that NAC's stimulation of GSH and ROS scavenging is the main mechanism by which it attenuated the negative effects of GB. Also the stimulation of Nrf-2 and its dependent enzymes may underlie sulbutiamine's specificity in attenuating the negative effects of serum deprivation. However further experiments are needed to provide the necessary additional information to understand more precisely the mechanism by which NAC and sulbutiamine act as neuroprotectants.

The mitochondria may also be an important target of NAC and sulbutiamine. Studies of their effects on mitochondrial targets in mitochondrial injury need to be further evaluated. Also compared to sulbutiamine, the high concentrations required for NAC to act as a neuroprotectant could be a problem for use in a chronic manageable disease like glaucoma but might find a use in an acute disease affecting ganglion cells, such as ischaemic optic neuropathy.

Chapter 4

Comparative effects of two different H_2S releasing sulphur compounds on retinal cell death



Chapter abstract

H_2S is one of three gaseous signaling molecules after nitric oxide and carbon monoxide. The interaction and role of these gaseous molecules in oxidative stress is complex. Studies with other gaseous molecules, such as carbon monoxide and nitric oxide, have generated a lot of data elucidating their physiological roles owing to the availability of donor drugs. Similarly various H_2S donor compounds have been synthesized to study its physiological function. Among these compounds NaHS, a rapid donor of H_2S gas has been shown to be protective in certain neuronal cell line but several *in vivo* studies have generated conflicting data. Furthermore several slow releasing H_2S donors have been shown to have positive effects on cells in culture. The intracellular concentration of H_2S and hence its rate of production may be a factor in keeping the balance between its neuroprotective and toxic effects. The present study was undertaken to deduce how a rapid H_2S donor (NaHS) as opposed to a slow donor (ACS1), affect oxidative stress related intracellular components and survival of RGC-5 cells. It was concluded that when RGC-5 cells are exposed to the toxic effects of GB, ACS1 was more efficacious at inhibiting apoptosis, scavenging ROS and at stimulating GSH and GST. Western blot and qPCR analysis showed ACS1 increased the levels of Nrf2, HO-1, PKCα, p-Akt, Bcl-2 and XIAP but decreased Nfκβ and xCT greater than NaHS. Nrf2 activity may regulate a feedback system limiting oxidative load during GB-induced stress while H_2S plays significant role in its regulation.

4.1. Introduction

4.1.1 Aim

The purpose of this study was to investigate the differences in neuroprotective effects of two different H_2S-releasing sulphur compounds ACS1 and NaHS, against glutamate or GB induced cell death. ACS1 (ADTOH) is a slow releaser and NaHS a fast releaser of H_2S.

4.1.2 Brief Overview

Glutamate excitotoxicity and oxidative stress may contribute to neurodegenerative diseases such as glaucoma (Bonne et al. 1998; Ko et al. 2000; Agardh et al. 2006; Tezel 2006; Kaur et al. 2008; Zanon-Moreno et al. 2008). To further elucidate the complex mechanisms underlying RGCs degeneration in glaucoma, the cellular signaling pathways involved and the role of signaling molecules in the pathophysiology of RGCs apoptosis are currently being investigated. In addition to nitric oxide (NO) and carbon monoxide (CO), hydrogen sulfide gas (H_2S) has now emerged as a third important neuromodulator or signaling molecule in cells (Abe and Kimura, 1996). H_2S is a ubiquitous gas produced endogenously in the human body from the transulfuration enzyme cystathionine β-synthase (CBS) in the brain mainly and cysthionine γ-lyase (CGL) enzymes in the liver mainly (Moore et al. 2003). H_2S is thought to play a role in oxidative stress regulation, inflammation, vasodilation and neuronal survival (Szabó 2007, Lefer 2007, Kimura et al. 2005, Zhang & Bhatia 2008). H_2S also stimulates the formation of cAMP in primary neuronal cultures (Shao et al. 2010). Furthermore the activity of the enzyme CBS has been shown in the

human eye to be present more in the anterior segment than in the retinas and optic nerves (Persa et al. 2006). In the mouse retina CBS was not detectable, but CGL although low was present (Pong et al. 2007). Although the activity of H_2S has been implicated in countless physiological functions (Szabó 2007, Lefer 2007), it's role in the eye and specifically retinal disease such as glaucoma is not well understood. However a recent study has shown that the latanoprost acid derivative (ACS 67, that is able to release H_2S) reduced intraocular pressure (IOP) in ocular hypertensive rabbits to a greater extent than latanoprost acid on its own (Perrino et al. 2009). Moreover, ACS67 as opposed to latanoprost acid markedly stimulated the formation of GSH and cGMP (Perrino et al. 2009). It has also been reported that H_2S protects cultured mouse hippocampal neurons from cell death (oxidative glutamate injury) partly by upregulation of glutathione (Kimura et al. 2006). Certain *in vivo and vitro* studies however have shown that H_2S can promote cell death rather than survival (Qu et al. 2006, Wong et al. 2006, Chung et al. 2007). Given the known toxic chemical properties of H_2S, it is probable that the physiological role (s) of H_2S is highly dependent on its local concentration in situ. The differences reported seen in experimental studies is therefore probably related to nature of H_2S donor compound used, where donors that release H_2S rapidly increase *in situ* levels of H_2S to a degree to cause toxicity. Moreover, in cell culture studies the cell-type and culture conditions might be critical factors that determine what level of exogenous H_2S is neuroprotective rather than neurodestructive. It might also be worth noting that it remains to be clearly demonstrated both how H_2S elicits its neuroprotective action in different situations and whether the donor compounds play a part in the process (Jacob et al. 2008).

In this chapter (as in chapter 3), the effect of adding GB to simulate oxidative stress conditions in RGC-5 cells in culture (Maher et al. 2005) is used to deduce whether two different H_2S donating compounds attenuate its detrimental effects in distinctive ways. This study therefore aims at trying to understand how the unique nature of the dithiolethione ACS 1 moiety, that releases H_2S in a controlled and sustained way, (Perrino et al. 2009; Li et al. 2007, Sparatore et al. 2009, Muzzafar et al. 2008), compares with NaHS as neuroprotective agents. NaHS is a rapid donor of H_2S which exerts a cytoprotective action partly causing an upregulation of GSH and by interfering with glutamatergic neurotransmission (Li and Moore 2007). Here the effectiveness of NaHS and ACS1 are compared by their ability to increase the basal GSH, GST and HO-1 levels in RGC-5 cells and also on their capacity to scavenge different forms of ROS. The mechanism of neuroprotection of the two donors was also explored by investigating their effects on the prosurvival proteins Bcl-2, p-Akt and XIAP, the stress marker HO-1 and the oxidative stress related signal transduction molecules PKCα, NfkB and Nrf2. System X_c^- regulates the concentration of intracellular cystine, a component substrate for GSH synthesis and in addition, the expression of the glutamate/cystine exchanger xCT is controlled by Nrf2. Thus the effect of GB, NaHS and ACS1 on the regulation of the cysteine/glutamate exchanger (xCT) was also investigated.

In this study it was concluded that the stimulation of the oxidative stress-induced transcription factor Nrf2 activity by NaHS was sufficient to protect RGC-5 cells from

glutamate but not GB toxicity. This is probably because the addition of BSO irreversibly inhibits GSH upregulation and NaHS rapid loss of H_2S is not able to sustain this positive effect. However ACS1 which sustains H_2S production over a longer duration activates Nrf2 more significantly and also more efficiently increased GSH synthesis and was thus able to attenuate the effects of both glutamate and GB by modulating key antioxidant enzymes and prosurvival mediators.

4.1.3. Summary of results

The results of the studies in this section can be summarised as follows:

a) Reduction of RGC-5 cell viability caused by exposure to GB is attenuated by the slow releasing sulphur compound ACS1 and not NaHS.

b) Reduction of RGC-5 cell viability caused by exposure to glutamate is attenuated by both ACS1 and NaHS.

c) Since ACS1 is able to sustain an increase in H_2S levels over longer periods than NaHS, ACS1 provides enhanced neuroprotection.

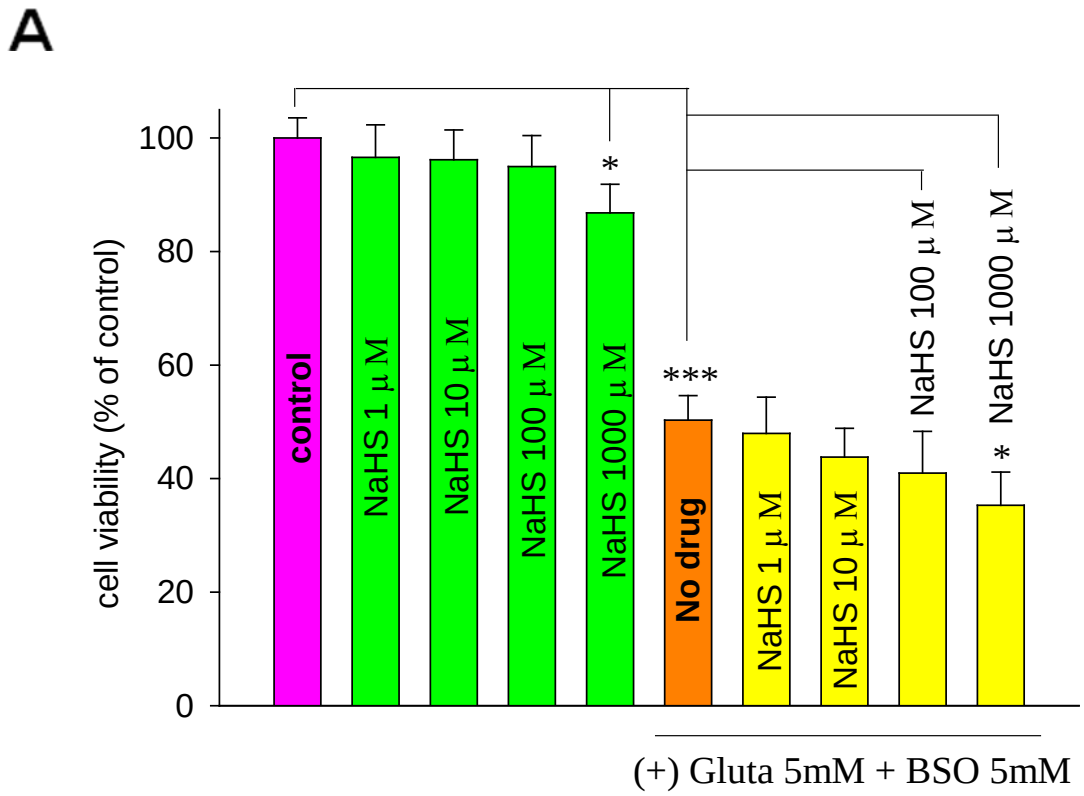
d) Both ACS1 and NaHS exert variable effects on key antioxidant and antiapoptotic markers which probably correlate with their ability to release H_2S gas.

e) The cystine/glutamate exchanger xCT is significantly up-regulated in GB induced oxidative injury and by reducing the oxidative load, H_2S donors down-regulate its expression.

4.2. Results

4.2.1 GB decreased RGC-5 cells viability while H_2S donating compounds influence these negative effects differently.

As in chapter 3 cell viability studies (RRA) demonstrated the detrimental effect of GB on RGC-5 cells. NaHS a rapid H_2S donor showed no significant toxicity to RGC-5 cells in culture up to 100 µM concentration (Figure 4.1 A). Concentrations of NaHS up to 1000 µM were not able to nullify the detrimental effects of GB 5mM to RGC-5 cells in culture over 24 hours (Figure 4.1 A) but 100 µM attenuated the effects of glutamate 1 mM (Figure 4.1 C). However ACS 1 (5 to 50 µM) was able to nullify the detrimental effects of 5mM GB to RGC-5 cells in culture over 24 hours (Figure 4.1 B). ACS 1 (1 to 50 µM) also nullified the effects of 1 mM glutamate over 24 hours (Figure 4.1 D).


A
cell viability (% of control)
0
20
40
60
80
100
control
NaHS 1 µ M
NaHS 10 µ M
NaHS 100 µ M
NaHS 1000 µ M
No drug
NaHS 1 µ M
NaHS 10 µ M
NaHS 100 µ M
NaHS 1000 µ M
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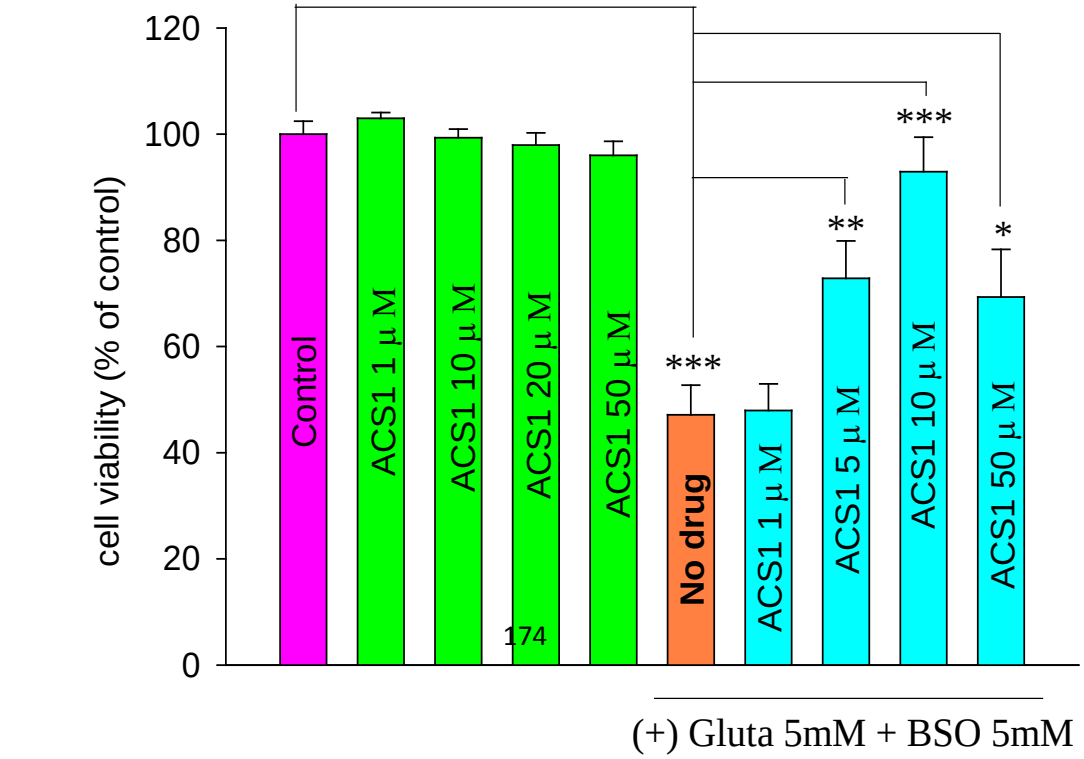
*
(+) Gluta 5mM + BSO 5mM


B
cell viability (% of control)
0
20
40
60
80
100
120
Control
ACS1 1 µ M
ACS1 10 µ M
ACS1 20 µ M
ACS1 50 µ M
No drug
ACS1 1 µ M
ACS1 5 µ M
ACS1 10 µ M
ACS1 50 µ M
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*
(+) Gluta 5mM + BSO 5mM

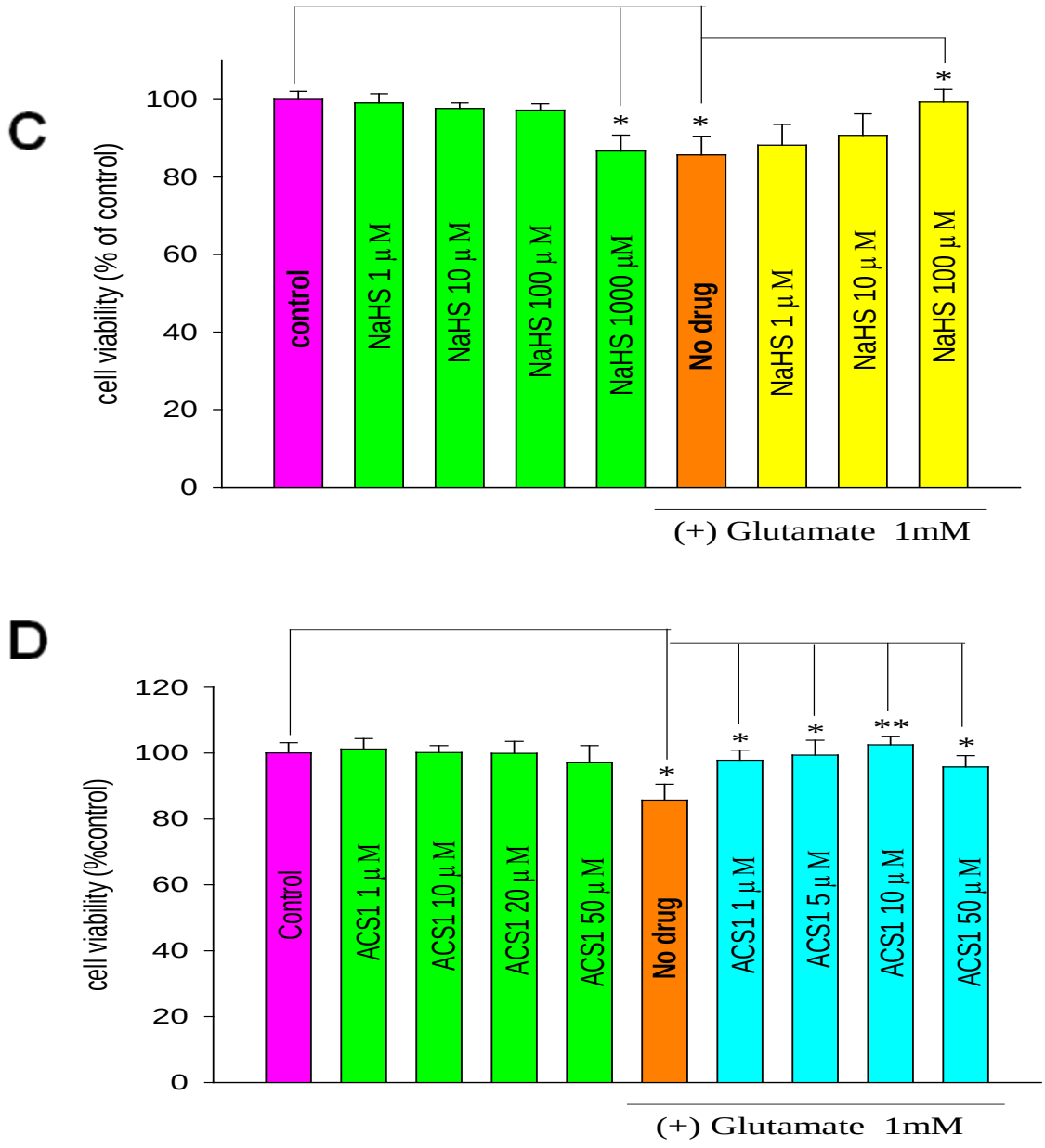


Figure 4.1: Cell viability analysis (RRA) of RGC-5 cells exposed to 5 mM of GB (A,B) or 1 mM glutamate (C,D) for 24 hours. 1 mM NaHS significantly decreased RGC-5 cell viability while ACS1 had no effect even at concentration of 50 µM. GB induced toxicity to RGC-5 cells was attenuated by ACS1 (up to 50 µM) (B) but not by NaHS (up to 1000 µM) (A). Glutamate toxicity was attenuated by NaHS at 100

µM (C) and ACS 1 (1-50 µM) (D). Data are expressed as mean ± SEM where n=6 (*p < 0.05 **p < 0.01 ***p < 0.001)

4.2.2 Cytological evidence to show that ACS1 attenuates GB-induced death to RGC-5 cells.

Following exposure of RGC-5 cells to an insult of 5 mM GB, cell death was revealed by FDA/PI staining where live cells appear green and dead cells appeared red (I and J). The death process was by apoptosis as phosphatidylserine could be revealed by use of the Apopercentage^{TM} dye procedure (A and B). Moreover, cell death by apoptosis results in a stimulation of ROS that could be detected by use of DHE (E and F). The negative effect on cell survival induced by 5 mM GB in terms of cell death (J), apoptosis (B) and generation of ROS (F) is unaffected by the presence of 100 µM NaHS (C,G,K) while ACS1 (10 µM) significantly blunted the negative effects of GB (D,H,L).

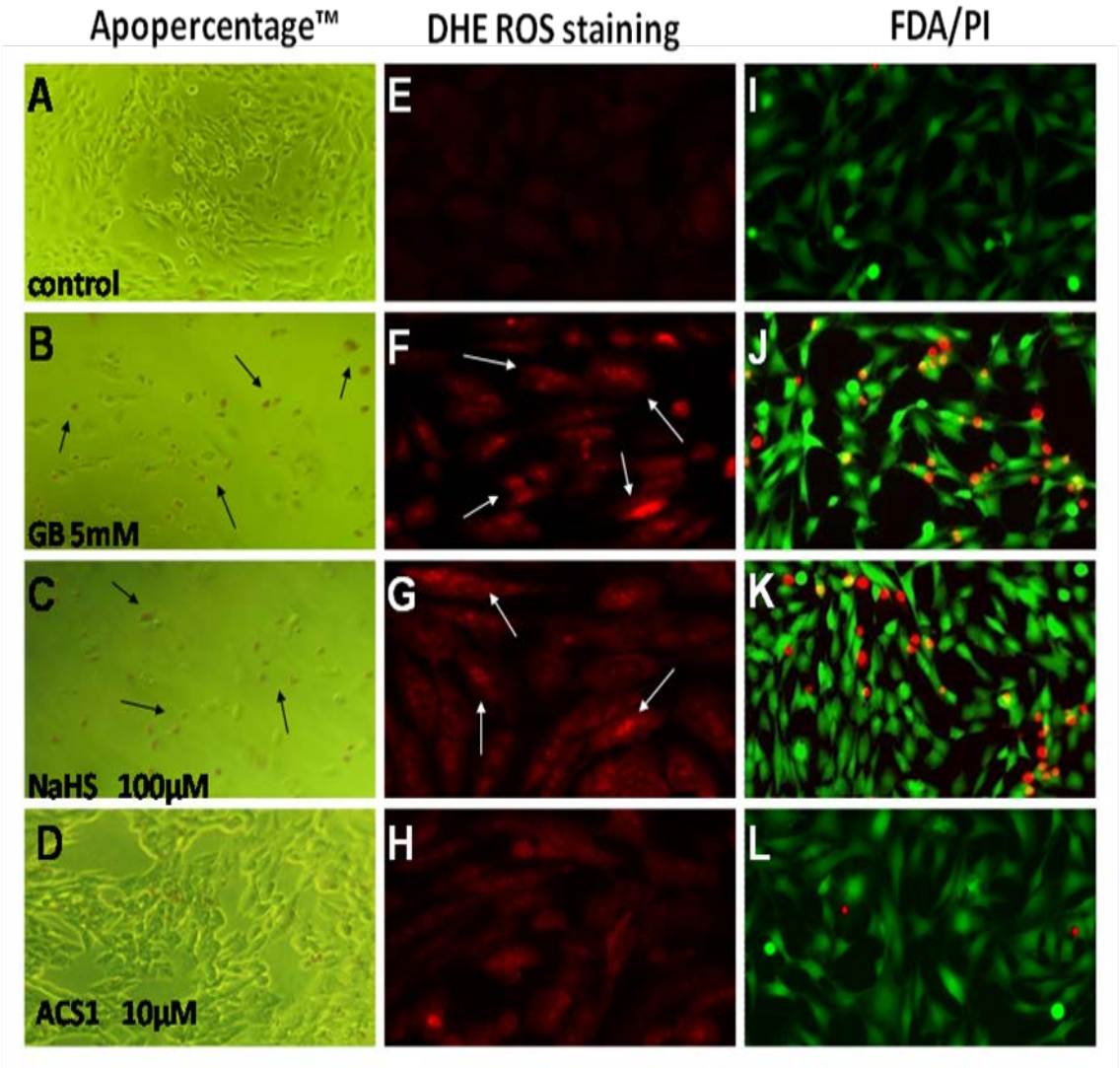
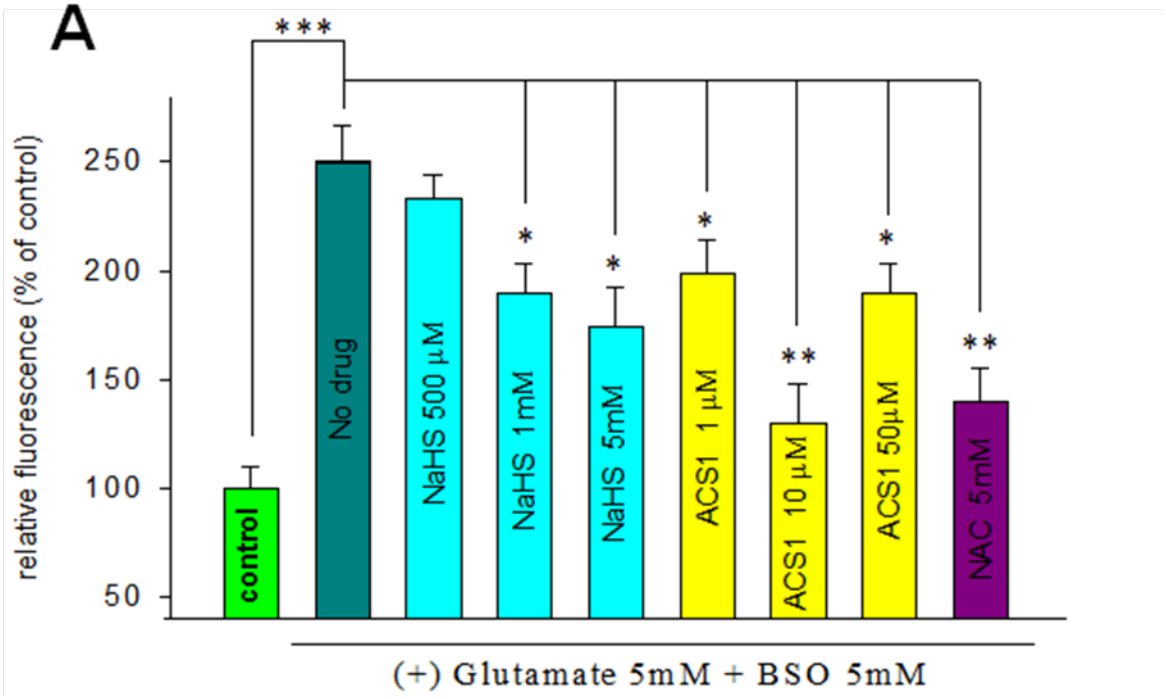


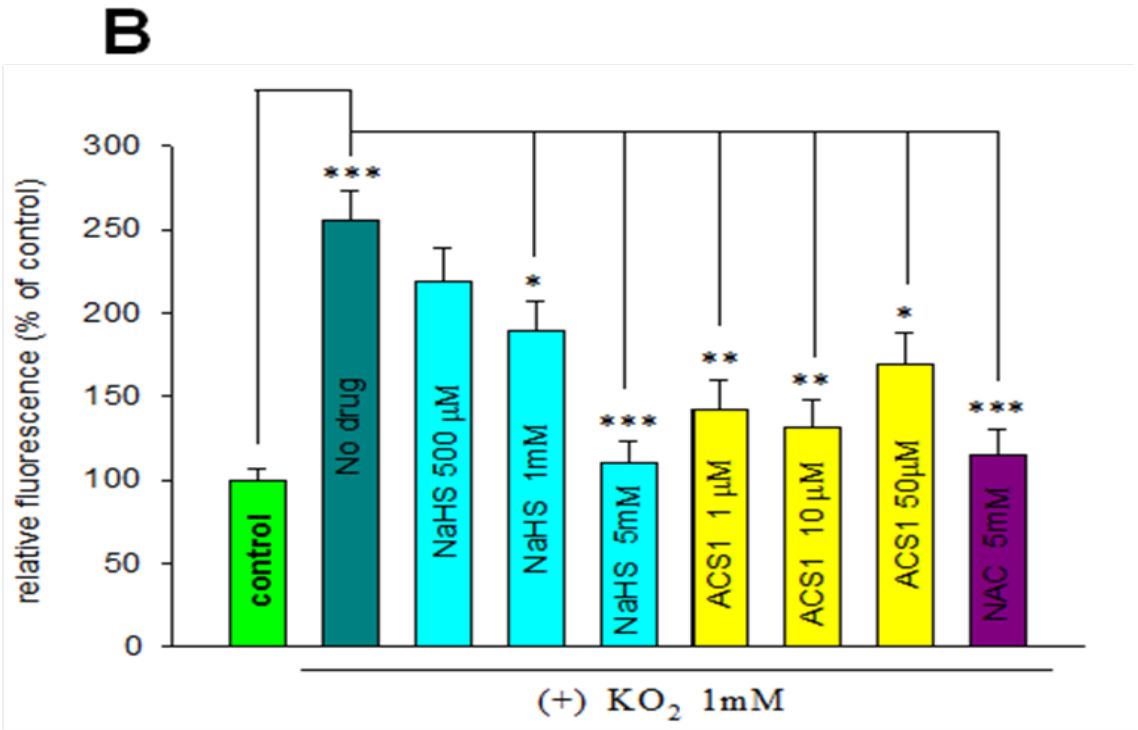
Figure 4.2: Detection of apoptosis, ROS and cell death. Cultures maintained in normal culture medium were confluent and did not stain red for apoptosis (phosphatidylserine) (A), red for ROS (E) or red rather than green for cell death (I). However, cultures incubated with 5 mM GB for 24 h resulted in reduced cell numbers

and morphology with some cells staining positively for ROS (F) and cell death (J). Inclusion of 1 mM NaHS did not appear to nullify the effects of GB (C,G,K). However, when 10 µM of ACS1 was included it reduced the negative effects of GB in causing apoptosis (D), stimulating ROS (H) and causing cell death (L). These experiments were repeated in three separate experiments.

4.2.3 ACS1 is more effective than NaHS in scavenging different types of ROS

The effect of GB 5 mM on ROS levels was subsequently quantified where 5 mM of GB was found to significantly increase ROS production and where ACS1 was more effective than NaHS at scavenging ROS. ACS1 as low as 1 µM was able to significantly reduce ROS levels (Figure 4.3 A). When H_2O_2 at 1 mM was added to RGC-5 cells, ROS activity (relative fluorescence units: RFU) was significantly increased (Figure 4.3 C). ACS1 between 1 µM and 50 µM significantly scavenged the H_2O_2 induced ROS. ACS1 also scavenged $O_2\cdot^-$ radicals generated by 1 mM KO_2 (Figure 4.3 B) and hydroxyl radicals produced by adding 1 mM H_2O_2 plus 100 µM Ferrous(II) perchlorate in the similar manner (Figure 4.3 D). Using rat brain homogenates, ACS1 was also found to be an effective inhibitor of SNP-induced lipidperoxidation with an IC50 value of 4.4 µM. In contrast, NaHS was less potent with a ten-fold greater EC50 value of 452 µM (Figure 4.4 E-G)


A
relative fluorescence (% of control)
250
200
150
100
50
***
control
No drug
NaHS 500 µM
NaHS 1mM
NaHS 5mM
ACS1 1 µM
ACS1 10 µM
ACS1 50µM
NAC 5mM
(+) Glutamate 5mM + BSO 5mM


B
relative fluorescence (% of control)
300
250
200
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100
50
0
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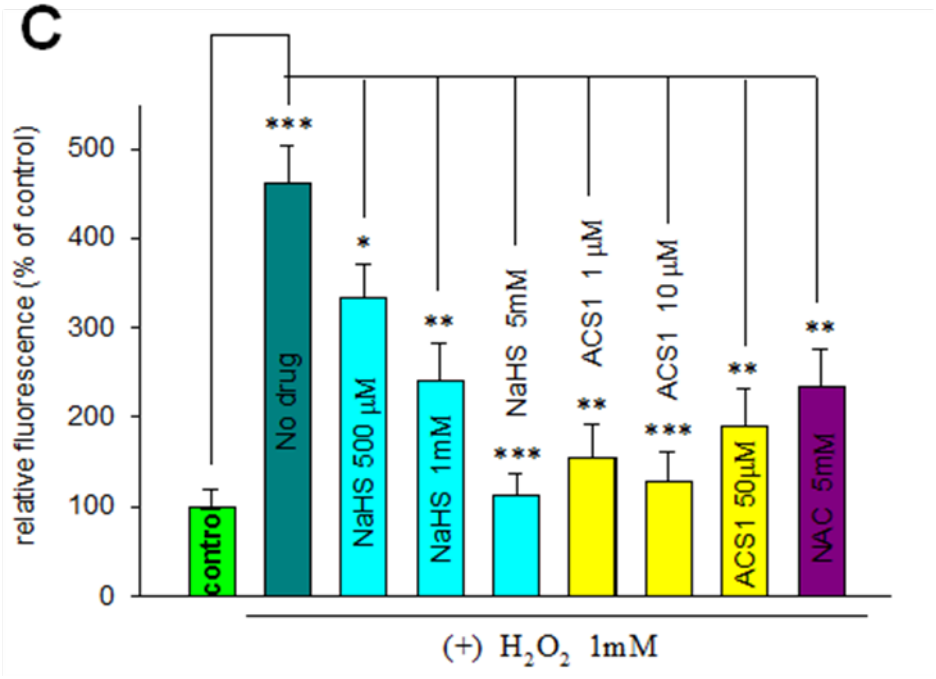
control
No drug
NaHS 500 µM
NaHS 1mM
NaHS 5mM
ACS1 1 µM
ACS1 10 µM
ACS1 50µM
NAC 5mM
(+) KO2 1mM


C
relative fluorescence (% of control)
0
100
200
300
400
500
control
No drug
NaHS 500 µM
NaHS 1mM
NaHS 5mM
ACS1 1 µM
ACS1 10 µM
ACS1 50µM
NAC 5mM
(+) H_2O_2 1mM

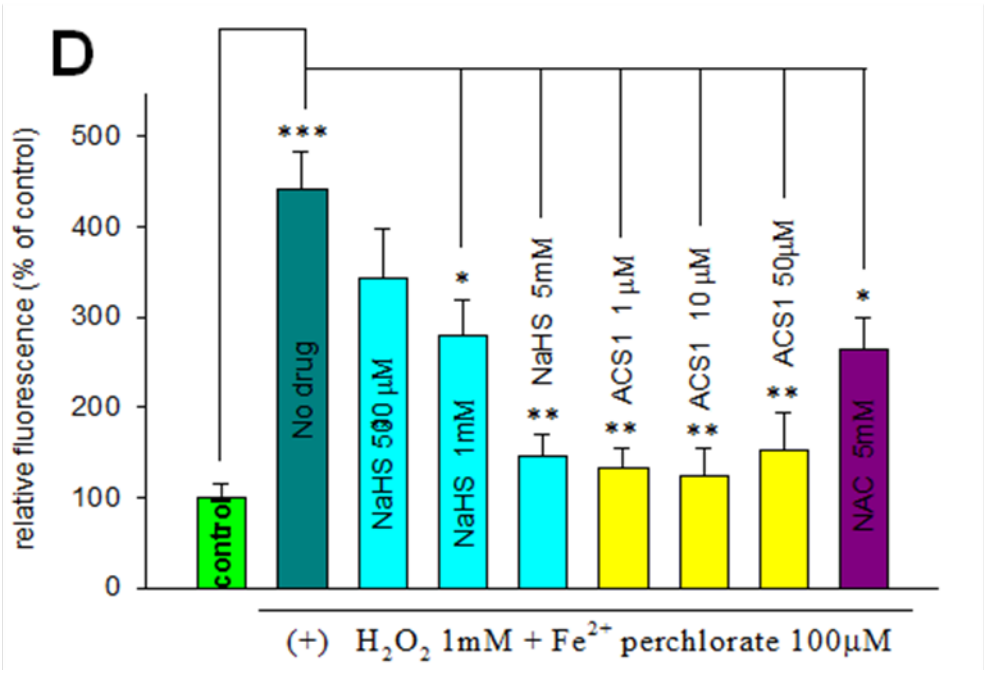
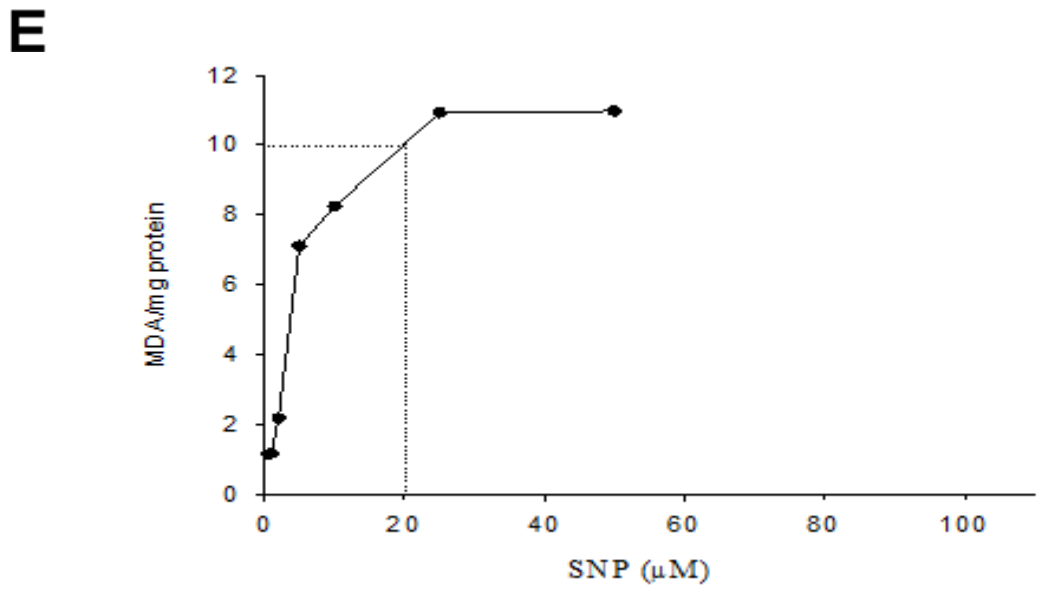

D
relative fluorescence (% of control)
0
100
200
300
400
500
control
No drug
NaHS 500 µM
NaHS 1mM
NaHS 5mM
ACS1 1 µM
ACS1 10 µM
ACS1 50µM
NAC 5mM
(+) H_2O_2 1mM + Fe^{2+} perchlorate 100µM

Figure 4.3: The effect of GB on ROS activity in RGC-5 cells using DCFH-DA, showed 5 mM of GB increased ROS production by more than two folds (A). NaHS from 1 mM and ACS1 from 1 µM significantly reduced GB induced ROS levels. Furthermore the addition of H_2O_2 at 1 mM to RGC-5 cells increased ROS activity significantly from the basal level without H_2O_2. NaHS from 500 µM and ACS1 from 1 µM significantly scavenged the H_2O_2 (C). NaHS and ACS1 also scavenged $O_2\cdot^-$ radicals generated by 1 mM KO_2 (B) and hydroxyl radicals produced by adding 1 mM H_2O_2 plus 100 µM Ferrous(II) perchlorate in the similar manner (D). Data are expressed as mean ± SEM where n=4 (*p < 0.05 **p < 0.01 ***p < 0.001)



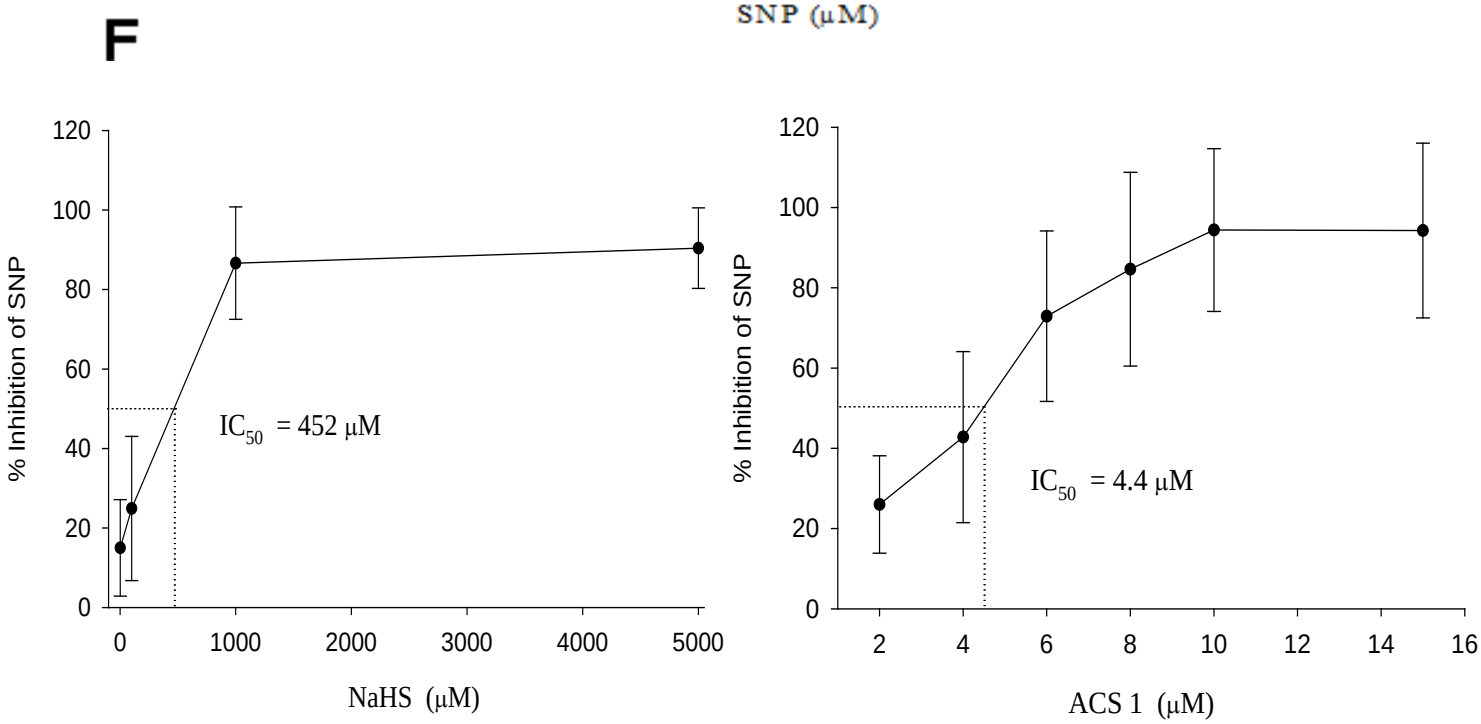
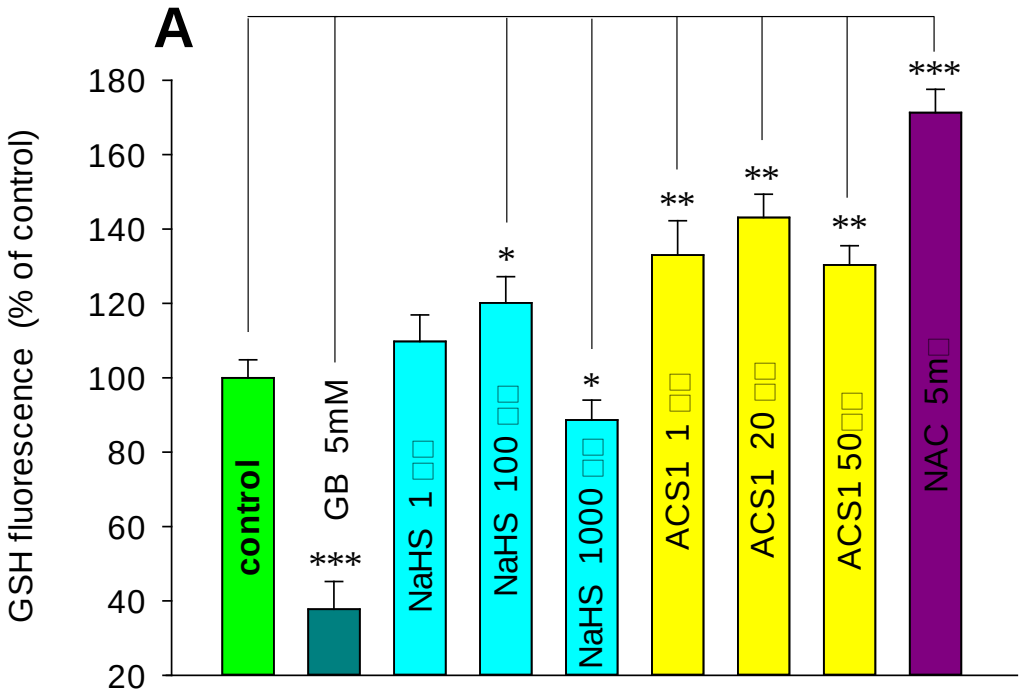


Figure 4.4: The effect of NaHS and ACS1 in inhibiting lipid peroxidation induced by SNP. (E) shows the effect of SNP in stimulating TBARS (which is related to malondialdehyde or MDA) formation in rat brain homogenates. SNP at a concentration of 20 µM caused a sub-maximum stimulation of TBARS. The effectiveness of ACS1 or NaHS in reducing the effect of 20 µM SNP is shown. NaHS inhibited 50% of SNP induced lipid peroxidation at 452 µM (F) while ACS1 at 4.4 µM (G). Data are expressed as mean ± SEM where n=6

4.2.4 The effect of GB on the antioxidant defense process of RGC-5 cells and influence of H_2S donating compounds

To further evaluate the beneficial effects of H_2S releasing sulphur compounds, their influence on key intracellular antioxidant components were investigated. The relative fluorescent intensity which corresponds to reduced glutathione levels in RGC-5 cells was measured after 24 h exposure to 5 mM GB. Treatment of RGC-5 cells with 5 mM GB resulted in significant depletion of GSH levels compared to control (Figure 4.5 A). The GSH levels in RGC-5 cells treated with defined concentrations of NaHS (100 µM) and ACS1 (1-50 µM) over 24 hours were significantly increased in both instances (Figure 4.5 A). However 1 mM NaHS caused a significant decrease in the basal level of GSH. When RGC-5 cells were pre-incubated with NaHS (100 µM), GST activity increased by approximately 20 % after 60 mins (Figure 4.5 B). ACS 1 even at very low concentration (1 µM) significantly stimulated GSH and GST in RGC-5 cells i.e. at 1 µM and 10 µM after 24 hours incubation (Figure 4.5 A and 4.5 B) respectively. As previously shown the sulphur compound NAC also stimulated GSH and GST significantly but at much higher concentration (Figure 4.5 A and 4.5 B).


A
GSH fluorescence (% of control)
180
160
140
120
100
80
60
40
20
control
GB 5mM
NaHS 1 □□
NaHS 100 □□
NaHS 1000 □□
ACS1 1 □□
ACS1 20 □□
ACS1 50□□
NAC 5m□
***
*
*
**
**
**
***

B

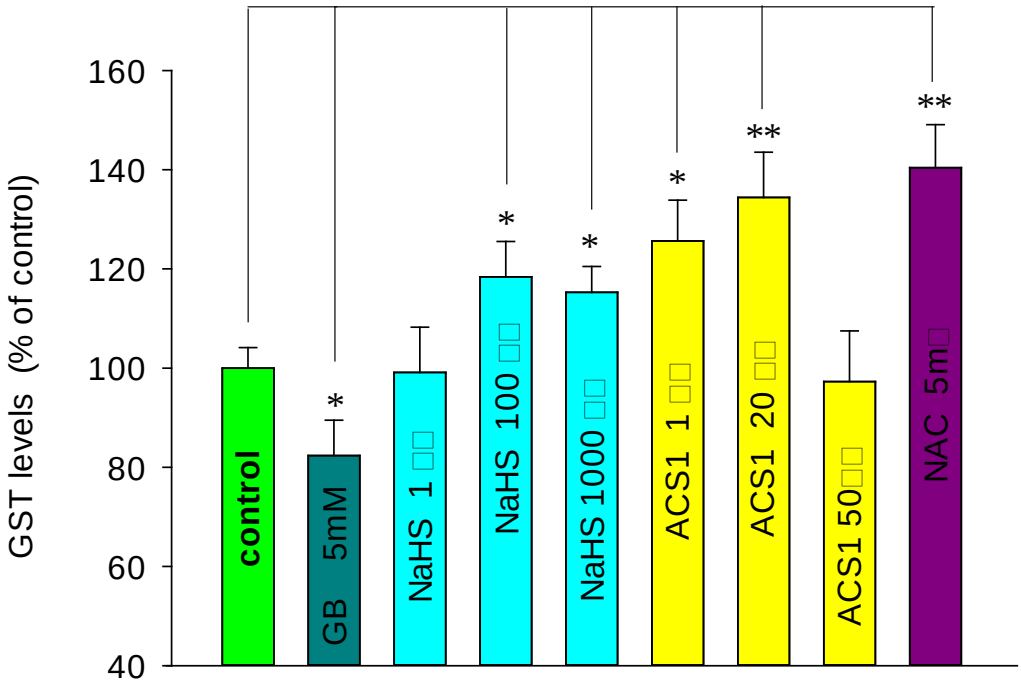


Figure 4.5: Effect of 5mM GB, NaHS and ACS1 on intracellular antioxidants levels. GSH intensity of RGC-5 cells increased significantly when the cells were treated with 100 µM NaHS over 24 hours (A) and an increased GST levels (B). ACS 1 significantly stimulated GSH, and GST at 1 µM and 10 µM. NAC as a positive

control also stimulated GSH and GST significantly (A and B). Data are expressed as mean ± SEM where n=4 (*p < 0.05 **p < 0.01 ***p < 0.001**)**

4.2.5 Effect of NaHS versus ACS1 on intracellular H_2S content over 24 hours

To compare the rates of H_2S production between NaHS and ACS1, H_2S concentration was measured over 24 hours at different time intervals. Exposure of RGC-5 cells to NaHS at 100 µM and 1 mM for 6 and 12 hours produced a rapid rise in intracellular H_2S production (Figure 4.6) However, after 24 hours no significant increase in basal levels of H_2S occurred with NaHS. In contrast, ACS1 is particularly effective at elevating H_2S after a period of 24 hours even at the lower concentration of 1 µM. At 6 hrs ACS1 showed no significant increase in H_2S levels and only 10 µM begins to stimulate H_2S production only at 12 hrs (Figure 4.6).

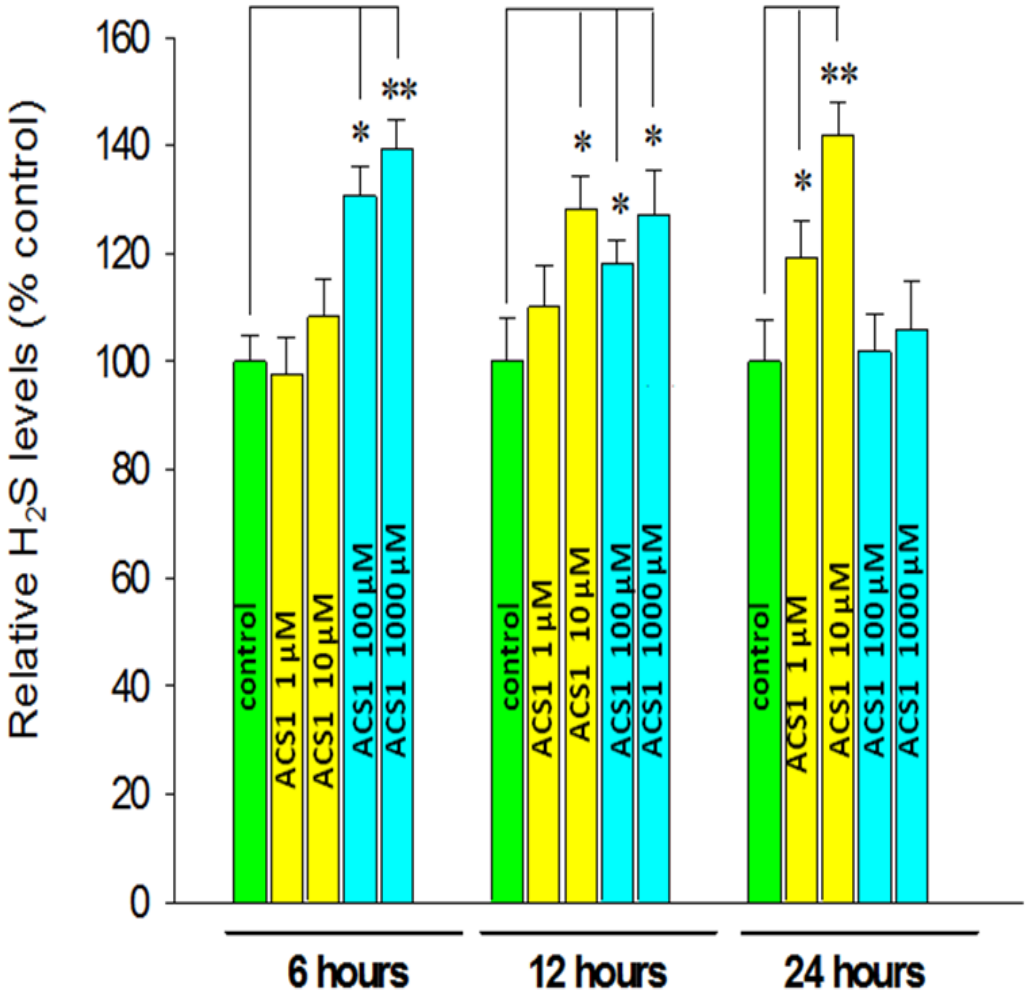

Relative H2S levels (% control)
160
140
120
100
80
60
40
20
0
control
ACS1 1 µM
ACS1 10 µM
ACS1 100 µM
ACS1 1000 µM
*
**
6 hours
12 hours
24 hours

Figure 4.6: Effect of NaHS and ACS1 on intracellular H_2S activity. H_2S levels were detectable with NaHS incubation at 100 µM and 1 mM up to 12 hours but not at 24 hours whereas ACS1 treatment at 1 µM and 10 µM significantly increased H_2S production only at 24 h. Data are expressed as mean ± SEM where n=4 (*$p < 0.05$ **$p < 0.01$ ***$p < 0.001$)

4.2.6. GB and NaHS increase Nrf2, but inclusion of ACS1 causes its inhibition in RGC-5 cells

RGC-5 cells were exposed to NaHS (100 µM), GB (5 mM) or ACS1 (10 µM) for 24 h and processed for the localization of Nrf2 immunoreactivity. Nrf2 immunoreactivity throughout the cell with a possible slight relative increase in the nucleus was noted when compared with the cytoplasm. Use of DAPI to locate the nucleus suggested this to be the case. The effect of GB on Nrf2 immunoreactivity was partially mimicked by 10 µM ACS1 but not by 100 µM NaHS (Figure 4.7 L, I) Western blot and qPCR studies of fractionated RGC-5 cells exposed to the various substances provided support for the idea that GB (5 mM) and to a lesser extent ACS1 (10 µM) both increase Nrf2 protein (Figure 4.7M) and mRNA (Figure 4.7 O) contents. In contrast NaHS only at a concentration of 1 mM had a minor but significant effect at increasing Nrf2 mRNA and protein levels.

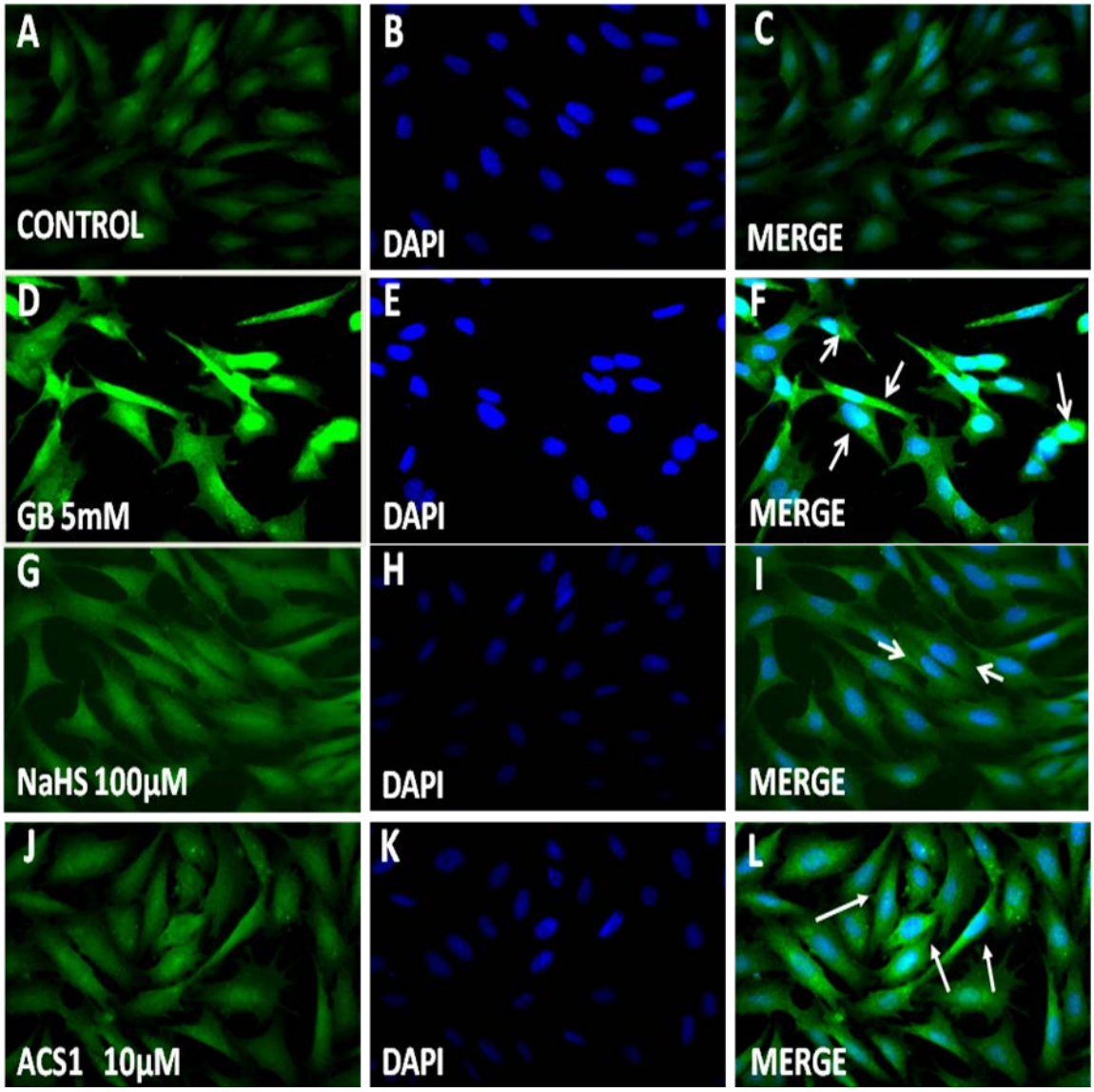


Figure 4.7(A-L): Effect of GB, ACS1 and NaHS on Nrf2 expression via immunocytochemical detection of Nrf2 cellular localization. GB (5mM), ACS1 (10 µM) and NaHS (100 µM) treated RGC-5 cells were cultured over 24 h and stained with Nrf2 antibody and counterstained with DAPI. Increased Nrf2 immunoreactivity is seen in the cytosol and nucleus (white arrow) confirmed by blue DAPI-stained nucleus (F and L) especially in ACS1 and GB treated groups. NaHS treatment showed little difference in immunoreactivity (I) compared to control. These experiments were repeated in three separate experiments.

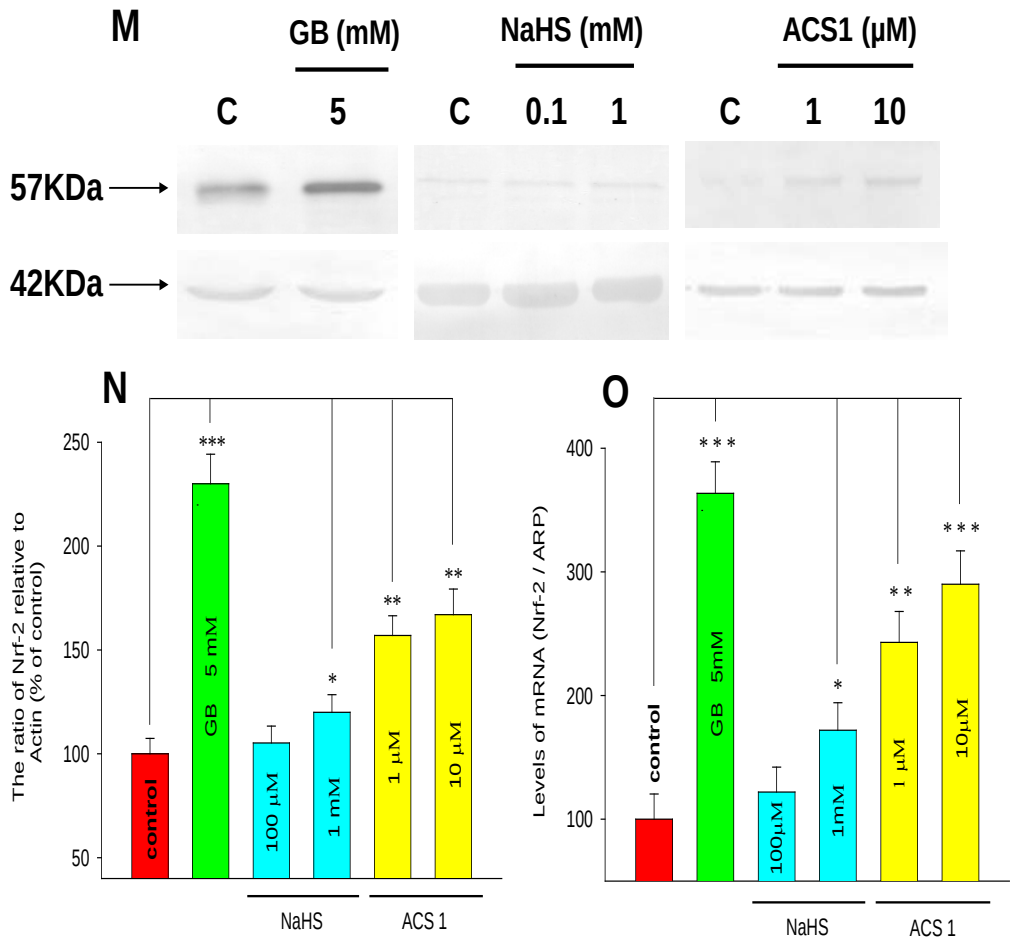


Figure 4.7 (M-O): Effect of GB, ACS1 and NaHS on Nrf2 expression. Western blot analysis also shows that GB 5 mM as well as NaHS 1mM significantly increased Nrf2 protein relative to actin when compared to control. ACS1 at 1 and 10 µM also significantly caused an upregulation of Nrf2 protein. qPCR studies also show similar increase in Nrf2 mRNA levels with 10 µM ACS1 significantly stimulating Nrf2 mRNA. Data are expressed as mean ± SEM (n=4), *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$ vs control, Bonferroni test.

4.2.7 H_2S donors stimulates Nrf2 dependent HO-1 expression

Hemeoxygenase protein and mRNA levels in RGC-5 cells were determined by Western blotting and qPCR studies. An increase in HO-1 protein levels and mRNA levels was noted with ACS1 (1µM) but only the protein levels and not the mRNA was detectable with 10 µM ACS1 inclusion. NaHS (1 mM) and GB (5 mM) exposure also significantly increased HO-1 levels (Figure 4.8)

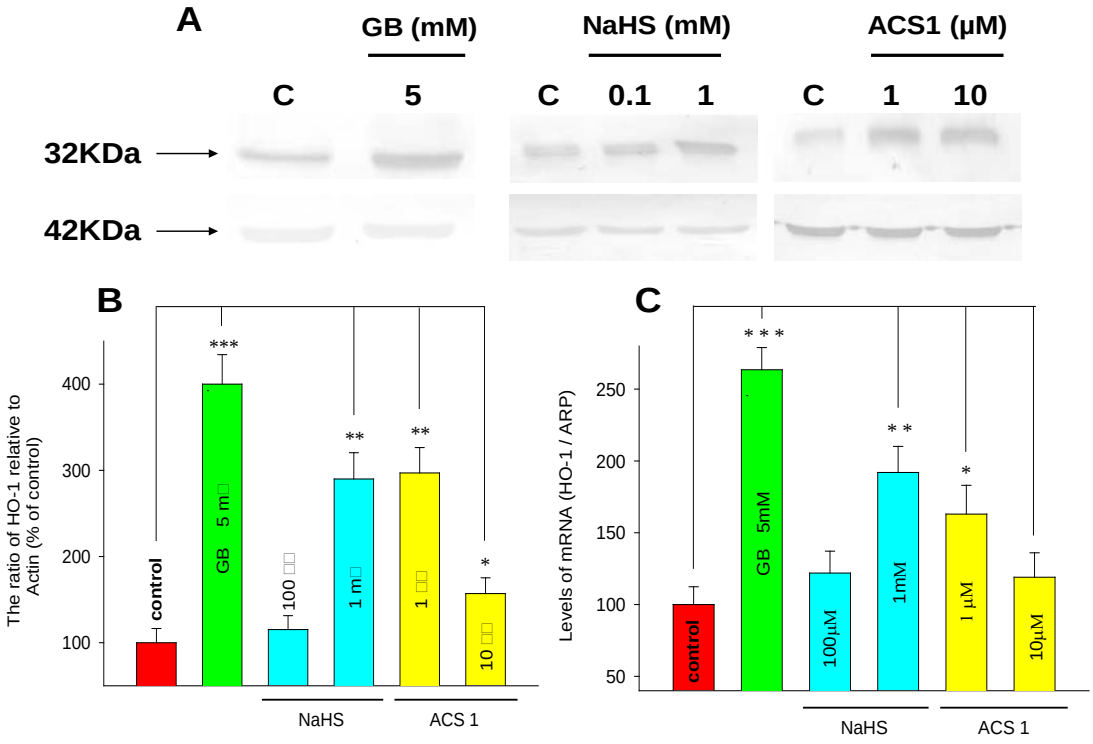


Figure 4.8 A-C: Effect of GB, ACS1 and NaHS on HO-1 levels in RGC-5 cells quantified using western blotting and qPCR. Both studies confirmed the presence of HO-1 in RGC-5 cells (A-C). Cells treated with GB, ACS1 and NaHS over 24 hours were compared to control. ACS1 1 µM significantly stimulated HO-1 expression, while NaHS at 1 mM also stimulated HO-1 expression. GB 5 mM significantly increased the protein expression of HO-1 by more than 3 folds in Western blotting studies (A) and HO-1 mRNA activity increased more than 2 folds (C). Each value is

the mean ±SEM where n=4 (*p < 0.05 **p < 0.01 ***p < 0.001), vs control, Bonferroni test.

4.2.8 The effect of GB, ACS1 and NaHS on protein and mRNA levels of PKC*α* NFkB, Bcl2, PARP, XIAP and p-AKT

To investigate the differences between ACS1 and NaHS in influencing the levels of selected markers, the exposure of RGC-5 cells to these substances for 24 h were investigated. The effects of GB were also investigated independently. Exposure of RGC-5 cells to NaHS 100 µM or ACS1 (1 and 10 µM) resulted in a significant stimulation of PKC*α* at protein and mRNA levels (Figure 4.9 A-C). GB also caused a significant decrease in protein and mRNA levels of PKC*α* (Figure 4.9 A-C) and an increase of NfkB (Figure 4.9 D-F) respectively.

Immunocytochemistry studies also support the involvement of PKC*α* in H_2S signaling with NaHS at 100 µM and ACS1 at 10 µM significantly stimulated the levels (Figure 4.10 C and D). GB also clearly inhibits the levels of PKC*α* (Figure 4.10 B).

The effects of 24 h GB, NaHS or ACS1 on antiapoptotic proteins were also investigated. Bcl-2 was significantly decreased 24h post GB treatment (Figure 4.11 A-C). ACS1 (10 µM) significantly increased the antiapoptotic proteins Bcl-2 expression. Consistently increased expression of Bcl-2 mRNA levels was also noticeable with ACS1 (10 µM) and also 100 µM of NaHS inclusion but not NaHS at 1 mM which caused a significant decrease in both protein and mRNA levels (Figure 4.11 B, C). Only ACS1 had positive effects on the antiapoptotic protein XIAP levels (Figure 4.11 D-F) and NaHS had no significant influence on XIAP levels. Studies on the effect of GB and the influence of ACS1 and NaHS on p-AKT levels showed

significant decrease in p-AKT levels with 24 h GB treatment. NaHS (100 µM) significantly increased p-AKT protein levels but no increase in mRNA levels was detected (Figure 4.11 G-I). In contrast ACS1 (1 µM and 10 µM) increased both p-AKT protein and mRNA levels.

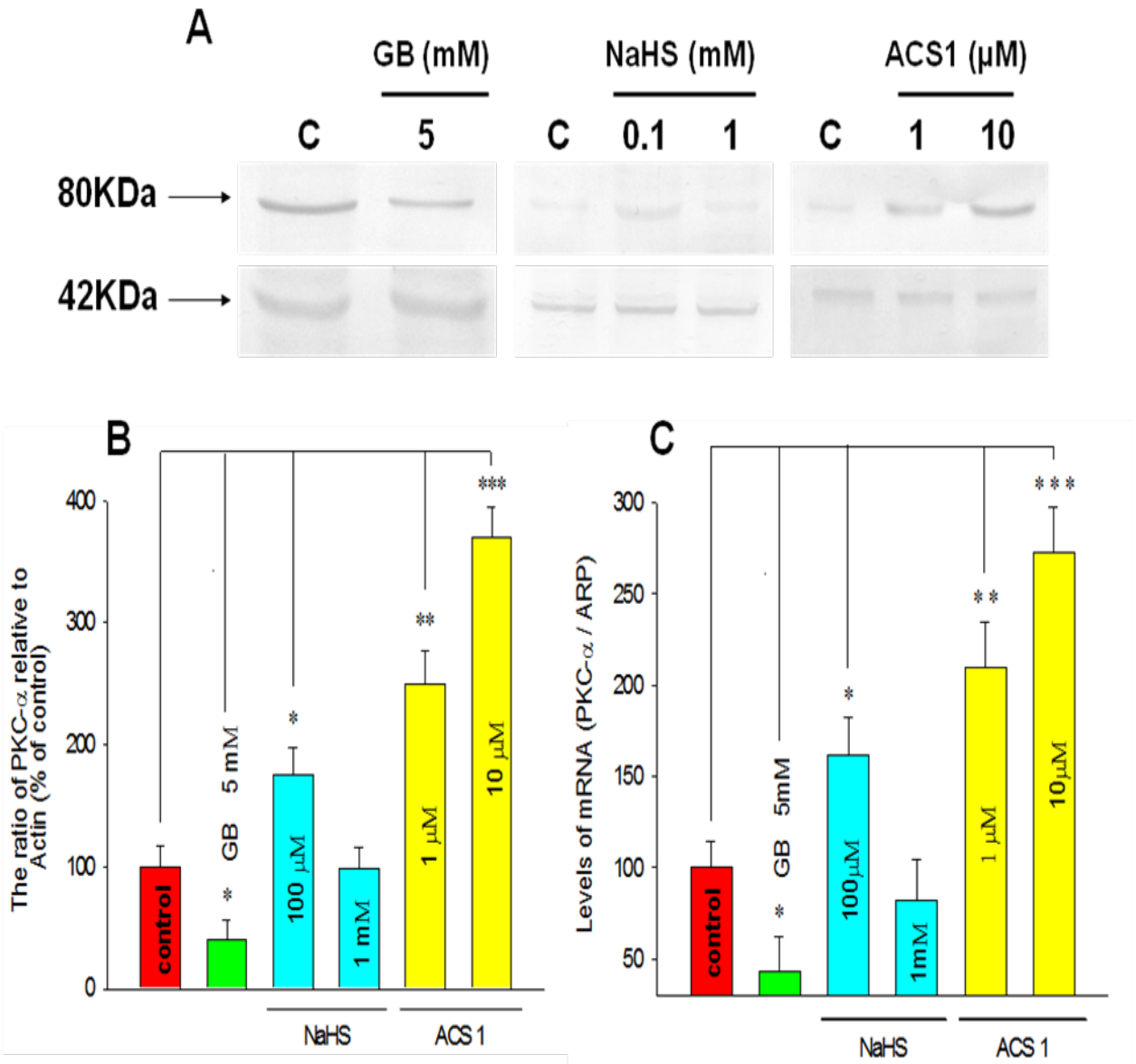


Figure 4.9 (A-C): Western blot analysis shows that GB decreased PKCα protein (relative to actin) (A and B) and mRNA (relative to ARP) (C) levels compared to

control. ACS1 (1 and 10 µM) and NaHS 100 µM significantly stimulated PKCα levels. Data are expressed as mean ± SEM where n=4, (*p < 0.05 **p < 0.01 ***p < 0.001) vs control, Bonferroni test.

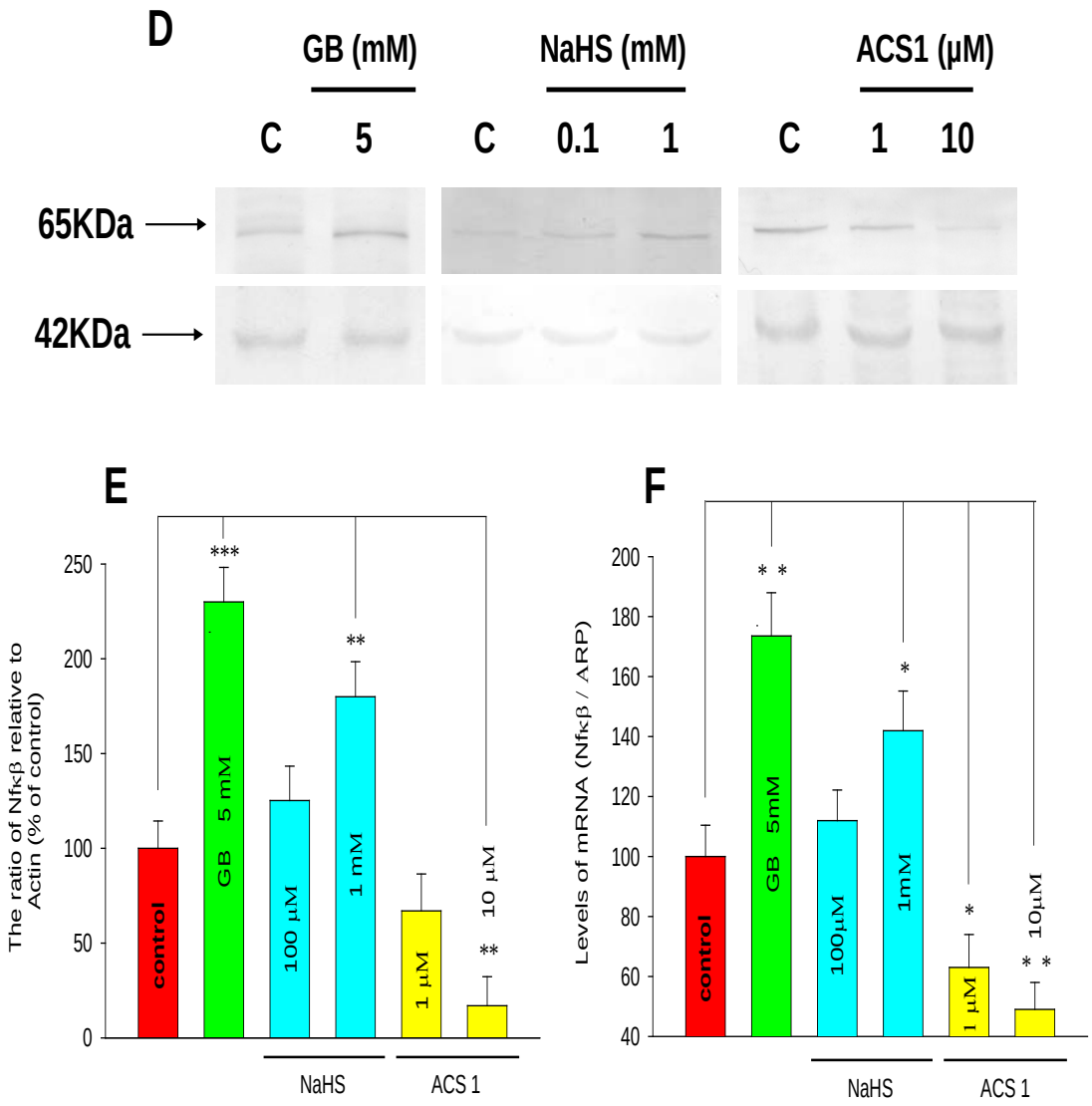


Figure 4.9 (D-F): Western blot analysis shows that GB treatment stimulated NfkB protein expression relative to actin (E) and also the mRNA levels compared to control (F). However ACS1 10 µM significantly decreased NfkB protein and mRNA levels (E, F). NaHS at toxic levels (1000 µM) significantly increased NfkB expression both

at protein and mRNA levels but had no effect at 100 µM. Data are expressed as mean ± SEM where n=4 (*p < 0.05 **p < 0.01 ***p < 0.001).

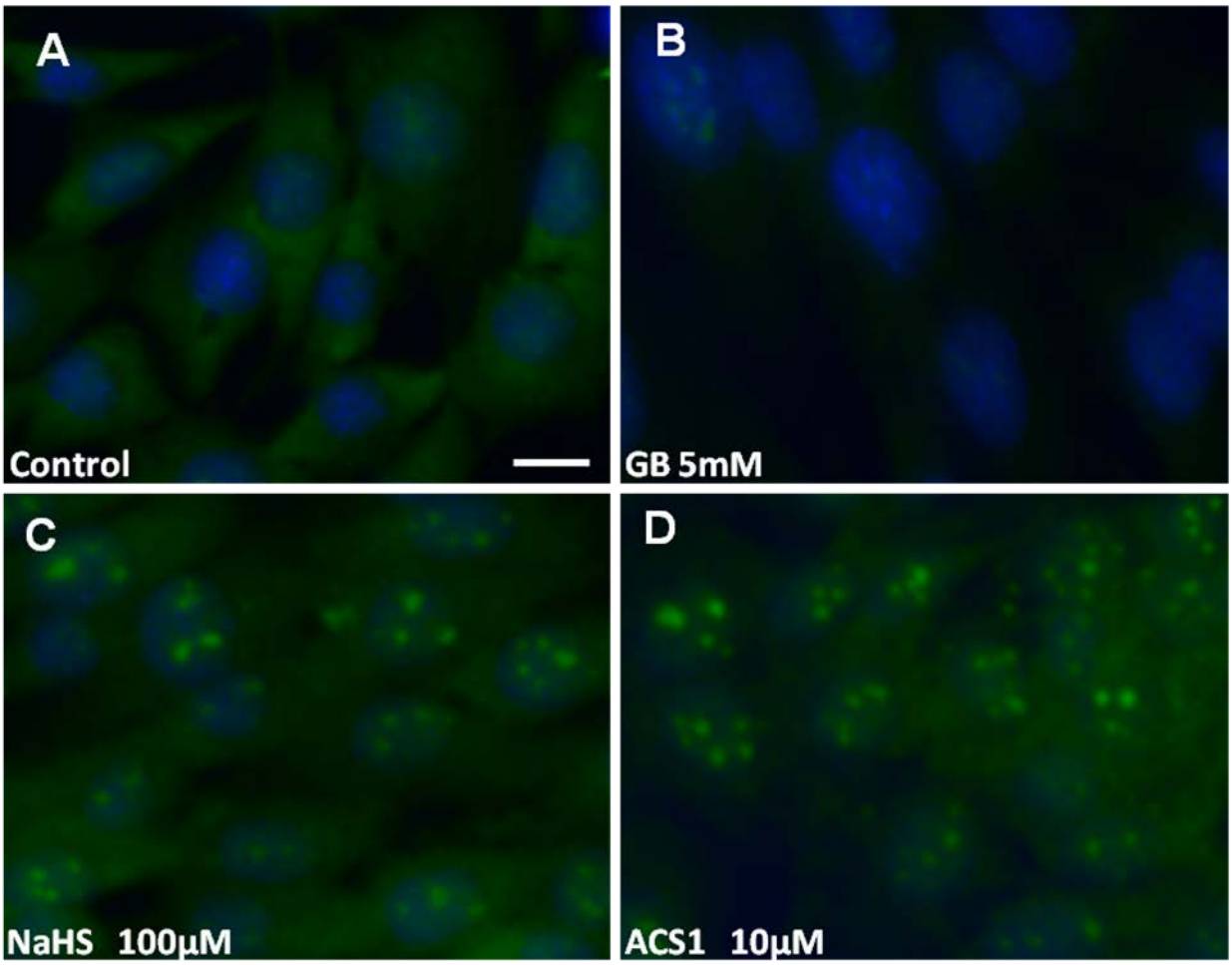


Figure 4.10 (A-D): Effect of GB, ACS1 and NaHS on PKCα levels. Immunocytochemical detection shows PKCα translocation to the nucleus (C and D). Control, GB 5mM, ACS1 10 µM and NaHS 100 µM treated RGC-5 cells were cultured over 24 h and stained with PKCα antibody and counterstained with DAPI. Increased PKCα activity from the cytosol to the nucleus was observed principally as increasing immunoreactivity within the nuclear region and the nucleus was confirmed by blue DAPI-stained nucleus (A). NaHS at 100 µM and ACS1 10 µM clearly increased the immuoreactivity of RGC-5 cells to PKCα especially within the nucleus

but GB exposure showed a significant down regulation in immunoreactivity compared to control. Immunocytochemistry studies were repeated three times, and representative results are shown. Scale bras=60µm

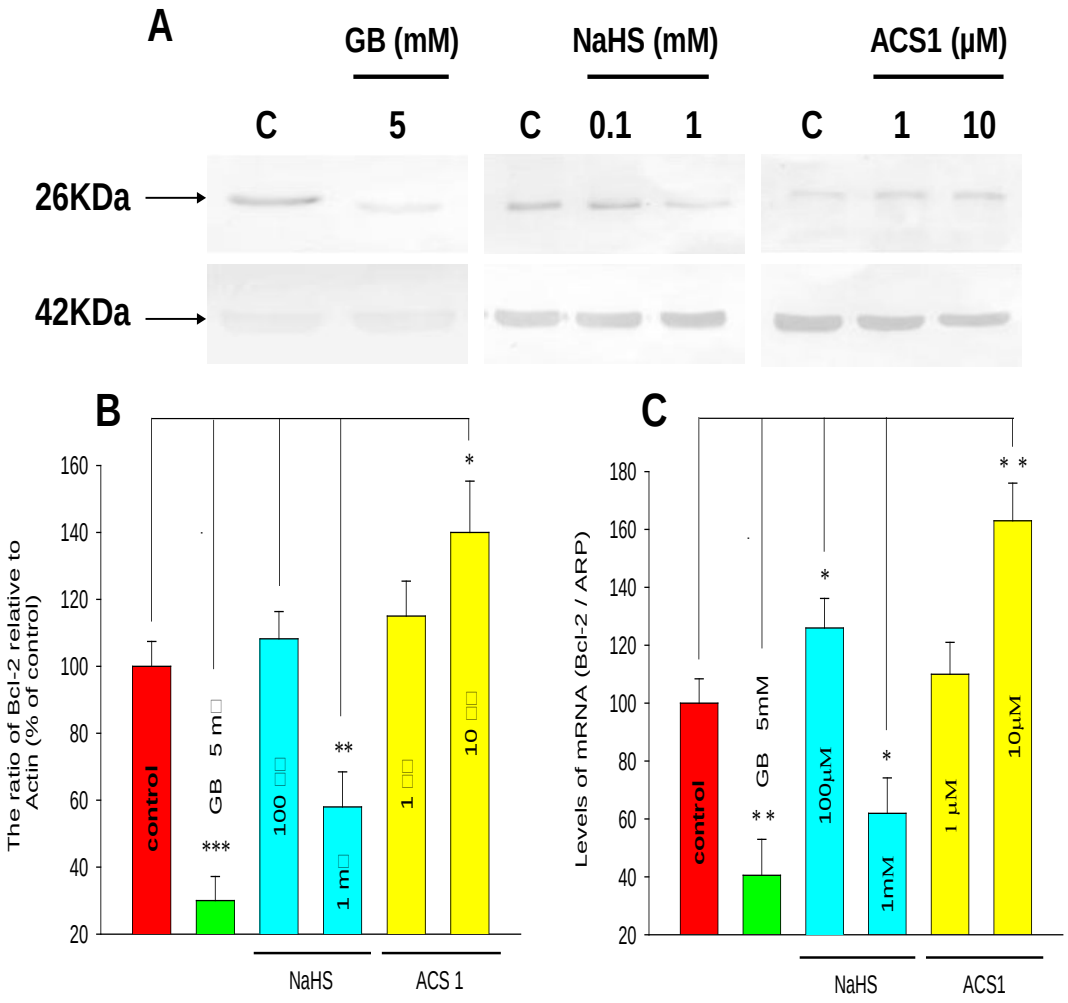


Figure 4.11 (A-C): Western blot analysis shows that GB and NaHS (1 mM) both inhibited Bcl-2 expression relative to actin and mRNA levels compared to control (B, C). However NaHS at non toxic concentration (100 µM), appears to be stimulating its expression at mRNA level but not at protein level. ACS1 at 10 µM significantly

increased both its protein and mRNA levels. Data are expressed as mean ± SEM where n=4 (*p < 0.05 **p < 0.01 ***p < 0.001), vs control, Bonferroni test.

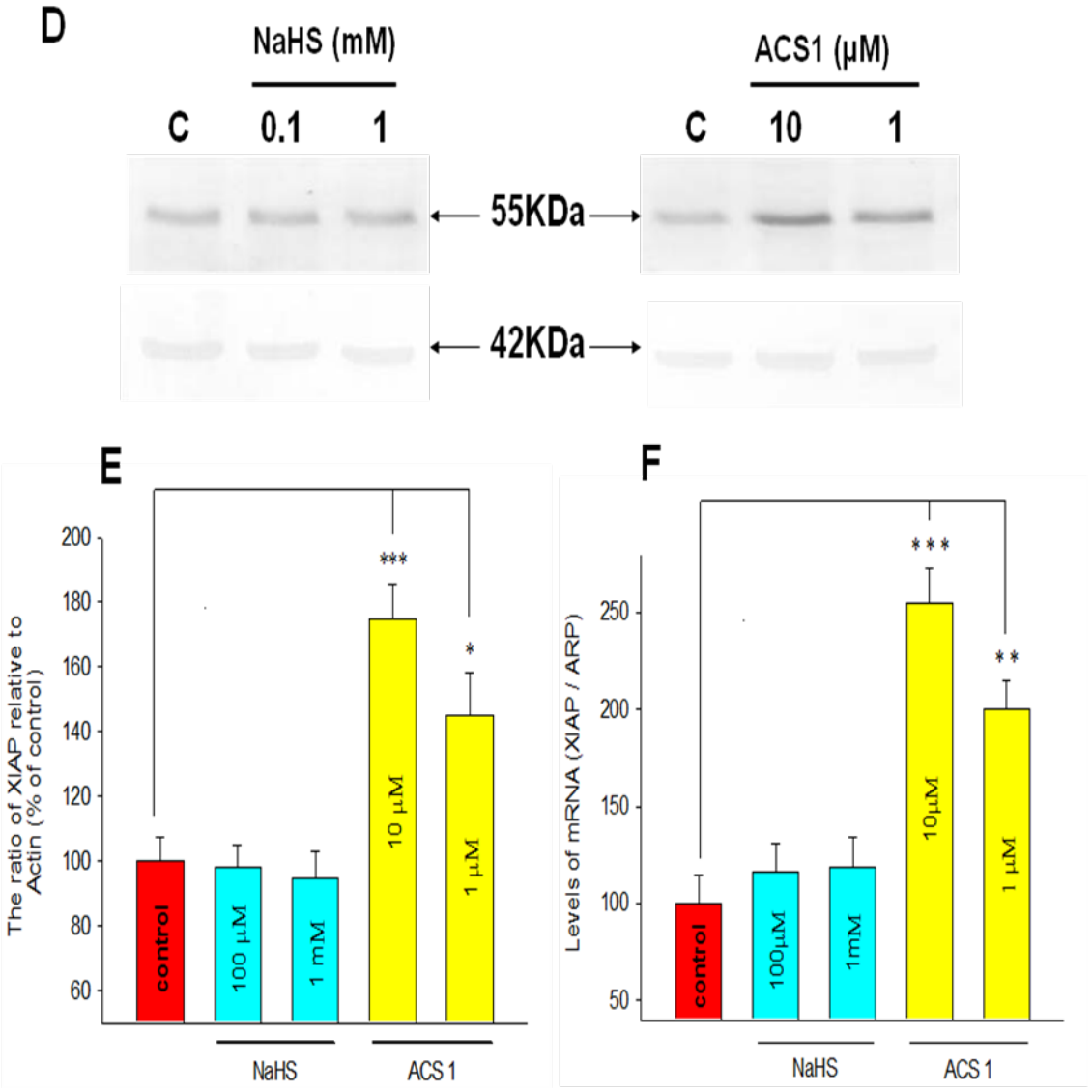


Figure 4.11 (D-F): Western blot analysis shows that NaHS treatment did not affect the levels of XIAP protein and mRNA levels relative to actin and ARP respectively

compared to control (E,F). However ACS1 at 1 µM and 10 µM significantly increased both its protein and mRNA expression. Data are expressed as mean ± SEM where n=4 (*p < 0.05 **p < 0.01 ***p < 0.001), vs control, Bonferroni test.

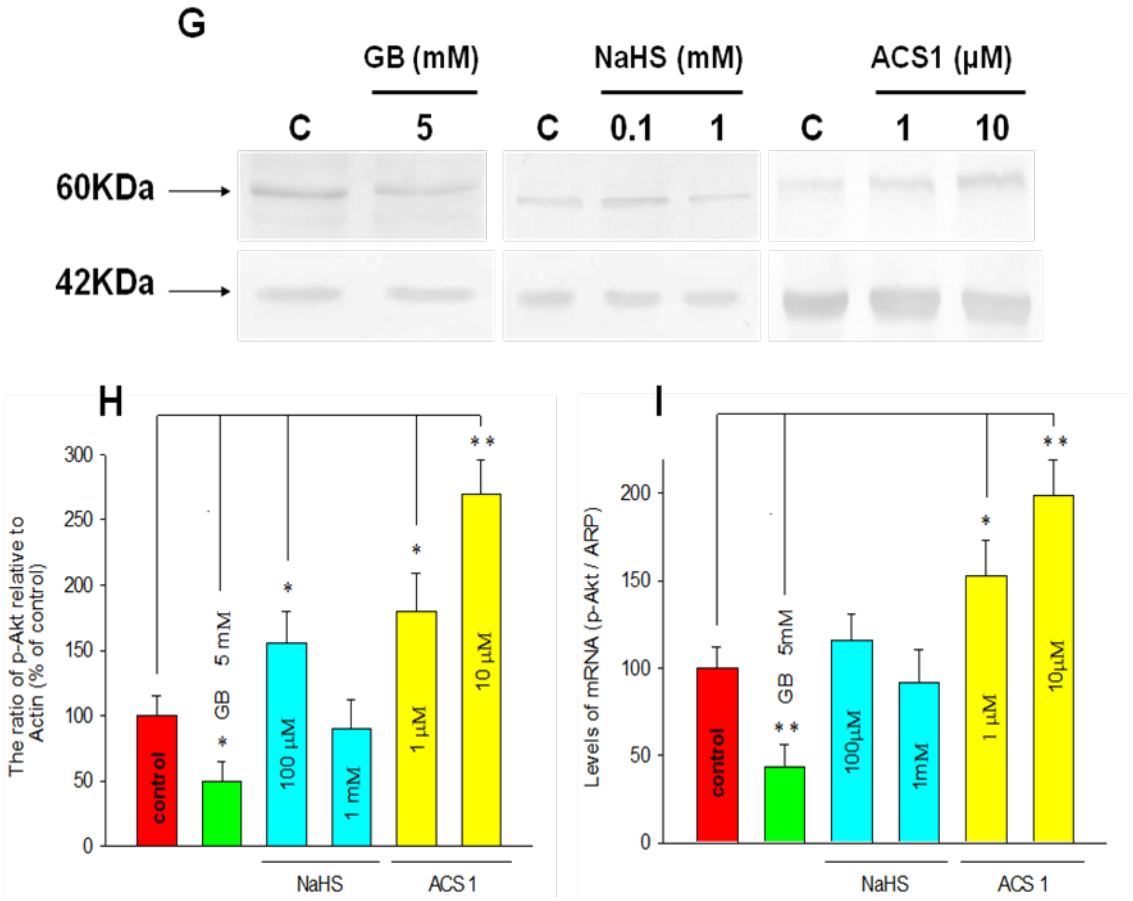


Figure 4.11 (G-I): Western blot analysis shows that GB treatment effectively decreased the expression of p-AKT protein and mRNA levels relative to actin and ARP respectively compared to control (G,I). ACS1 at 1 µM and 10 µM significantly increased both its protein and mRNA expression. NaHS only at 100 µM significantly

increased p-AKT at protein level. Data are expressed as mean ± SEM where n=4 (*p < 0.05 **p < 0.01 ***p < 0.001), vs control, Bonferroni test.

4.2.9 GB treatment stimulates system X_c^- by upregulation of xCT expression but H_2S donors significantly downregulated xCT expression

The expression of the glutamate/cystine exchanger xCT was investigated 24h post ACS1, NaHS or GB 5mM treatment. GB significantly stimulated xCT expression both at protein and mRNA levels however both NaHS and ACS1 at all concentrations significantly inhibited its expression at both protein and mRNA levels (Figure 4.13 (A-C).

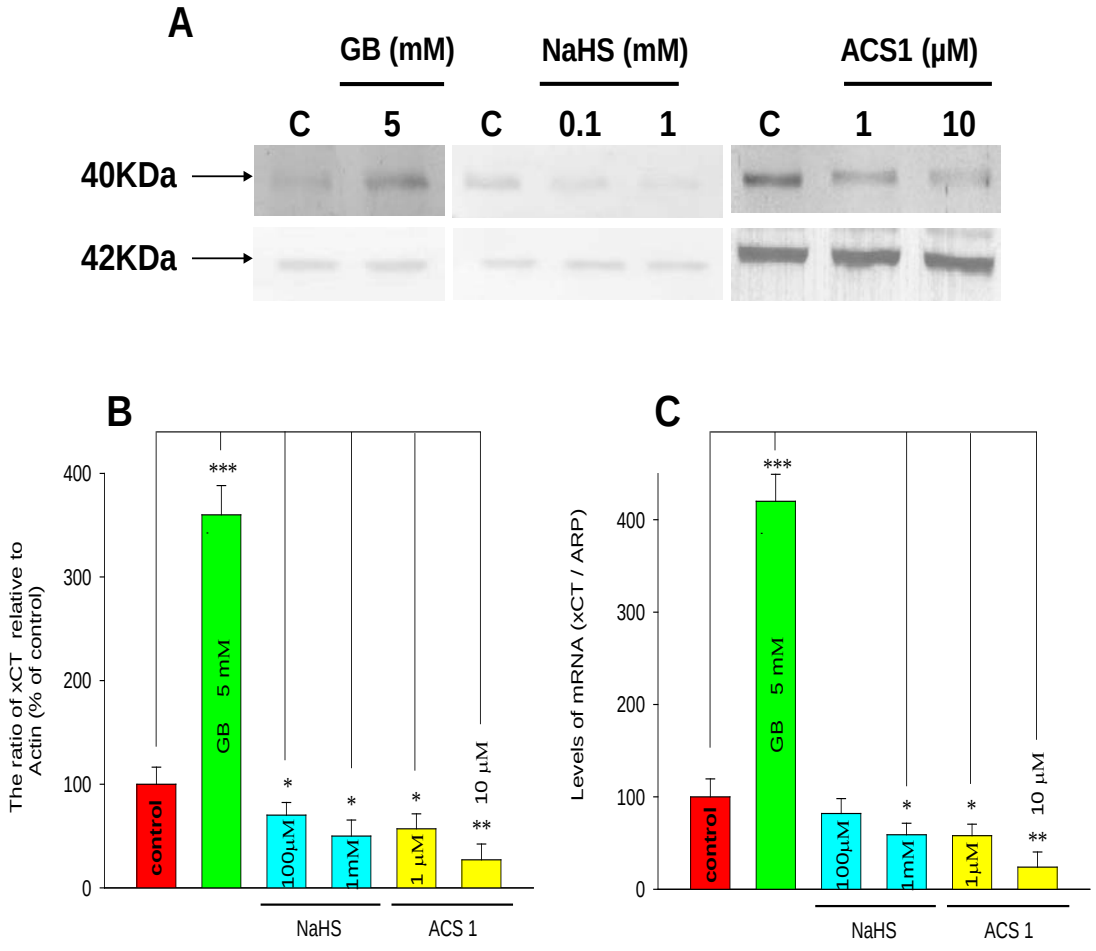


Figure 4.12 (A-C): Western blot analysis shows that GB treatment stimulated xCT receptor protein levels relative to actin (A and B) and also the mRNA levels compared to control (C). However both ACS1 and NaHS significantly decreased xCT significantly both at protein and mRNA levels (B and C). Data are expressed as mean ± SEM where n=4 (*p < 0.05 **p < 0.01 ***p < 0.001), vs control, Bonferroni test.

4.2.10 Schematic summary of the results in section 4.2

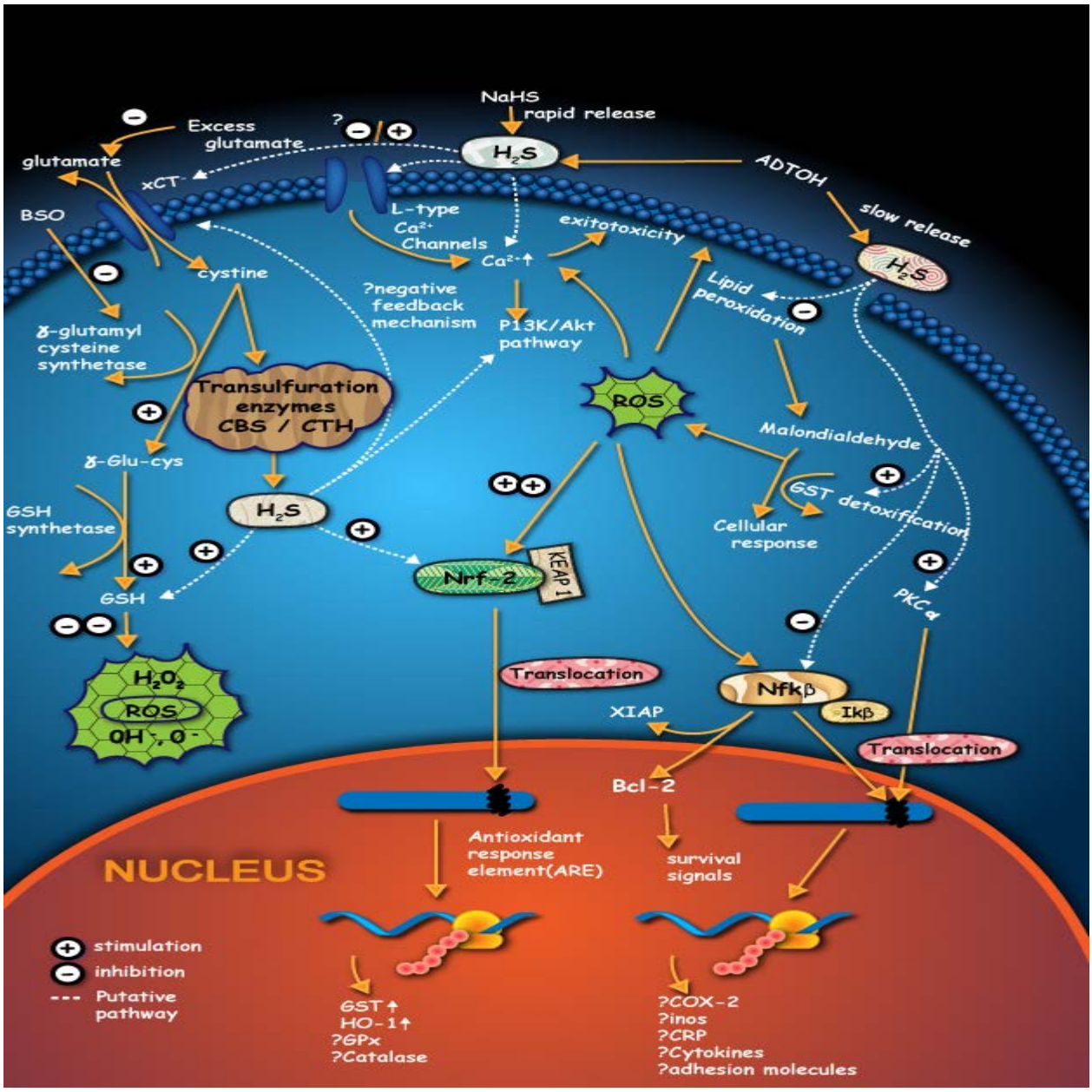


Figure 4.13: The putative signaling pathways of H_2S released via two different donors ACS1 (ADTOH, a slow donor) and NaHS (a rapid donor) in GB induced cell death are shown. Cysteine is metabolised from methionine (not shown) or from and exogenous source of cystine which enters the cell via xCT transporter and can be converted via CBS/CSE enzymes to form H_2S. Cysteine is also the rate limiting substrate to produce GSH. Excess extracellular glutamate can cause inhibition of cystine uptake via xCT and hence reducing H_2S and GSH levels. It is proposed that H_2S levels may trigger a negative feedback mechanism on xCT to regulate its expression. H_2S may also cause stimulation of PI3K/p-Akt pathway. This can also be activated in response to increase ca2+ levels via its stimulation of L-type calcium channels. GSH formation can be inhibited by the action of BSO on the rate limiting enzyme γ-glutamyl-cysteine-synthetase. GSH has various metabolic functions including scavenging excessive production of ROS such as H_2O_2, OH^- and O_2^-. The level of ROS or H_2S generated or H_2S levels can both cause redox modification of the transcription factor Nrf2 and stimulate its translocation to the nucleus leading to increased production of phase II antioxidant enzymes including GST, HO-1, catalase and GPx. Expression of these enzymes protects cells from oxidative damage and can prevent apoptosis. Transcription of their genes is coordinately regulated through antioxidant response elements (AREs). Inactive Nrf2 is retained in the cytosol by forming a complex with the cytoskeletal protein Keap1. Also shown is the GST detoxification reaction with the lipid peroxidation product malanodialdehyde which is inihibited by the H_2S released from ACS1. H_2S at low level may also activate PKCα, induce its translocation to the nucleus to cause changes in gene expression or inhibition of NfkB to the nucleus. NfkB activity in turn can modulate pro-survival proteins such as Bcl2, XIAP and inflammatory mediators.(own source)

4.3. Discussion

Various theories have been proposed to explain the pathophysiology of glaucoma that results in ganglion cells dying by apoptosis. Oxidative stress in particular has been proposed as one of the causes for ganglion cells dying in glaucoma animal model (Moreno et al., 2004). There is good reason to believe that oxidative stress can be caused by raised extracellular glutamate levels (Simonian and Coyle 1996). Glutamate is a major excitatory neurotransmitter in the brain and retina, and has also been implicated in nerve cell death after acute neurologic insults and in several different ocular diseases. High level of extracellular glutamate is toxic to nerve cells

in culture elicited by two processes, excitotoxicity and by affecting the glutamate/cystine transporter to cause oxidative stress. In excitotoxicity, ionotropic glutamate receptors in particular are over-activated, resulting in disruption of calcium homeostasis and cell death (Rothman 1985). Excess extracellular glutamate can also cause a reversal of the glutamate/cystine transporter system to mediate a series of disturbances to the intracellular redox system that includes the elevation of mitochondrially generated ROS (Simonian and Coyle 1996, Maher and Hanneken 2005). Since adequate cystine uptake into cells is essential for the biosynthesis of GSH and H_2S, inhibition of the glutamate-cystine antiporter system (X_c^-) by high levels of exogenous glutamate, is detrimental to cell survival (Tomi et al. 2002). This process is exacerbated by addition of BSO to excess extracellular glutamate in cell culture studies resulting in a significant depletion of GSH to cause oxidative stress and cell death (Maher et al 2005). It has been demonstrated in rat retinal capillary endothelial cell line that xCT mRNA level, L-cystine transport activity, and GSH levels were enhanced under oxidative stress conditions (Tomi et al. 2002).

Thus in this study as in chapter three, the effect of simulated oxidative stress conditions by the exposure of RGC-5 cells to high concentrations of exogenous glutamate in combination with an inhibitor of GSH biosynthesis, BSO, which consequently leads to endogenous over production of ROS was used as a method to induce cell death. This was demonstrated in chapter three to cause both the depletion of GSH from the cells within 24 hours of treatment and subsequent increase in ROS 2.5 fold compared to control. It is for these reasons why GB is much more toxic to RGC-5 cells in culture than glutamate on its own.

GSH synthesis is dependent on ATP-dependent enzymes, γ-glutamylcysteine synthetase and glutathione synthetase. It was reported that the levels of both these key enzymes are affected by oxidative stress responsive transcription factor Nrf2 (Chan and Kwong 2000). As mentioned earlier system X_c^- regulates the concentration of intracellular cystine, a component substrate for GSH synthesis. Moreover the expression of system X_c^- is controlled by Nrf2 (Ishii et al. 2000). In this study GB caused a significant upregulation of xCT whereas ACS1 and NaHS decreased the levels of xCT. These findings suggest Nrf2 is able to regulate intracellular GSH levels. Furthermore Nrf2 activity also regulates the sensitivity of death receptor signals by means of regulating intracellular GSH levels (Morito et al. 2003). In the present study GB treatment caused altered antioxidant status because of elevated ROS, GSH depletion and effects on the antioxidant enzyme HO-1 and GST. In addition GB treatment causes alterations in Nrf2 and prosurvival proteins. GB also caused a significant increase of xCT levels while H_2S donating compounds reduced xCT significantly.

The current study also shows that GSH depletion in RGC-5 cells following GB treatment influences PKCα levels. In the present study GB decreased PKCα protein and activated NfkB. Similarly Kim et al. (2002) showed that expression of PKCα in articular chondrcytes blocked both NfkB activation and apoptosis. Furthermore inhibition of PKCα or -zeta activities potentiated NO-induced apoptosis, whereas expression of these isoforms significantly reduced the number of apoptotic cells (Kim et al. 2002). Expression of PKCα or zeta did not affect p38 kinase or ERK but

inhibited the p53 accumulation and caspase-3 activation that are required for NO-induced apoptosis of chondrocytes (Kim et al. 2002). When NfkB is activated it is translocated to the nucleus because of the phosphorylation and proteolytic degradation of the inhibitory protein IkB (Ghosh et al, 1998). This then can cause changes in transcriptional activity and changes in gene expression. In the present study it was shown that GB treatment of RGC-5 cells causes the activation of NfkB (Figure 4.9). Ishige et al. 2005 also showed glutamate activates distinct NF-kB/Rel proteins, which play pivotal roles in enhancing or repressing cell death pathways, and glutamate-induced neuronal death in HT22 cells (Ishige et al. 2005). In resting cells, NF-kB/Rel factors are retained in the cytoplasm by association with the inhibitory IkB proteins. In stimulated cells, IkB is phosphorylated, ubiquitinated, and then degraded through a proteasomal pathway, thus allowing its release and leading to nuclear translocation of NF-kB/Rel dimmers (Ishige et al. 2005). In the current study, the H_2S donor NaHS at a non-toxic concentration (100 μM) significantly increased PKCα but had no effect on NfkB at the same concentration. In fact NaHS increased NfkB significantly at toxic concentrations of 1 mM. In contrast, the slow releaser ACS1 increased PKCα, inhibited the expression of NfkB and upregulated Bcl2 and XIAP levels. However it is not clear if ACS1 is acting directly on XIAP or by modulating NfkB. The data presented here demonstrate that the activation of distinct NF-kB proteins can result in a modulation of the survival of RGC-5 cells that had been exposed to GB.

The class 1 phosphoinositide 3 kinases (PI3K) are a ubiquitously expressed family of proteins that are central to cell survival and proliferation. Akt, a serine/threonine kinase, is a key mediator of PI3-kinase (Anderson and Jackson 2003). Activated Akt

is then responsible for the phosphorylation of numerous downstream targets, regulating apoptosis and survival, cell growth and the cell cycle (Vivanco and Sawyers 2002). In the CNS Akt is a critical mediator of growth factor-induced neuronal survival (Dudek et al. 1996). In the present study the effect of GB and the influence of different H_2S donors were also investigated. It was clear that ACS1 was more effective than NaHS at increasing p-AKT levels and that GB significantly caused a decrease in its levels. Thus H_2S levels may play an important role in the regulation of p-AKT activation.

With regard to NaHS and ACS1 both have also been shown to increase GSH levels (Perrino et al. 2009, Osborne et al. 2010, Kimura 2004). In addition to this, released H_2S can directly scavenge molecules that are implicated in oxidative stress, such as hydrogen peroxide, superoxide anions, and the reactive chlorine species, HOCl (Pong et al. 2007). Therefore these compounds can potentially protect GSH depleted neuronal cells under oxidative stress *in vitro and in vivo*. Furthermore it is generally agreed that naturally-occurring neuronal death in developing animals is dependent on protein synthesis (Castagné et al. 1998) and that oxidative stress involves protein thiol oxidation (Wood et al. 2007). It may also well be that the endogenous H_2S and H_2S donating compounds protect protein thiol oxidation in oxidative stress by providing a substitute for the oxidation of their sulphur containing molecules. It is likely that ACS1 confers better neuroprotection to RGCs possibly due to its sustained stimulation of GSH over time. Although studies with these sulphur-containing compounds have shown their remarkable GSH stimulating properties and ability to protect cells against oxidative injury, however their exact mechanisms of action

compared to other different sulphurated compounds may vary and remains controversial (Wood et al. 2007). Putative mechanisms how ACS1 and NaHS may modulate certain cellular components are shown in figure 4.13.

The present study supports the working hypothesis that inhibition of endogenous GSH is detrimental to neuronal cell survival and its stimulation via H_2S releasing compounds can support cells exposed to glutamate induced toxicity to RGC-5 cells. Also the rate of release of H_2S to cells by donor drugs could possibly determine their degree of neuroprotection *in vitro*. NaHS which rapidly releases H_2S attenuates glutamate induced toxicity but does not blunt the effect of GB, whereas ACS 1, being a more potent GSH inducer, effectively blunts GB toxicity induced oxidative stress and apoptosis. Furthermore ACS1 shows greater potency in inhibiting SNP induced lipid peroxidation in rat brain homogenates and scavenges different forms of free radicals at much lower concentrations compared to NaHS. One possible explanation as to why NaHS is not able to attenuate the toxic effects of GB to RGC-5 cells is likely due to the unregulated rapid release and rapid loss of H_2S by evaporation. Also its is likely that the highly reactive property of NaHS thiol group moiety unlike the ACS1 moeity, renders rapid oxidation and loss of NaHS thiol group function upon addition of GB.

Several studies, however, have shown that inhibition of endogenous production of H_2S can be protective. One study reported that administration of NaHS or L-cysteine

exacerbated the volume of brain infarct induced by unilateral occlusion of the middle cerebral artery, and the addition of H_2S metabolizing enzymes CBS or CSE inhibitors, reduced it (Qu et al. 2006). In addition, H_2S concentration in the cerebral cortex was shown to increase in an experimental stroke model and that high concentration of cysteine associated with H_2S worsens the clinical outcome in patients with ischaemic stroke (Wong et al. 2006). In another study H_2S was shown to induce neuronal death via glutamate receptor and is associated with calpain activation and lysosomal rupture in mouse primary cortical neurons (Cheung et al. 2007). Further, using CBS knock-out mice, the authors demonstrated that CBS produces endogenous H_2S in the brain **and** that H_2S production is greatly enhanced by the excitatory neurotransmitter L-glutamate, as well as by electrical stimulation (Eto et al. 2002). Investigations on the effects of endogenous H_2S on organ injury caused by acute severe endotoxemia in rats, showed that pretreatment with an inhibitor of H_2S synthesis, PAG, attenuated the liver injury, neuromuscular injury and pancreatic injury (Collin et al. 2005). In contrast there are also some data suggesting that H_2S may be protective in neurons and thus its deficiency in the brain may be detrimental i.e. in particular, H_2S protects neurons against neurotoxicity of glutamate independently of the stimulation of excitatory amino acid receptors (Kimura et al. 2006). The present study supports the latter study and confirms that H_2S donor drugs are able to increase the basal levels of GSH, and additionally its related detoxification enzyme GST and the phase II oxidative stress related antioxidant enzymes HO-1. This is likely to occur via the induction of the transcription factor Nrf-2 antioxidant response element (ARE) pathway.

It is known that under conditions of oxidative stress, the transcription factor Nrf2 binds to the antioxidant response element (ARE) to induce antioxidant and phase II detoxification enzymes and its stimulation has been postulated to be neuroprotective in a mouse model of Parkinson's Disease (Chen et al. 2009). Since NaHS does not attenuate the effect of GB and that BSO irreversibly inhibits the phase II enzyme γ-GCS, essential for GSH synthesis (Griffith & Meister 1979), this could imply that NaHS may be up regulating GSH via the Nrf2 dependent pathway, and ACS1 is able to stimulate GSH both via Nrf2 pathway and other unknown mechanism. Interestingly immunocytochemistry and protein studies on Nrf2 levels in RGC-5 cells showed a significant difference between NaHS and ACS1 treatment. The more efficacious neuroportectant ACS1 showed a greater increase of Nrf2 levels and its dependent phase II enzymes HO-1 and GST.

In conclusion the slow releasing H_2S donor ACS1 confers better neuroprotection, and is able to attenuate glutamate toxicity and GSH depletion at defined concentrations in culture. On the other hand, the inclusion of high levels of exogenous H_2S as generated by NaHS, is not able to blunt the negative effects and morphological changes induced by GB on RGC-5 cells in culture. It is also suggestive from the current data that the observed neuroprotective effects of slowly regulated H_2S release via compounds such the ACS1 against this form of cell death may also be due to both its direct anti-apoptotic and anti-oxidative effects. Controlled released H_2S compounds may act as potential neuroprotectants by up-regulation of GSH, and modulation of related antioxidant enzymes via their action on the Nrf-2 pathway and also their actions on

the modulation of PKCα activity and its interaction with the NfkB signaling pathways. H_2S releasing compounds may play a future role in the prevention of RGC cells degeneration for the treatment of neurodegenerative diseases such as Glaucoma.

Chapter 5

ACS67, a H_2S–Releasing Derivative of Latanoprost Acid, Attenuates Retinal Ischaemia and Oxidative Stress to RGC-5 Cells in Culture

Chapter Abstract

The neuroprotective property of a latanoprost derivative (ACS67) that donates the gas hydrogen sulphide (H_2S) was examined by *in vivo* and *in vitro* procedures. Ischaemia was delivered to the adult rat retina by elevation of intraocular pressure and ACS67 injected intravitreally immediately thereafter. Electroretinograms (ERGs) were recorded five days after ischaemia and two days later retinas were dissected and analysed by immunohistochemistry, western blotting and RT-PCR methods. Ischaemia/reperfusion caused damage to the retina characterised by a reduction in the a- and b-wave amplitudes of the ERG, a clear reduction in the retinal localisation of nNOS, GFAP and Thy-1 immunoreactivities and a significant reduction (NF-L) and elevation (GFAP and PARP) of specific proteins. Moreover, a clear reduction (NF-L and Thy-1) and an elevation (GFAP, PARP, caspase-3 and caspase-8) of certain retinal mRNAs occurred. In addition, the optic nerve proteins associated with ganglion cell axons (NF-L and tubulin) was drastically reduced. Importantly, most of the effects caused by ischaemia/reperfusion were significantly blunted by ACS67.

In vitro studies on RGC-5 cells in culture were also carried out to confirm that ACS67 is a neuroprotective agent and also to deduce whether latanoprost acid lacking H_2S donating properties functions in a similar way. The influence of latanoprost, ACS67 and NAC (an antioxidant) on RGC-5 cultures exposed to GB for 24 hours were therefore assessed using a variety of procedures (described in the methods section). The results show that ACS67 is powerful neuroprotectant at low concentration (1-20 μM) but at higher concentration becomes less effective while NAC is in comparison only effective as a neuroprotectant at very high (5mM) concentrations. In contrast, latanoprost was toxic at all concentrations tested. In addition, exposure of RGC-5 cells to various substances showed ACS67 and NAC stimulated GSH levels while latanoprost was ineffective. It is concluded that the H_2S-donating properties of ACS67 are neuroprotective and that latanoprost alone is toxic in the conditions of the experiments conducted here. It is also proposed that the latanoprost moiety of ACS67 is the cause for ACS67 being less effective as a neuroprotectant at elevated concentrations rather than in low amounts.

5.1 Introduction

5.1.1 Aims of study

To deduce whether ACS67, a] attenuates the negative effect of an ischaemic insult to the intact rat retina, b] blunts an insult of GB to RGC-5 cells in culture and c] is able to stimulate the production of GSH in RGC-5 cells.

5.1.2 Brief Overview

Glaucoma is a neurodegenerative disease resulting in retinal ganglion cells dying at different rates (Quigley 1999, Whitmore et al. 2005; Weinreb and Khaw 2004). The cause of glaucoma is thought to be due to a number of risk factors which results in ischaemia to the optic nerve head (Osborne et al. 2001; Osborne et al. 2006; Pache and Flammer 2006; Flammer et al. 2002). One definite risk factor is raised intraocular pressure (IOP) which often occurs and can be lowered by surgery or by IOP lowering agents (Flammer and Orgul 1998, Weinreb and Khaw 2004). However, despite adequate lowering of elevated IOP loss of vision still can continue in many cases (Osborne et al. 2006, Pache and Flammer 2006; Weinreb and Khwa 2004). It is clear that in order to treat glaucoma more successfully it is necessary both to reduce elevated IOP and reduce the rate of the death of ganglion cells. The term “neuroprotection in glaucoma” implies the possible use of a drug to do the latter. For this aim to be achieved the substance will have to reach the retina and/or optic nerve head to be effective. Theoretically, some of many topical agents used to lower IOP should reach the retina/optic nerve head via the systemic system. As a consequence if one of two substances with equal capacity to lower IOP also has powerful

neuroprotective properties then it could logically be argued that it would be more effective for the treatment of glaucoma.

One of the most potent IOP lowering agents used to treat glaucoma is the prostaglandin latanoprost (isopropyl ester of latanoprost acid, (Figure 1.14, Chapter 1) (Stjernschantz et al. 2000). A derivative of this substance, ACS67 has now been developed which maintains the IOP effectiveness associated with latanoprost but also releases H_2S (Li et al. 2007; Perrino et al. 2009). H_2S is a gas, produced in biological tissues from cysteine and now recognised as a signalling molecule that participates in many functions (Szabó 2007; Lefer 2007). It is produced from cystathionine β-synthase (CBS) and cystathionine γ-lyase (CSE) enzymes that are present in various tissues (Moore et al. 2003). H_2S is implicated to participate in functions as diverse as inflammation, vasodilation and neuronal survival (Szabó 2007; Lefer 2007; Kimura et al. 2005; Zhang and Bhatia 2008). Actual endogenous concentrations of H_2S in human, rat and bovine brain have been estimated to be significant and elicits biological effects between 10-1000μM (Szabó 2007, Kimura and Kimura 2004, Abe and Kimura 1996). H_2S stimulates the formation of cAMP in primary neuronal cultures or glial cell lines (Kimura 2000) and is also a regulator of the NMDA receptor and shown to play a part in long-term potentiation (Kimura 2000, Boehning and Snyder 2003). H_2S also regulates the activities of serotonergic neurons, (Kombian et al. 1993) induces a release of corticotrophin-releasing hormone, (Dello Russo et al. 2000) causes an activation of neuronal calcium channels, (Nagai et al. 2004) increases intracellular GSH levels and affects smooth muscle relaxation and vasodilation by acting on K_{ATP} or Cl^- channels (Szabó 2007; Lefer 2007; Kimura et al. 2005). Historically, H_2S is known to be a noxious gas

with wide ranging cytotoxic effects (Abe and Kimura 1996) but recent studies suggest that its positive and negative effects are probably related to its concentration. In homogenates, H_2S generation is between 1-10 pmoles/second/mg protein (Doeller et al. 2005) and given its rapid turnover, extracellular active sulphide concentration is probably in the low micromolar range (Szabó 2007). This might explain some apparent contradictions in the literature where agents that donate H_2S have generally been found in culture studies to protect neurons and other cell-types from oxidative stress (Kimura and Kimura 2004; Elrod et al. 2007; Kimura et al. 2006; Sivarajah et al. 2006; Umemura et al. 2007; Lu et al. 2008) or damage caused by β-amyloid to neurons (Tang 2008). Moreover, the effect of ischaemia/reperfusion insults to the heart and liver are attenuated by H_2S donors (Elrod et al. 2007; Jha et al. 2008; Bliksøen et al. 2008). However, reports from other studies show that H_2S can be toxic to brain neurons in culture (Cheung et al. 2007) and exacerbate the negative effect of ischemia to the cerebral cortex (Qu et al. 2006). The derivative of latanoprost, ACS67 belongs to a class of compounds known as dithiolethiones (Figure 1.14 Chapter 1) which release H_2S in a controlled long-lasting way (Li et al. 2007). Dithiolethione compounds of diclofenac, (Rossoni et al. 2008; Sidhapuriwala et al. 2007) sildenafil (Muzaffar et al. 2008) and aspirin (Sparatore et al. 2009) have all been shown to maintain their well known endogenous properties but have additive effects associated with H_2S. It has also been reported that ACS67 is more effective than latanoprost at reducing IOP in rabbits as well as stimulating cGMP and GSH levels (Perrino et al. 2009). The ability of ACS67 to increase GSH suggests that the compound has neuroprotective properties.

5.1.3. Summary of results

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1. ACS67 attenuates the negative effects to RGC-5 cells exposd to GB.
2. ACS67, as opposed to latanoprost stimulates the production of GSH in RGC-5 cells.
3. ACS67 counteractes the negative effect of ischaemia/reperfusion insults to the intact rat retina.

5.2 Results

5.2.1. Studies on RGC-5 cells in culture

Figures 5.1 A and B show data from RRA viability assays where RGC-5 cells in the absence or presence of 5mM GB were exposed to ACS67, NAC (a known antioxidant acting as a positive control) or latanoprost over a period of 24 hours. It can be seen that ACS67 had no effect on cell survival in the absence of GB up to concentrations of 50µM (Figure 5.1A). In Figure 5.1B, latanoprost is only toxic at 20µM). Figures 5.1A and 5.1B, also show that approximately 50% of RGC-5 cells die following exposure to GB and that ACS67 attenuates the death process in a dose-dependent manner between 10-50µM while latanprost exacerbates the toxic insult of GB dose-dependently between 10-50µM. It can also be seen that NAC at 5mM nullified the influence of GB but it is far less effective than ACS67.

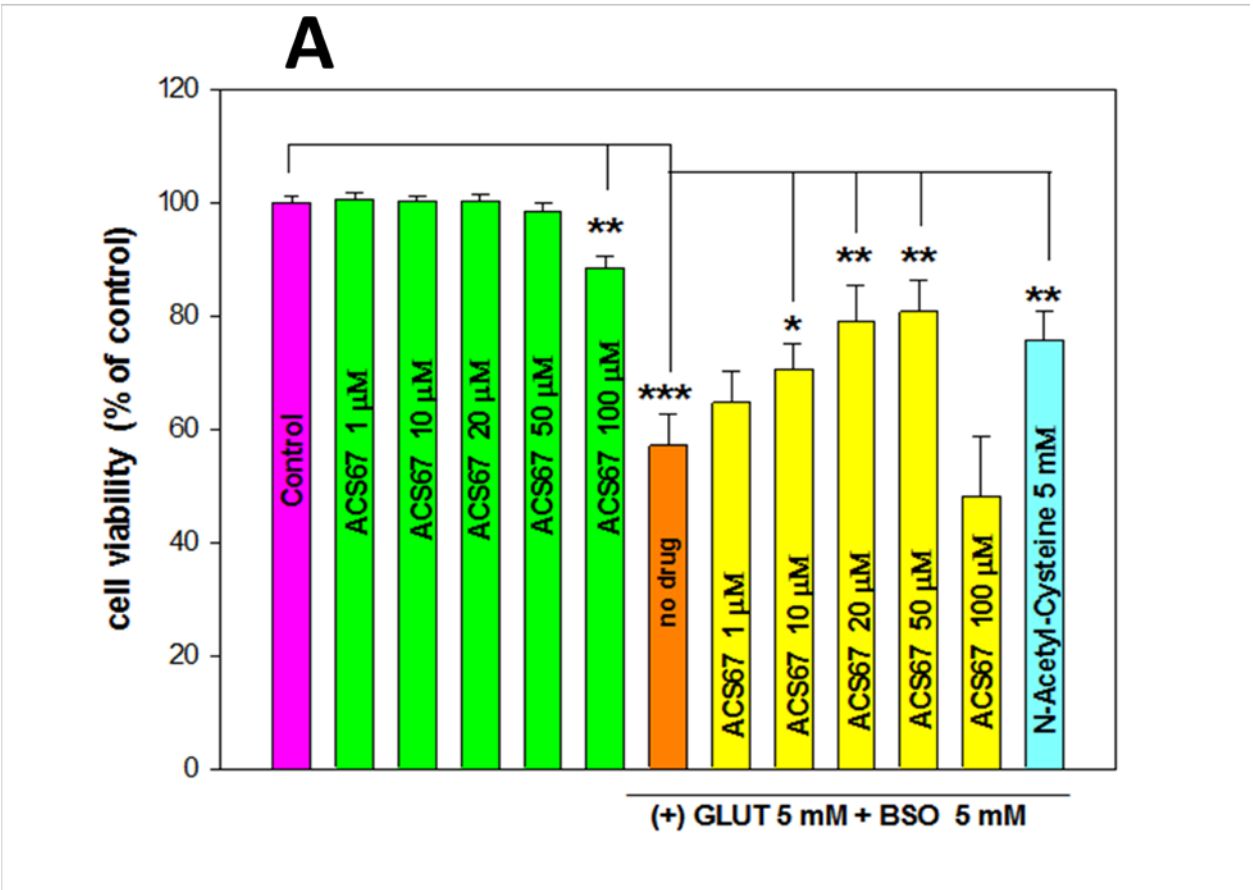


Figure 5.1 A: The effect of different concentrations of ACS67 in the absence or in the presence of GB 5 mM (after 24 hours) on the viability of RGC-5 cells in culture as measured by the RRA assay. Results are mean values with error bars indicating ±SEMs where n=6 (*p<0.05, **p<0.01, ***p< 0.001).

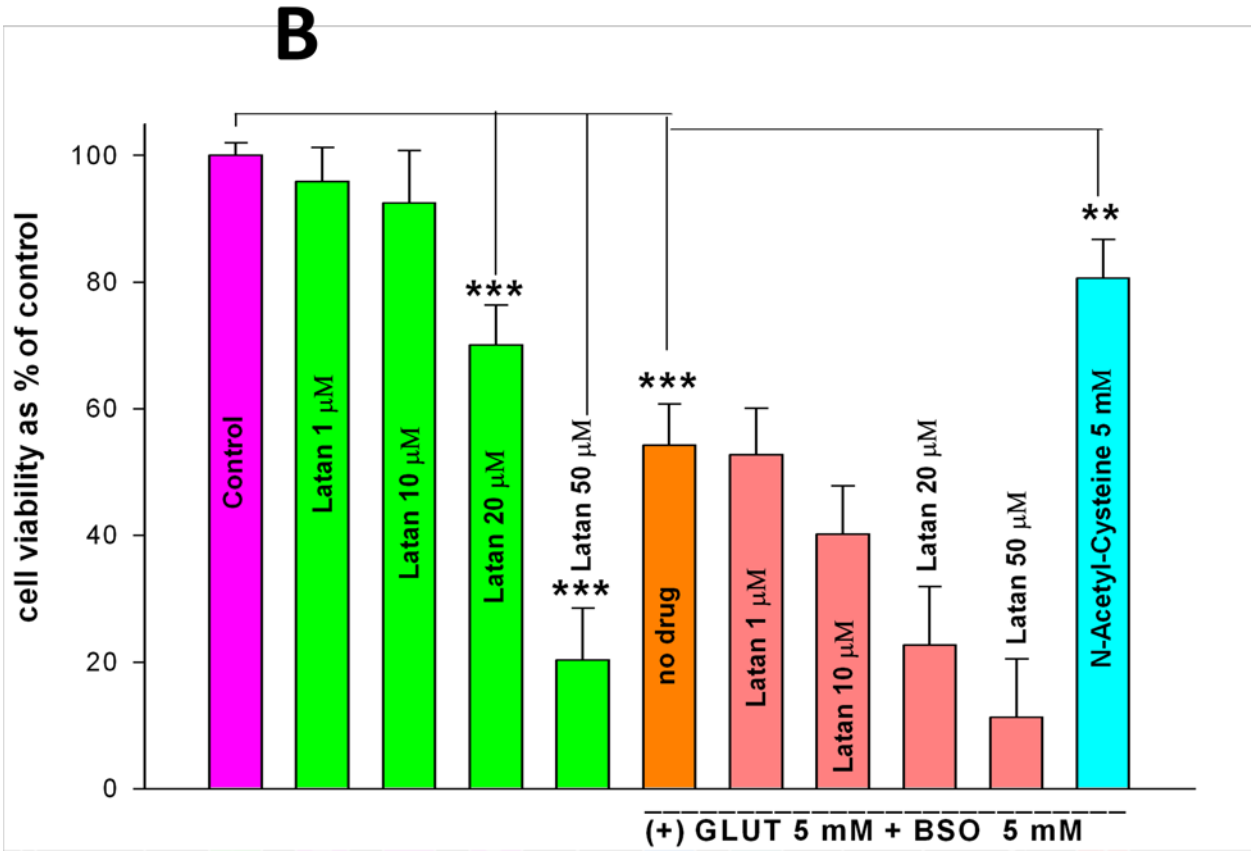


Figure 5.1 B: The effect of different concentrations of latanoprost (Latan) in the absence or in the presence of GB 5 mM (after 24 hours) on the viability of RGC-5 cells in culture as measured by the RRA assay. Results are mean values with error bars indicating ±SEMs where n = 6 (**p<0.01, ***p< 0.001).

5.2.1.1 Cytological evidence to show that ACS 67 attenuates GB-induced death to RGC-5 cells.

Hoechst 33258 solution stains the nuclei of dying RGC-5 cells bright fluorescent blue as shown in Figure 5.2. It can be seen that in comparison to controls many bright fluorescent cell nuclei stained positively in cultures exposed to GB and this was much reduced in cultures where 10µM ACS67 or 5 mM NAC were included.

As shown in chapters 3 and 4, exposure of RGC-5 cells to 5 mM GB results in a number of cells staining for phosphatidylserine by the APOP*ercentage*™ method. Figure 5.3 shows that this positive staining, an indicator for apoptosis, is clearly attenuated by the presence of 10µM ACS67.

Cell cultures in the presence and absence of 10µM ACS67 exposed to GB were also stained for ROS (Figure 5.3 D,E,F) or FDA/PI where live cells appear green (Figure 5.3 G) and dead cells appear red (Figure 5.3 H). It can be seen that GB caused a generation of ROS and a number of dead red PI stained cells and that this was significantly reduced in the presence of 10µM ACS67 (Figure 5.3 I).

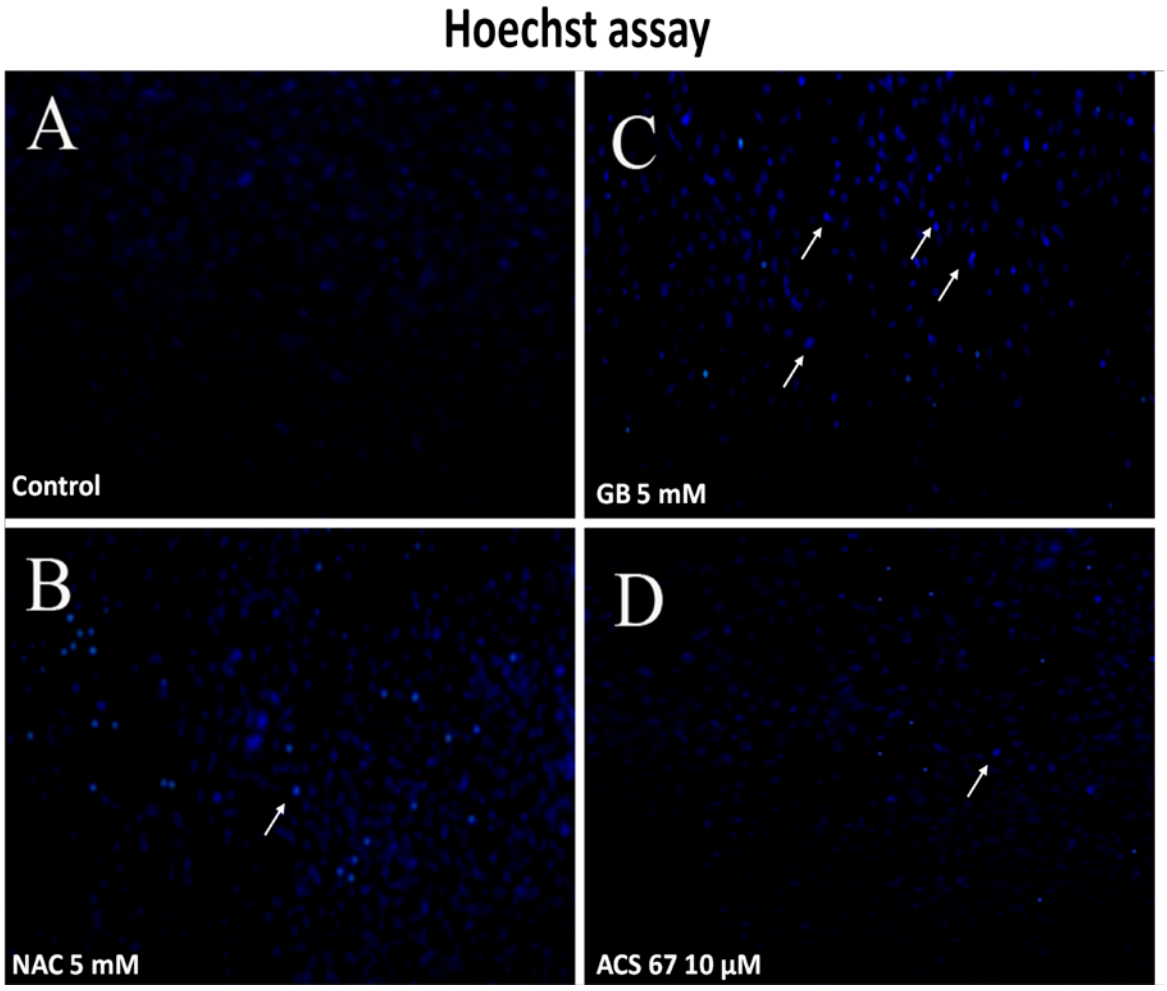


Figure 5.2: Effect of 10µM ACS67 (D) and 5 mM NAC (B) on RGC-5 cell apoptosis induced by exposure to 5 mM GB for 24 hours (C). Figure 5.1 A is a control culture. Staining of cultures was by use of Hoechst which reveals damaged DNA (arrows) as an indicator for apoptosis. It can be seen that a number of cells stain for apoptosis following GB treatment (C) which is blunted by both ACS67 (D) and NAC (B) when compared with untreated control cells (A).

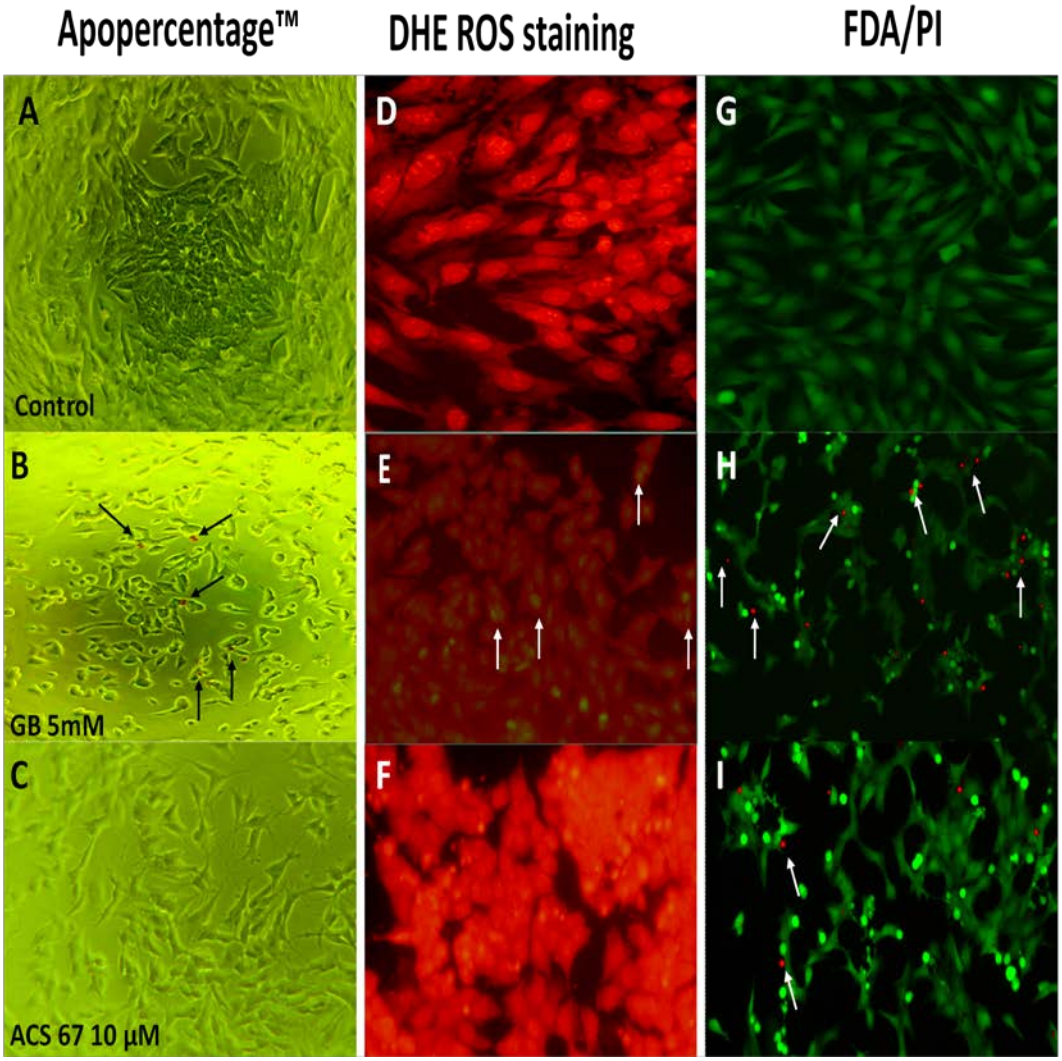


Figure 5.3: Cultures maintained in normal culture medium were confluent and did not stain red for apoptosis (phosphatidylserine) (A), red for ROS (D) or red rather than green for cell death (G). However, cultures incubated with 5 mM GB for 24 h resulted in reduced cell numbers and morphology with some cells staining positively for phosphatidylserine (B), ROS (E) and cell death (H). Inclusion of 10 µM ACS67 reduced the effects of GB in causing apoptosis (C), stimulating ROS (F) and causing cell death (I)

5.2.1.2 Effect of ACS67 on GSH

Exposure of RGC-5 cells to ACS67 (1 and 10µM), NAC 5 mM or latanoprost (10µM) for 24 hours showed ACS67and NAC to significantly cause an elevation of GSH while latanoprost was without effect (Figure 5.4).

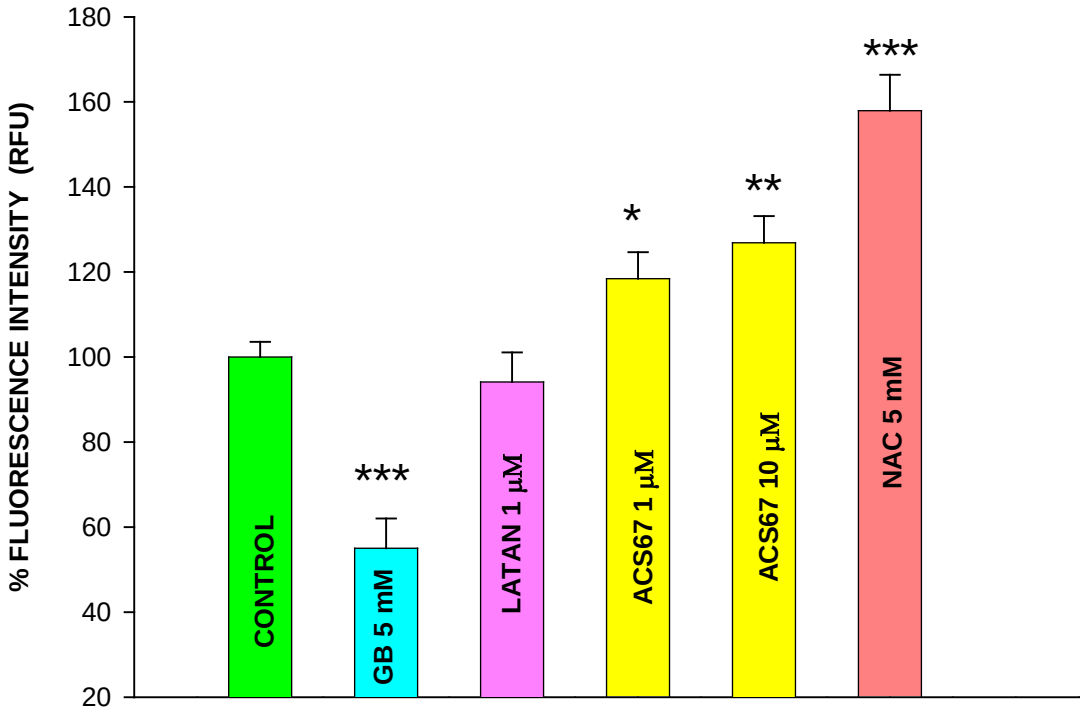


Figure 5.4: Relative amounts of GSH fluorescence in RGC-5 cultures following exposure to ACS67, NAC and latanoprost (LATAN) after 24 hours when compared with untreated control cultures. Results are mean values with error bars indicating ±SEMs where n=5 (*p<0.05, **p<0.01, ***p< 0.001).

5.2.1.3 Effect of ACS67 on intracellular H_2S content over 24 hours

Figure 5.5 shows that exposure of RGC-5 cells to ACS67 (1 or 20 µM) for 6 hours results in no significant increase in basal levels of H_2S. However, H_2S is detected in significant amounts after 12 hours exposure to 20 µM ACS67. Moreover, H_2S is generated in significant amount when RGC-5 cells are exposed to as little as 1 µM ACS67 after a period of 24 hrs.

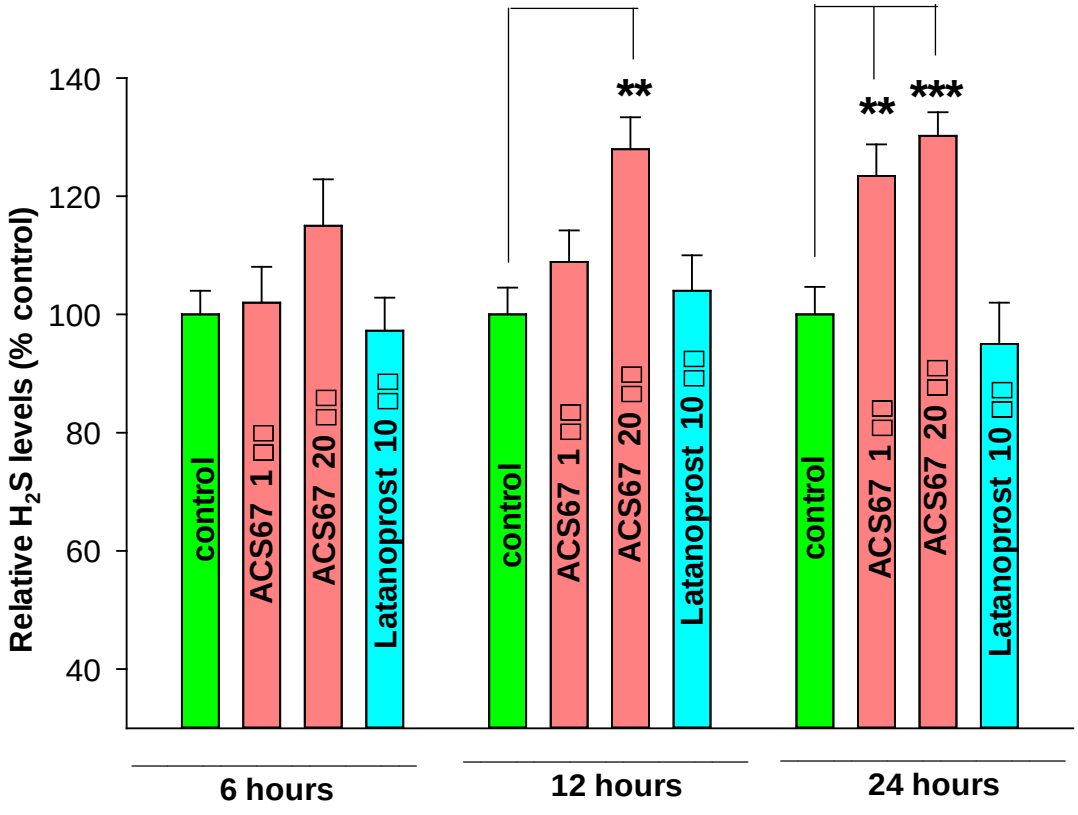


Figure 5.5: Effect of ACS67 on intracellular H_2S levels. H_2S levels were detectable with ACS67 incubation at 1 µM only at 24 hours whereas ACS67 treatment at 20 µM significantly increased H_2S production at 12 h and 24 h. Latanoprost (10 µM) showed no stimulation of H_2S over 24h. Data are expressed as mean **±** SEM where n=5 (*p < 0.05 **p < 0.01 ***p < 0.001).

5.2.2 Effect of ACS67 on retinal ischaemia/reperfusion (*in vivo studies)*

Elevated intraocular pressure-induced ischaemia for 50 minutes followed by 5 days reperfusion caused on average a 36% and 63% reduction in the a- and b-wave amplitudes, respectively in ACS67-treated rats (n=14). In animals treated with vehicle (n = 14), the reduction in the a- and b-wave amplitudes was approximately 49% and 76% respectively. The differences in amplitudes of both the a- and b-waves between the two groups of animals were statistically significant. Neither ACS67 nor its vehicle (DMSO) affected the ERG recordings of untreated (non-ischaemic) eyes (Figure 5.6).

Ischaemia for 50 minutes followed by seven days of reperfusion caused a change in the content of selected retinal and optic nerve proteins as shown in Figure 5.7 and 5.8. These data show that ischaemia/reperfusion statistically decreased the protein level of NF-L but increased the level of PARP and GFAP proteins in the retina (Figure 5.7). Importantly, the effects of ischaemia/reperfusion on the protein level of NF-L, PARP and GFAP are all significantly blunted by ACS67 treatment (Figure 5.7). In addition NF-L and tubulin proteins in the optic nerve were found to decrease by 53% and 42%, respectively after ischaemia/reperfusion (Figure 5.8). This level of decrease in optic nerve NF-L and tubulin proteins caused by ischaemia/reperfusion was significantly nullified in rats treated with ACS67 (Figure 5.8).

Ischaemia for 50 minutes followed by 7 days reperfusion also caused changes in the content of selected mRNAs. mRNA levels (normalized to cyclophilin) of Thy-1 and NF-L in vehicle-treated rat retinas were significantly reduced (Figure 5.9). In contrast, the mRNA levels of caspase-3, caspase-8, PARP and GFAP were elevated (Figure 5.10 and 5.11). Importantly, when ACS67 rather than vehicle was injected into the eye following ischaemia, the reductions of

retinal mRNA Thy-1 and NF-L were significantly attenuated. ACS67 treatment did not however affect the increased mRNA levels of caspase-8, caspase-3 or PARP but blunted the increase in GFAP mRNA.

Analysis of retinal tissues from vehicle treated animals for the localisation of NOS (neuronal nitric oxide synthase), GFAP and Thy-1 immunoreactivities seven days following ischaemia showed clear changes when compared with the controls. Normal Thy-1 staining in the retinas of rats is associated with the ganglion cell layer and the whole of the inner plexiform layer (Figure 5.12 A). Normal NOS immunoreactivity is associated with certain amacrine cells and their processes appearing as three defined bands in the inner plexiform layer (Figure 5.12 D). Normal GFAP immunoreactivity is primarily associated with astrocytes and end feet of Müller cells in the ganglion cell/nerve fibre layer (Figure 5.12 G). Ischaemia/reperfusion in vehicle-treated eyes caused a significant thinning of Thy-1 immunoreactivity (Figure 5.12 B), a marked elimination of NOS immunoreactivity (Figure 5.12 E) and an intense staining of GFAP in Müller cells throughout the retina (Figure 5.12 H). In contrast, in the ischaemia/ACS67-treated rats, the reduction of Thy-1 and NOS immunoreactivities was less pronounced (Figure 5.12 C and 5.12 F) and the enhancement of GFAP staining was clearly reduced (Figure 5.12 I). This conclusion was reached following analysis of many retinal sections from areas of the retina that were of similar eccentricity.

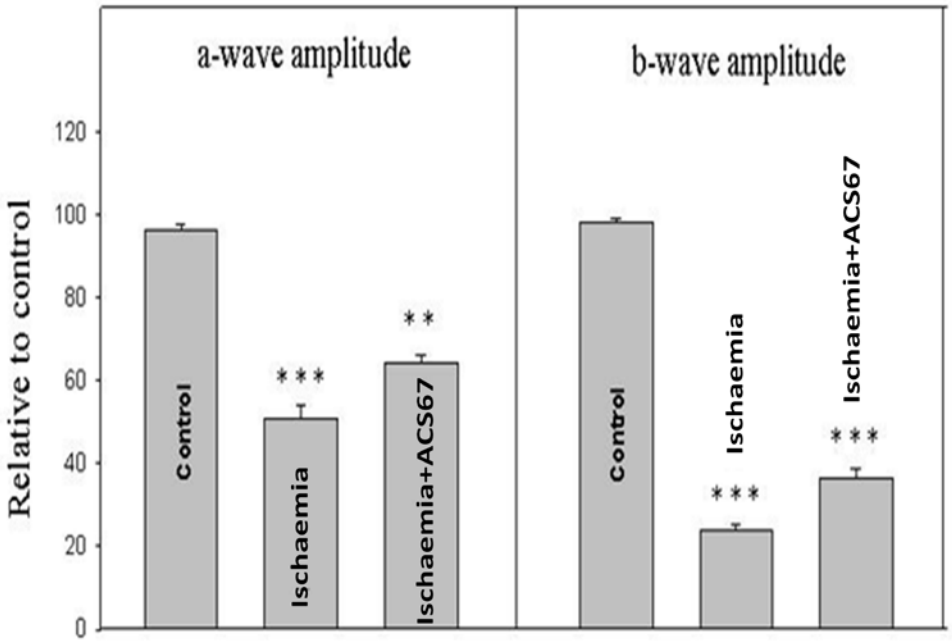


Figure 5.6: ERG studies on the effect of ACS67 in ischaemia. In these studies ERGs from both eyes were recorded. One eye was subsequently given ischaemia. Five days later the ERGs from the ischaemia and non-ischaemic eyes were recorded and the percentage changes in the a- and b-wave amplitudes were compared between the ischaemic and control eyes. It can be seen that both the a- and b-wave amplitudes of the ERG are clearly reduced by ischaemia/reperfusion (*** $p< 0.001$ compared with control). Moreover, compared with vehicle treated animals ACS67 significantly attenuated the reduction of the a- and b-wave amplitudes (** $p< 0.01$ compared with ischaemia). Results are means ± SEM, n = 14.

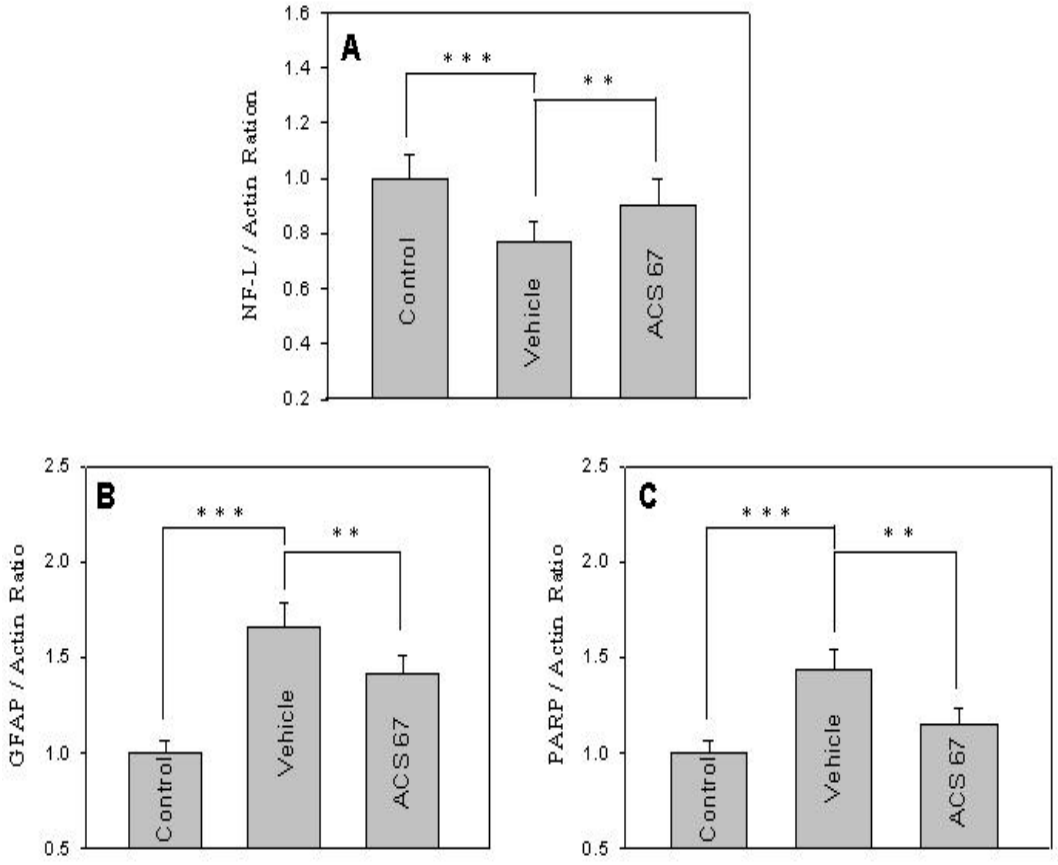


Figure 5.7: NF-L (A), GFAP (B) and PARP (C) proteins relative to actin in the non-ischaemic retina (control) and following ischaemia/reperfusion with vehicle-treatment (vehicle) or ACS67-treatment (ACS67). Ischaemia/reperfusion caused a significant change in the amounts of all proteins compared with control (*** $p < 0.001$) and treatment with ACS67 significantly (** $p < 0.01$) counteracted this effect. Data are means with error bars indicating ± SEMs where n= 12.

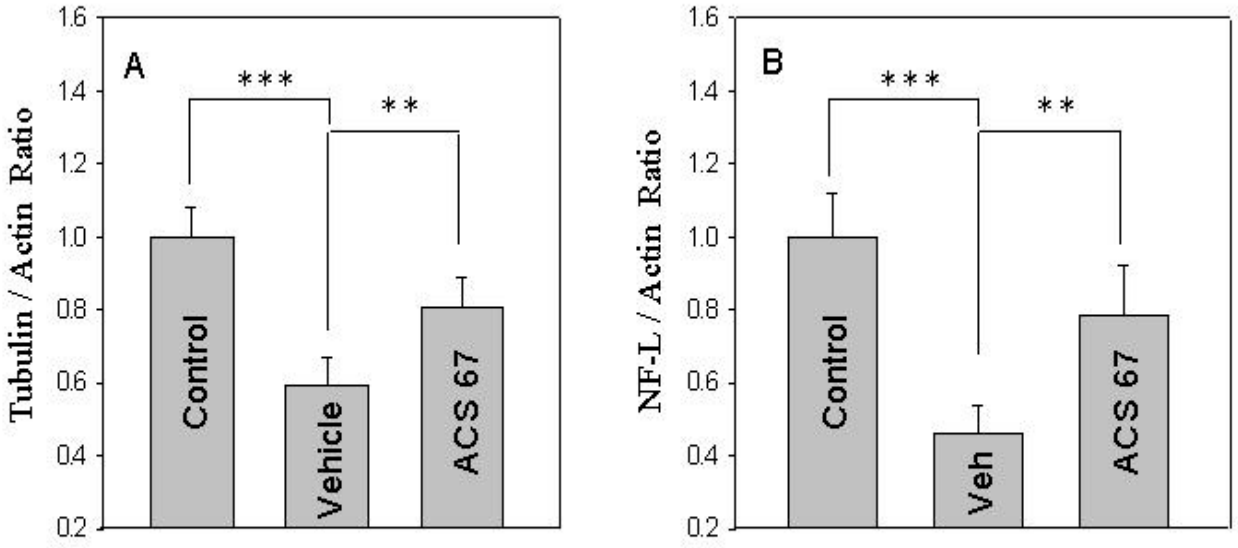


Figure 5.8: Tubulin (A) and NF-L (B) proteins relative to actin in the non-ischaemic optic nerves (control) and following ischaemia/reperfusion with vehicle-treatment (vehicle) or ACS67-treatment (ACS67). Ischaemia/reperfusion caused a significant (*** $p < 0.001$) decrease in both NF-L and tubulin proteins. ACS67 treatment significantly attenuated (** $p < 0.01$) this reduction. Data are means with error bars indicating ± SEMs where n= 12.

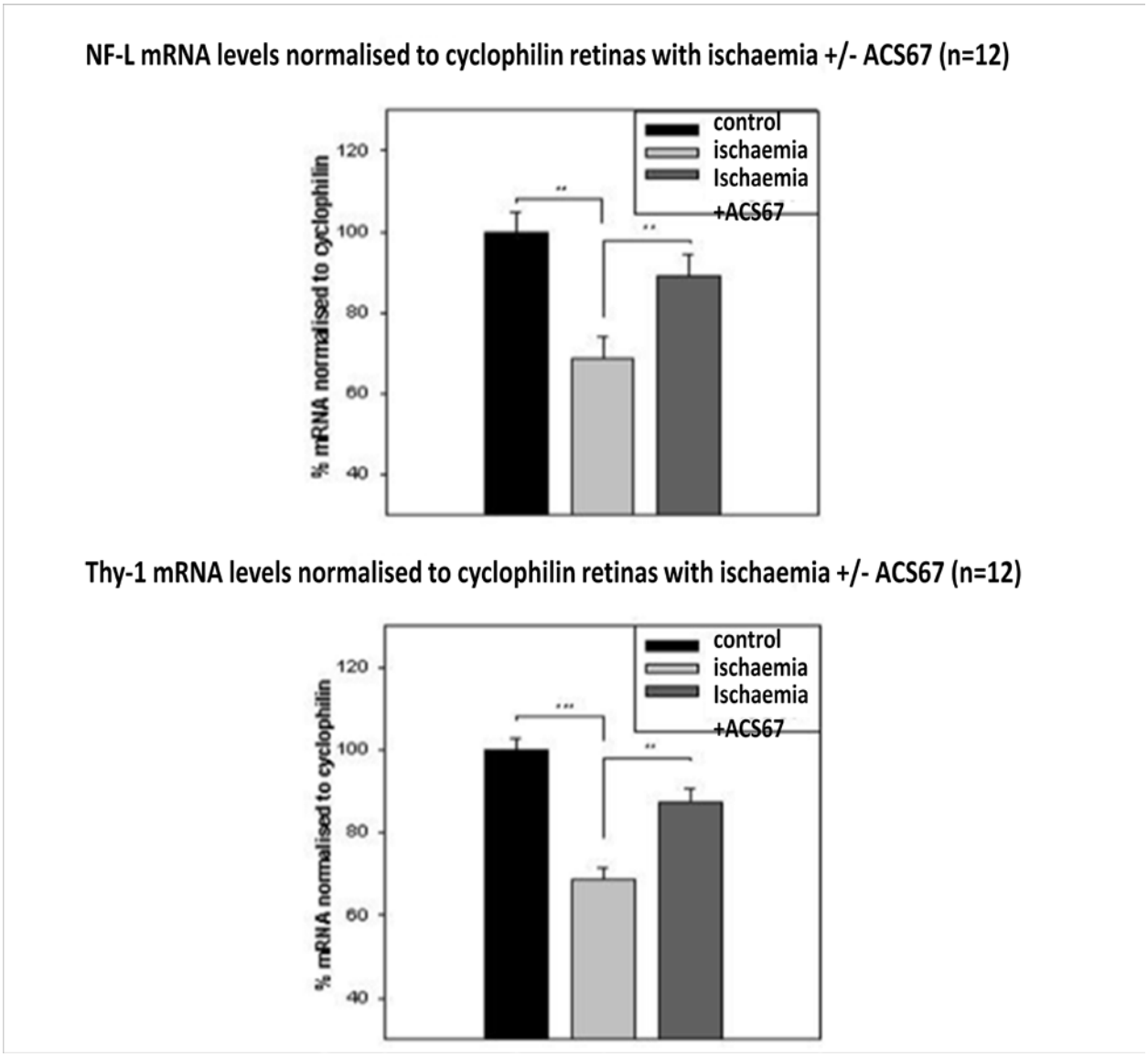


Figure 5.9: NF-L and Thy-1 mRNA levels relative to cyclophilin mRNA in non-ischaemic retina (control) and following ischaemia/reperfusion with treatment of vehicle (ischaemia) or ACS67 (ischaemia + ACS67). It can be seen that ischaemia/reperfusion caused significant decrease of the mRNA level in vehicle-treated animals and these changes were all significantly blunted by treatment with ACS67. Data are means with error bars indicating ± SEMs. n= 12 in each case, ** $p< 0.01$, *** $p< 0.001$.

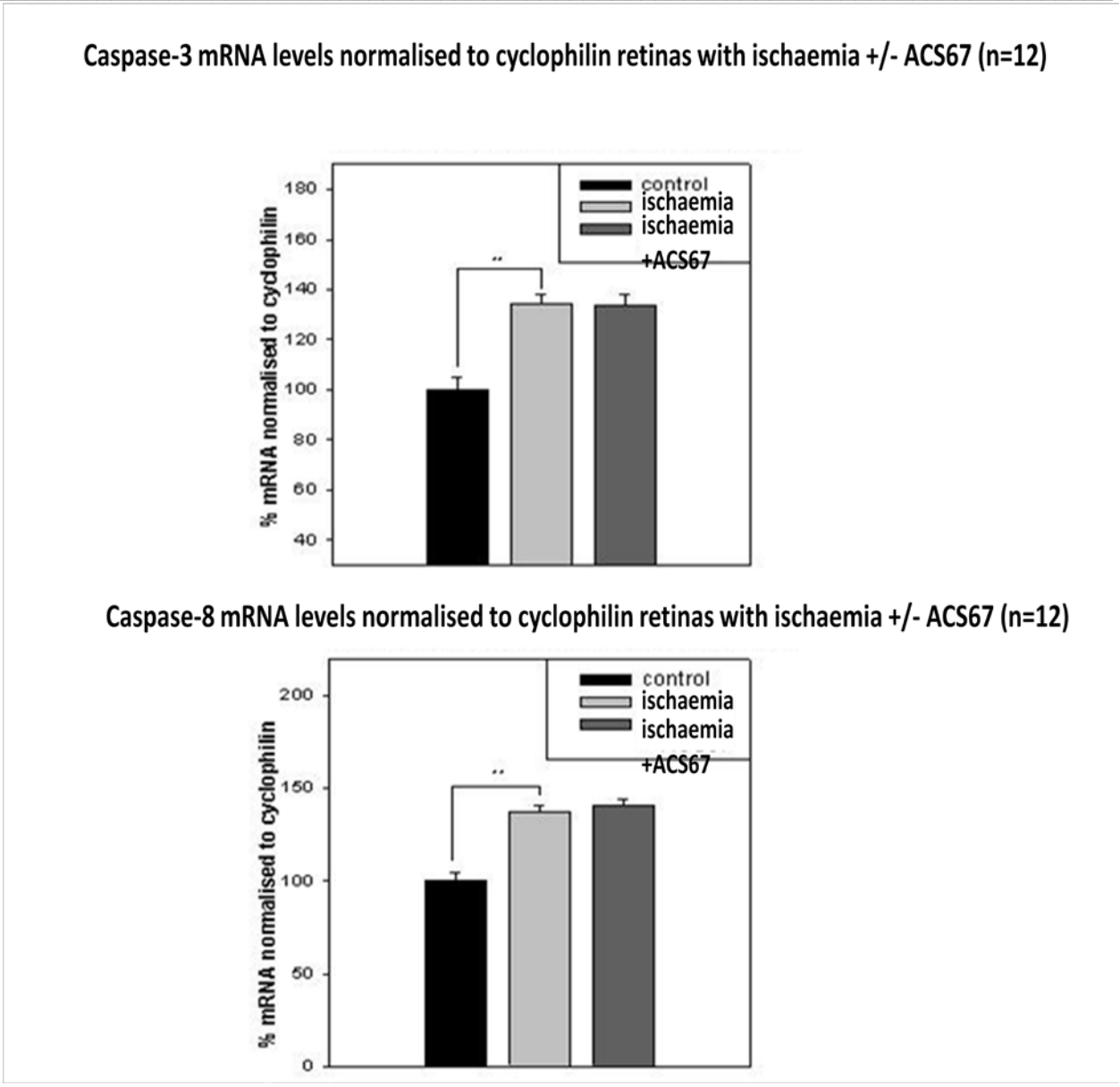


Figure 5.10: Caspase-3 and caspase-8 mRNA levels relative to cyclophilin mRNA in non-ischaemic retina (control) and following ischaemia/reperfusion with treatment of vehicle (ischemia) or ACS67 (ischaemia + ACS67). It can be seen that ischaemia/reperfusion caused significant elevation of the mRNA level in vehicle-treated animals but these changes were not significantly blunted by treatment with ACS67. Data are means with error bars indicating ± SEMs. n= 12 in each case, ** $p< 0.01$.

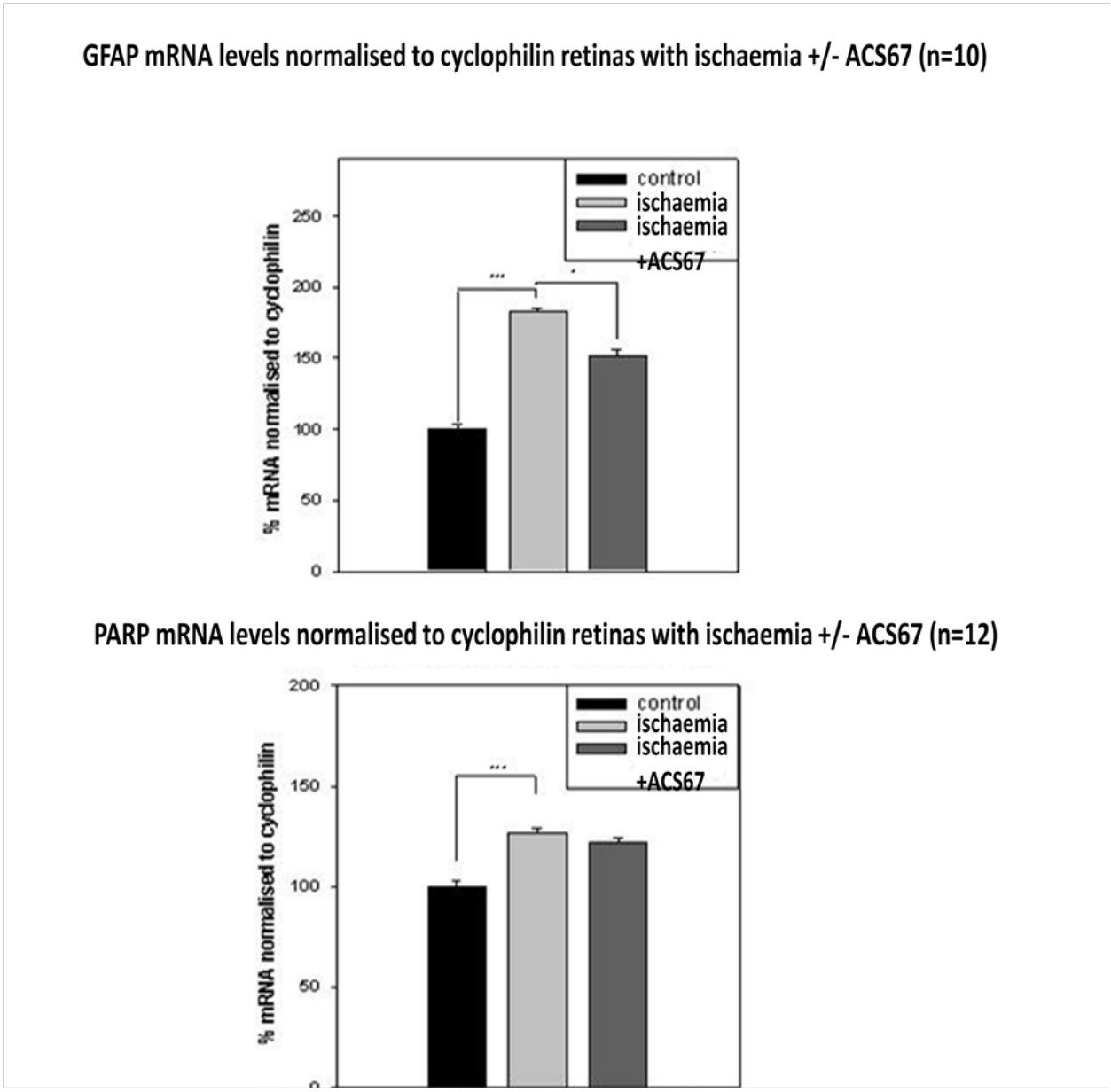


Figure 5.11: GFAP and PARP mRNA levels relative to cyclophilin mRNA in non-ischaemic retina (control) and following ischaemia/reperfusion with treatment of vehicle (ischaemia) or ACS67 (ischaemia + ACS67). It can be seen that ischaemia/reperfusion caused significant elevation of the mRNA level in vehicle-treated animals but these changes were only significantly blunted for GFAP by treatment with ACS67. Data are means with error bars indicating ± SEMs. n= 12 in each case, *** $p< 0.001$, * $p< 0.05$.

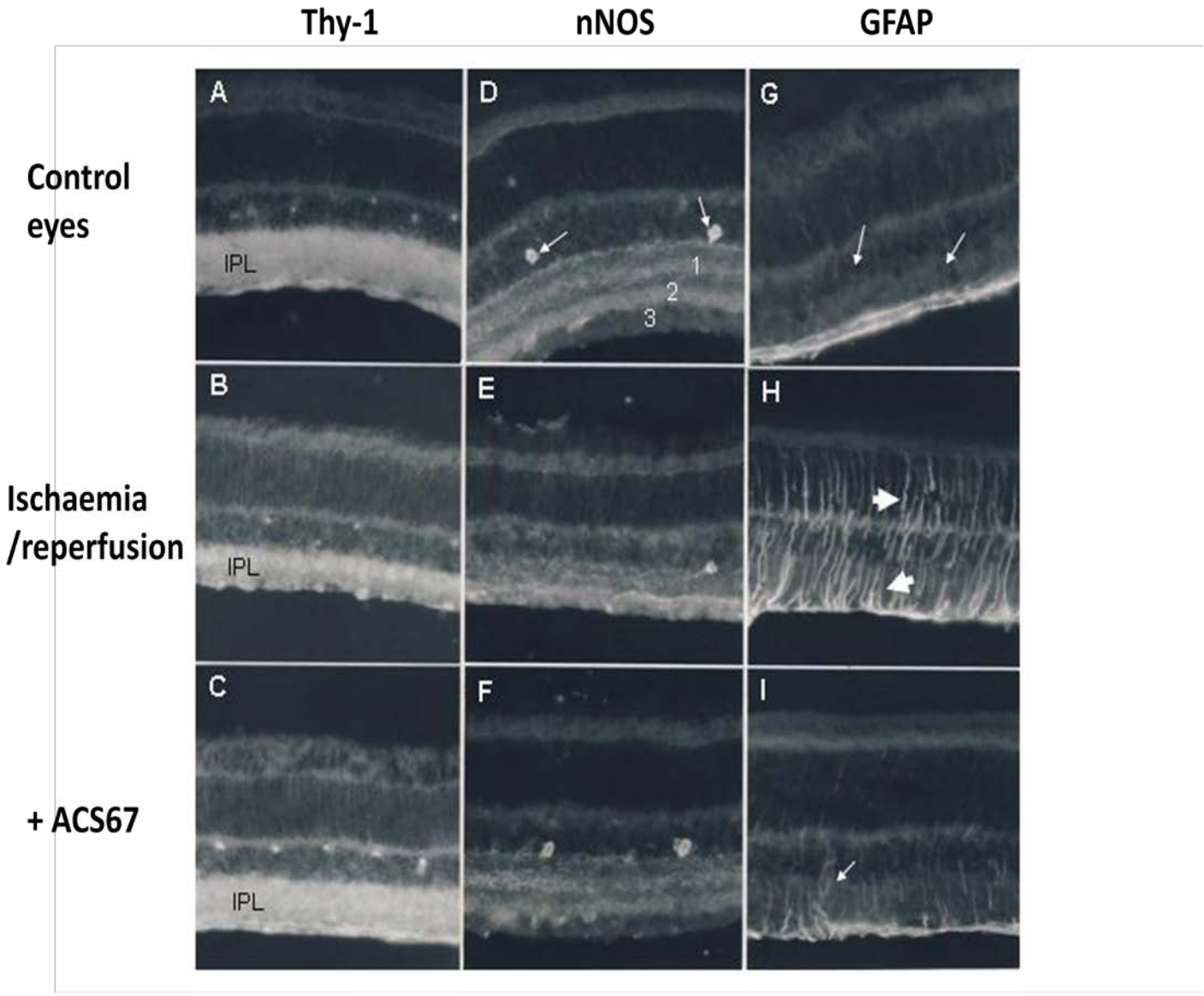


Figure 5.12: Effect of ischaemia/reperfusion on retinal cells (immunohistochemistry). This figure shows that ischaemia/reperfusion causes a clear change in the thickness of the Thy-1 immunoreactivity (B) in the inner plexiform layer (IPL), an almost a complete loss of the nNOS immunoreactive amacrine cells (E) and distinctive strong staining of GFAP (thick arrows, H) associated with retinal Müller cells. The effect of ischaemia on the localisation of retinal Thy-1, nNOS and GFAP immunoreactivities are attenuated in animals treated with ACS67 (C, F and I). Figure 5.12 A shows the broad band of Thy-1 immunoreactivity in the IPL of a control retina. Figure 5.12 D shows the nNOS immunoreactive neurones (thin arrows) and three bands on immunoreactivity (1-3) in the IPL in control retina. Figure 5.12 G shows GFAP in the control retina to be located mainly to a single band (thin arrows), that make up astrocytes and Müller cell end feet.

5.3. Discussion

The results of the described study show that ACS67, a derivative of latanoprost acid, is a powerful neuroprotectant counteracting an ischaemic/reperfusion insult to the retina and an oxidative insult to RGC-5 cells in culture. ACS1, the parent compound of ACS67, has been demonstrated to release H_2S *in vitro* when incubated with rat liver homogenates (Li et al. 2007) Moreover, ACS1 esters containing diclofenac (ACS15) or aspirin (ACS14) are known to release H_2S *in vitro* and *in vivo* (Li et al. 2007, Sparatore et al. 2009). It can be hypothesized that the neuroprotective action of ACS67 is partly due to the H_2S release from the sulfurated moiety as found for ACS1 in Chapter 4 and the present study (Figure 5.5). Moreover, the finding that ACS67 stimulates the production of GSH in cultured cells (Figure 5.4) and in the aqueous humour when applied topically to the rabbit eye (Perrino et al. 2009) provides support for this notion. H_2S is known to stimulate GSH formation, (Kimura and Kmiura 2004; Qu et al. 2008) as well as other processes (Lefer 2007; Kimura et al. 2006) that could contribute to a neuroprotective action.

The present study provides biochemical, electrophysiological and immunohistochemical data to show that ACS67 counteracts an insult of ischaemia/reperfusion to the retina *in situ.* It can be concluded that this is partly due to H_2S being released from ACS67. Significantly, H_2S has been shown to attenuate the effects of ischemia to tissues such as the heart and liver (Szabó 2007, Elrod et al. 2007; Jha et al. 2008; Bliksøen et al. 2008) but not to the brain where it exacerbates the insult (Qu et al. 2006). The present study however demonstrates that H_2S blunts the influences of ischemia/reperfusion to CNS tissue. Ischaemia to the retina was induced by

elevation of the intraocular pressure above the systolic blood pressure for 50 minutes. Five days later the a- and b-wave amplitudes of electroretinograms are significantly reduced (Figure 5.6). The a-wave provides information about the photoreceptors while the b-wave provides information on the physiology of the ON-bipolar and Müller cells (Block and Schwarz 1998). Importantly, the reductions in the a- and b-wave amplitudes caused by ischaemia/reperfusion were significantly less in animals where ACS67 was injected into the vitreous humour straight after ischaemia rather than vehicle. Thus electrophysiological data provides evidence that physiological damage to the retina induced by ischaemia/reperfusion is attenuated by ACS67.

In the model of ischaemia/reperfusion used in this study it is known that the inner retina is particularly affected (Osborne et al. 2004). This is also shown in Figure 5.12 where nNOS and Thy-1 immunoreactivities associated with different sub-sets of amacrine and ganglion cells in the inner retina are significantly reduced by ischaemia/reperfusion. In contrast, GFAP immunoreactivity located to glial cells is clearly enhanced by the insult. It needs to be emphasized that it is important for sections of similar eccentricities to be compared when making comparisons between different retinas. However, by comparing sections from approximately the same retinal areas in a masked fashion it is possible to make an informed judgement. In this study it is concluded that nNOS, Thy-1 and GFAP immunoreactivities are much affected by ischaemia/reperfusion and the changes seen are blunted by intravitreal administration of ACS67.

Analysis of the level of retinal mRNAs (Figure 5.9 to 5.11) and proteins associated with the retina and optic nerve (Figure 5.7 and 5.8) of rats subjected to ischaemia corroborates the data derived by immunohistochemistry and electrophysiology. The degree of ganglion cell death after ischaemia/reperfusion was determined by relating the protein (NF-L) and mRNA (Thy-1 and NF-L) levels of ganglion cell specific markers with total retinal actin protein and cyclophillin

mRNA levels, respectively. The validity of this method to obtain information on ganglion cell death is documented in various publications (Nash and Osborne 1999; Casson et al. 2004). The results show that ischemia/reperfusion causes a drastic effect on ganglion cells resulting in a large decrease in NF-L mRNA/protein and Thy-1 mRNA levels. This effect is significantly blunted by injection of ACS67.

NF-L and tubulin proteins are present in ganglion cells and analysis of their levels in the optic nerve provides a measure of ganglion cell viability (Ji et al. 2008; Zhang et al. 2008). Ischaemia/reperfusion clearly reduces the levels of tubulin and NF-L proteins in the optic nerve, as well as NF-L in the retina, which are significantly blunted by ACS67 (Figure 5.7 and 5.8). Thus ischaemia/reperfusion causes both a reduction in retinal mRNA/proteins and optic nerve proteins associated with ganglion cells and this is nullified by intravitreal injection of ACS67.

GFAP is associated with retinal glial (astrocytes and Müller cells) and when these cells are activated by ischaemia the expression of GFAP is up-regulated (Bringmann et al. 2006). It remains unknown exactly why this occurs. In the present studies, GFAP protein and mRNA are up-regulated following ischaemia/reperfusion. This is consistent with the immunohistochemical findings (Figure 5.12) and other published data to show that GFAP immunoreactivity in retinal Müller cells is enhanced following ischaemia/reperfusion (Bringmann et al. 2006; Nishiyama et al. 2000; Barnett and Osborne 1995). Significantly ACS67 attenuates ischaemia/reperfusion-induced up-regulation of Müller cell GFAP protein and mRNA.

Various studies have shown that retinal neuronal death caused by ischaemia/reperfusion occurs by apoptosis (Osborne et al. 2004; Katai and Yoshimura 1999). Evidence for this is supported in the present study where it is shown that caspase-3 mRNA, caspase-8 mRNA and PARP mRNA/protein are up-regulated in the retina following ischaemia/reperfusion. During apoptosis caspase-3, caspase-8 and PARP are known to be up-regulated (Grütter 2000; Dawson and Dawson 2004; Hong et al. 2004; Kroemer 2001). The finding that ACS67 significantly attenuates the up-regulation of PARP protein caused by ischaemia/reperfusion provides further evidence that ACS67 is a neuroprotective agent. It is difficult to explain the lack of effect of ACS67 on the up-regulation of caspase-3, caspase-8 and PARP mRNAs caused by ischaemia/reperfusion. One possible explanation is that ischaemia/reperfusion caused too great an elevation of caspase3/8, PARP mRNAs and that the positive influence of ACS67 was insufficient to be detected.

Using RGC-5 cell line (Krishnamoorthy et al. 2001), this present study showed that GB-induced cell apoptosis was attenuated by ACS67. In contrast, latanoprost is ineffective. Oxidative stress caused by GB and apoptosis is indicated by a rise in intracellular ROS and the staining of cell membranes for phosphatidylserine or DNA using APOP*ercentage* or Hoechst dye, respectively. It is possible that the neuroprotective effect of ACS67 is caused partly, by the release of H_2S from the molecule. In contrast, there is no evidence that NAC acts as a neuroprotectant by such a process. It has been demonstrated that H_2S is very effective at counteracting an insult of oxidative stress to cells in culture (Kimura et al. 2006; Lu et al. 2008; Qu et al. 2008). The present study also shows that ACS67 stimulate GSH formation in RGC-5 cells (Figure 5.4).

There is also evidence to suggest that intracellular cysteine levels are enhanced indirectly to form GSH by H_2S stimulation of glutamate/cystine antiporters and γ-glutamylcysteine activity (Kimura and Kimura 2004). In conclusion, ACS67 attenuates, at least partially, the process of oxidative stress-induced RGC-5 cell death by the release of H_2S to cause an elevation of GSH. While evidence exists to show that raising intracellular GSH can influence oxidative stress and in consequence counteract apoptosis (Qu et al. 200;, Ben-Yoseph et al. 1996; Atkuri et al. 2007; Roh et al. 2007) it cannot be concluded from the present studies that the neuroprotective effect of ACS67 is solely due to its action on GSH. The actual process by which ACS67 act as a neuroprotectant probably involves a number of mechanisms that are affected by H_2S and not solely by a stimulation of GSH. Other mechanisms by which H_2S has been shown to have a distinctive action include the activation of ATP-dependent K^+(K_{ATP}) and Cl^- channels, postulated to be caused indirectly by oxidative stress to immortalised hippocampal cells (Kimura and Kimura 2004; Kimura et al. 2006). It has also been observed that H_2S protects astrocytes from injury caused by H_2O_2 by promoting glutamate uptake and in the process decreasing ROS generation, enhancing ATP production and suppressing ERK1/2 activation (Lu 2008). Moreover, H_2S stimulates adenylate cyclase and in the process modulates intracellular mechanisms (Qu et al. 2008) that could attenuate defined death mechanisms in neurones.

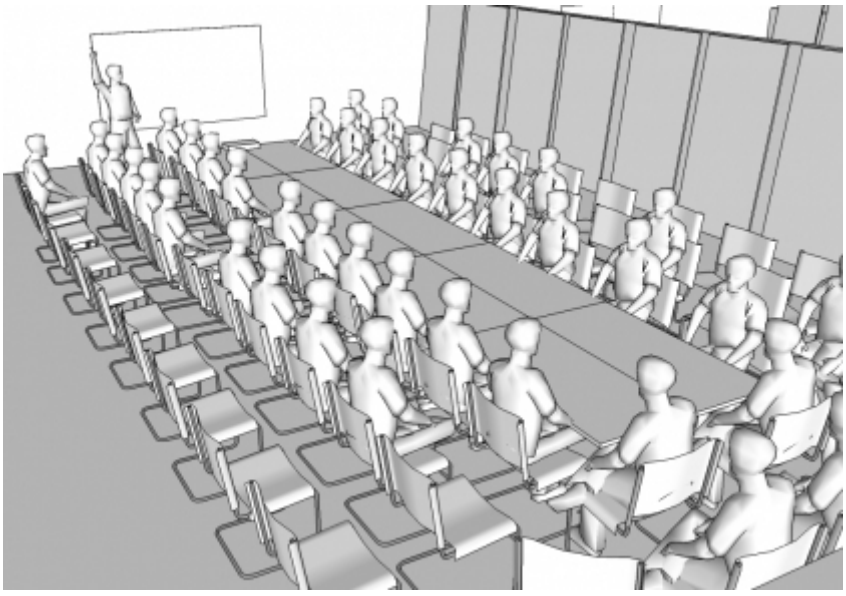
The present study also shows that latanoprost does not counteract GB-induced loss of RGC-5 cell viability in culture (Figure 5.1B). This observation provides support for the notion that the neuroprotective characteristics associated with ACS67 is partly due to H_2S being released from

the sulfurated moiety associated with latanoprost rather than latanoprost itself. Three publications have appeared which describe latanoprost to have neuroprotective properties. In the first study latanoprost was shown to protect retinal cells *in situ* (ischaemia) and *in vitro* (glutamate toxicity) by influencing cyclo-oxygenase and nitric oxide synthase activities (Drago et al. 2001). In the second study it was reported that ganglion cell death induced by axotomy or injection of NMDA into the vitreous humour are blunted by latanoprost administration (Kudo et al. 2006). Although minimal in effect, the third study latanoprost was reported to attenuate glutamate/BSO death to RGC-5 cells (Kanamori et al. 2009). The authors also demonstrated that topical application of latanoprost attenuated retinal ganglion cell death *in situ* caused by optic nerve crush (Kanamori et al. 2009). It is difficult to explain why this was not the case in the current study but it is possible that the latanoprost preparation that was used might have been of different formulation or due to variations in experimental conditions.

The conclusion reached from this study is that ACS67 is rapidly hydrolysed *in situ* or *in vitro* to release H_2S which has neuroprotective properties and results in an attenuation of the negative effects of retinal ischaemia or an oxidative insult to RGC-5 cells in culture. Once the sulfurated moiety is released from ACS67 the resultant latanoprost can have a negative effect on cell survival if present in too great a concentration. This idea is supported by the finding that latanoprost has no neuroprotective properties at low concentrations but negative effects at high concentrations.

Chapter 6

General Discussion



Glaucoma is a multi-factorial disease where raised IOP is an important risk factor (Leske et al. 1995). Until recently, IOP-lowering agents have been the mainstay of the treatment of the disease. Even with recent advances in glaucoma pharmacology, which have dramatically improved the efficacy and safety of topical glaucoma medications, there are still many patients who show progression of the disease despite treatment (Tezel et al. 2001). This may suggest that IOP-independent mechanisms could also contribute to the progression of the disease. Thus, the demand for new and better treatment strategies continues to rise and ample efforts have been devoted to finding new treatment modalities that confer direct protection to the RGCs that are damaged in glaucoma (Quigley et al. 1982; Nickells 1996). *In vivo* and *in vitro* studies involving the use of pharmacological agents with antioxidants, anti-apoptotic, anti-inflammatory and other neuro-protective properties to target IOP-independent mechanisms are currently ongoing.

The primary aim of this thesis is to provide evidence to support the hypothesis that sulphur-containing compounds are worthy of consideration as possible neuroprotectants in the treatment of glaucoma. The compounds studied include the thiolic thiamine derivative sulbutiamine and the procysteine GSH precursor drug NAC, and both have been used clinically as adjuvants for the treatment for a number of other human diseases (Perry and Shannon 1998; Walker et al. 2003; Tepel et al. 2000; Mandel et al. 1990; Berk et al. 2008; Ollat et al. 2007; Van Reeth et al. 1999; Tieve et al. 1999). Furthermore, the unique property of releasing H_2S gas by another category of sulphur compound dithiolethiones (i.e. ACS1 and its derivative ACS67, which

consists of a conjugate of latanoprost and dithiolethione molecules (Li et al. 2007)) was analysed for its ability to attenuate oxidative stress and retinal ischaemia. Thus neuroprotective studies with ACS1 were also carried out in comparison to the sulphur compound NaHS, which is also known to release H_2S.

It has been proposed that ischaemia is a major player in the initiation of the pathogenesis of glaucoma that ultimately results in RGC death and loss of vision (Osborne 2010; Osborne et al. 2001; Osborne et al. 2004). Ischaemic insults to the retina result in a number of effects that include oxidative stress, a cause for ganglion cell death in various animal glaucoma models (Levkovitch–Verbin et al. 2000; Yoles and Schwartz 1998). Significantly, evidence exists to show that the antioxidant status in glaucoma patients is markedly affected (Izzotti et al. 2003).

It is of importance to note that ACS67 – partly through the release of H_2S – has been shown to increase GSH and lower IOP in an ocular hypotensive rabbit glaucoma model (Perrino et al. 2009). As demonstrated in this study, the sulphur compounds sulbutiamine and NAC also stimulate GSH, but no evidence exists to suggest that they do so by releasing H_2S. It is of interest to note that, while studies have generally shown that substances that donate H_2S act as neuroprotectants (Kimura and Kimura 2004, Kimura et al. 2005, Kimura et al. 2006), there are reports which suggest that they are often toxic to cells (Wallace et al. 2007; Qu et al. 2006). One aim of the present studies was therefore to determine whether the sulphur compounds NAC and sulbutiamine (with no known ability to release H_2S like certain other sulphur-containing substances) act as neuroprotectants and to learn more about how this process might be elicited.

As shown in Chapter 3, serum deprivation and GB to RGC-5 cells in culture resulted in an increased level of ROS. However, how this ROS elevation relates to oxidative stress and whether ROS is primarily or secondarily involved in the cell death mechanism caused by the different insult remains a matter of debate. It is likely that the use of GB to induce cell death in cultures involves oxidative damage of some sort. It is known that excess extracellular concentrations of glutamate can cause oxidative stress by overwhelming the protective capacity of endogenous intracellular antioxidant defence systems in cells. This occurs because high levels of extracellular glutamate impair cystine uptake which is metabolically required for the production of GSH, H_2S and other important cellular substrates (Sen and Packer 2000; Kimura et al. 2010; McBean 2011). This can result in an endogenous production of free radicals such as H_2O_2 caused by superoxide, which is derived from mitochondria (Boveris et al. 1976). Once produced in defined amounts, H_2O_2 can, for example, generate hydroxyl radicals and hydroxide ions through the Fenton reaction (Winterbourn 1995; Sutton and Winterbourn 1989). Hydroxyl radicals are extraordinarily reactive and can mediate oxidation reactions to create protein carbonyls (Stadtman 1992). Moreover, H_2O_2 can directly damage DNA by producing a ferryl radical (Imlay et al. 1988). It can also oxidize protein cysteinyl (James and Murphy 2002) and methionine residues (Griffiths and Cooney 2002), which results in the modification of protein structures.

It has been suggested that, in glaucoma, elevated IOP or ischaemia reduces the availability of appropriate neurotrophic factors required by retinal ganglion cells for optimum survival (Quigley et al. 1982; Nickells 1996; Pease et al. 2000). Similar to the neurotrophic factor deprivation to ganglion cells *in situ* induced by raised IOP as occurs in glaucoma, serum withdrawal *in vitro* to

RGC-5 cells reduces the neurotrophins and antioxidants available to the cells. As a consequence, various experimental *in vitro* studies have been conducted in the past where serum withdrawal has been proposed to simulate conditions of axonal damage in some optic nerve injuries or elevated intraocular pressure (Quigley et al. 1982; Nickells 1996; Pease et al. 2000).

Many studies have also shown that ROS generation occurs in serum deprivation-induced cell death (Moon 2008; Zhuge and Cederbaum 2006; Pandey et al. 2003; Lee et al. 2010), but whether the ROS that are generated are the cause of cell death or a result of cells dying is a matter of speculation. Several studies have shown that serum deprivation results in an indirect production of ROS. For example, serum deprivation causes a change in mitochondrial membrane permeability and the collapse of the mitochondrial membrane potential that result in the production of ROS (Almeida et al. 2002; Fiers et al. 1999). Other studies have shown that the increase in ROS production caused by serum deprivation results from an activation of NADPH oxidase and xanthine oxidase (Pandey et al. 2003; Beswick et al. 2001; Tsujimoto et al. 1993). Thus, although serum deprivation or GB insults resulted in ROS production in RGC-5 cells and could therefore cause oxidative stress, it is however, unclear how they generate ROS. This is particularly true in the case of serum deprivation, where it could not be established whether other processes play more of a role in causing cell death rather than just the effects of oxidative stress.

Given that sulbutiamine is not as effective at scavenging certain types of ROS when compared with NAC (Figure 3.4), this might explain why sulbutiamine did not show any protective effect against an insult of GB, which may have generated more ROS through the depletion of GSH. This might also explain why sulbutiamine was able to protect cells from serum deprivation,

which generates less ROS (Figure 3.4). In the present study, all sulphur compounds tested quenched ROS (such as H_2O_2 and superoxide) with different efficiency, with the ACS compounds appearing to be the most effective. However, whether sulbutiamine may be effective at scavenging hydroxyl radicals at low concentrations cannot be ruled out. Furthermore, the sensitivity of the detection assay used may also be a factor in measuring the effectiveness of a particular substance in scavenging different types of ROS.

Part of the rationale for considering thiol-containing compounds as neuroprotective agents is that cellular thiol pools have been shown to be important in the regulation of the redox status of cells. For example, thiols provide a large antioxidant pool in cells that include free thiols, thiols associated with disulphide bonds and thiols bound to proteins (Wood et al. 2007; Sen and Packer 2000; Kimura et al. 2010; McBean 2011). Thiols and H_2S released from certain sulphur compounds can help maintain the redox balance, keep a reduced environment, scavenge reactive oxygen and nitrogen species and are involved in the detoxification of many other toxins and stress-inducing factors (Wood et al. 2007; Sen and Packer 2000; Kimura et al. 2010; McBean 2011). In most organisms, the major thiol is the tripeptide GSH. GSH is a major sulfhydryl (-SH) group antioxidant. In cellular defence systems, GSH acts as a nucleophile to donate electrons and is involved in redox reactions (Wood et al. 2007; Sen and Packer 2000; Kimura et al. 2010; McBean 2011). Since GSH and H_2S play important roles in protection against oxidative stress and many other functions, logic suggests that inducing an upregulation of GSH or regulating intracellular H_2S levels may help to protect RGCs against the damage of oxidative stress, as well as other insults where oxidative stress is implicated. Studies with thiol-containing compounds,

such as NAC and sulbutiamine, as well as thiols that release H_2S (such as NaHS and ACS) cause a remarkable upregulation of GSH and have the ability to protect cells from different types of oxidative insults. Their mechanisms of action are similar, but probably vary in detail. In the present studies, it is shown that sulbutiamine, NAC and the ACS compounds all induce a dose-dependent increase of reduced glutathione in RGC-5 cells. Furthermore, the ACS compounds were shown to regulate the release of H_2S in the cells. Apart from the maintenance of reduced GSH, the effect of these compounds in stimulating the activity of GST may also play an important role in their neuro-protective characteristics. Although the effects of these sulphur compounds on the activities of other GSH-related enzymes was not investigated, there is reason to believe they may also increase the activity of other GSH-related enzymes, such as glutathione reductase, glutathione peroxidase and other phase two detoxification enzymes. The data generated in this thesis shows that sulbutiamine, NAC, NaHS and ACS were able to stimulate GST activity with different efficacy. Similarly, biochemical assay showed they did not confer protection to RGC-5 cells equally. Thus, stimulation of GST activity, although playing a vital role in the antioxidant defence, may not be the only essential component in determining a cell's survival. Also, studies with sulbutiamine and NAC showed their different effects on the antioxidant catalase, which may contribute to their different detailed modes of action. Furthermore, in this study, it was shown that lipid peroxidation induced by SNP (a NO donor) is attenuated by the different sulphur compounds but with different efficiencies, with ACS1 being the most effective of the compounds investigated.

Excessive DNA damage results in over-activation of PARP-1 (a nuclear protein that is involved in the DNA-base-excision-repair system) (Hong et al. 2004; De Murcia et al. 1994). It was

initially thought that PARP-1 cleavage was catalysed by caspase-3 (Salvesen and Dixit 1997; Tewari et al. 1995) or caspase-7 (Germain et al. 1999). Thus, being a target molecule of effector caspases, PARP-1 cleavage has been regarded as evidence of caspase activation (Yang et al. 2004). However, other evidence has revealed that PARP-1 can also be cleaved in the absence of caspase-3 or caspase-7 (Masdehors et al. 2000). Interestingly, there was no evidence of caspase-3 activation when RGC-5 cells were treated with 5 mM of GB. Furthermore, the effects of GB were not attenuated by the inclusion of the pancaspase-inhibitor z-VAD-fmk (Figure 3.5). However, the inclusion of necrostatin-1 in GB-treated cultures rescued RGC-5 cells significantly. These findings do not show whether caspase-3 is involved in GB-induced insults to RGC-5 cells. Nevertheless, the results presented in this thesis suggest that cell death induced by serum deprivation results in the activation of caspase-3 and therefore is more apoptotic. GB-induced cell death, being attenuated by necrostatin-1, showed no involvement of caspase-3 and thus showed less features of classical apoptosis (Nicholson et al. 1997; Tewari et al. 1995).

No evidence exists to show that the selected sulphur compounds used in this study can reach the posterior parts of the human eye, although topical drug delivery does reach the retina in small laboratory animals such as the rat and the rabbit, probably via the systemic system (Osborne et al. 1999b). It has been shown, for example, that a beta blocker topically applied to a rabbit eye reached the retina of both the treated and untreated eyes, suggesting that this drug was distributed by the systemic system (Osborne et al. 1999b). While no evidence exists to suggest that topically administered latanoprost can reach the retina in the human eye, topically applied latanoprost via eye drops in rabbits, monkeys and humans has been reported to increase the optic nerve-head blood flow (Ishii et al. 2001). Whether this effect was caused by latanoprost directly reaching the

optic nerve or whether it was the result of an indirect action remains to be established.

The possible advantage of the proposed use of sulbutiamine, NAC and the ACS sulphur compounds as neuro-protective agents in glaucoma is that they can be taken orally. Indeed, sulbutiamine and NAC are already sold in tablet form as daily dietary supplements and derivatives of ACS are currently being evaluated as a chemo-preventive agent for certain types of cancer (Roebuck et al. 2003). However, an orally administrated sulphur compound for the treatment of glaucoma would have to reach the retina in significant amounts to counteract any detrimental effect to ganglion cells. Sulbutiamine has the added advantage that it is highly lipid soluble and easily crosses the BBB (Bettendorff et al. 1990). However, it remains to be clearly established whether orally administered sulbutiamine or any of the sulphur compounds used in this study can reach the human retina.

The molecular mechanism of how endogenous cellular antioxidants are generated in sufficient amounts to counteract oxidative stress sufficiently to enhance neuronal survival following a specific insult remains unknown (Ritch 2007). A recently elucidated pathway involving the Nrf-2 transcription factor that induces antioxidant enzymes involves transcriptional activation through the antioxidant-responsive element (ARE) (Lim et al. 2007). In this case, electrophilic agents including reactive oxygen species induce a set of genes encoding Phase II enzymes, including hemeoxygenase-1 (HO-1), NADPH quinine oxidoreductase 1, and γ-glutamyl cysteine ligase (γ-GCL). These enzymes provide efficient cytoprotection, in part by regulating the intracellular redox state (Lim et al. 2007). Sun et al. (2010) showed that pharmacological induction of HO-1 by cobalt protoporphyrin resulted in HO-1 expression in the whole of the retina. Furthermore,

HO-1 over-expression alleviated retinal apoptosis, preserved RGCs, and attenuated reduction of inner retinal thickness after ischaemia-reperfussion injury (Sun et al 2010). Experimental studies in this thesis show that sulbutiamine, NaHS and ACS1, although variable in degree, all significantly caused a stimulation of Nrf2 and the Phase II-dependent enzymes HO-1 and GST, while NAC had no significant effect. Interestingly Forresti et al. (1997) reported that co-incubation with NAC significantly attenuated HO-1 induction by SNP and SNAP, and a reduction in HO-1 activity was also observed when bovine endothelial cells were preincubated with NAC for 16 hours prior to exposure to NO donors. The effect of these sulphur compounds on the endogenous cellular antioxidant architecture thus varies. The differences in Nrf2 stimulation may partly explain the observed variation in their degree of neuroprotection. Sulbutiamine and ACS1 both stimulated Nrf-2 but only ACS1 significantly attenuated the effects of GB which decreases GSH significantly. Since ACS1 is more efficacious than sulbutiamine in stimulating GSH, this could explain why these substances which both stimulate Nrf-2 do not equally attenuate the detrimental effects of GB. Also neuroprotection via Nrf-2 induction may be specific to a particular death mechanism or stage of cell death. Furthemore the sulphur compounds ACS1 and NaHS influence the levels of H_2S release differently thus this may also possibly have an influence on their ability to stimulate Nrf2. ACS1, which slowly releases H_2S (Li et al. 2007), showed a greater efficacy at stimulating Nrf2 and HO-1 levels. In addition the sustained effect of H_2S release from ACS1 compared to NaHS might also determine its influence on certain signaling proteins like PKCα and NfkB. This might also explain why, unlike NaHS, ACS1 increased PKCα levels and stimulated its translocation to the nucleus.

In the *in vivo* rat experiments (Chapter 5), ischaemia was induced by elevating the IOP (up to

120 mm Hg for 50 minutes). In this rat ischaemia/reperfusion model, the magnitude of IOP elevation, the duration of the raised IOP and the reperfusion time determines the degree of damage to the retina. Although a large number of studies have been conducted using this model, it is not an experimental model of true glaucoma. For example, apoptosis of ganglion cells occurs slowly over many years in true glaucoma (Osborne et al. 1999a, e; Quigley 1999; Kerrigan-Baumrind et al. 2000), while in the rat ischaemia/reperfusion model ganglion cells die within a few days. The rat ischaemia/reperfusion model may relate more closely to what may occur in human acute angle closure glaucoma. In the rat ischaemia/reperfusion model, the raised IOP probably causes both ischaemia and pressure-induced injury to retinal ganglion cells (Gehlbach and Purple 1994). Indeed, it has been suggested that, in animal models of retinal ischaemia, less retinal injury occurs when ischaemia is induced by vascular ligation than elevation of IOP (Osborne et al. 2004a). However, the rat ischaemia/reperfusion model used in this study does provide consistent results, which are important when trying to demonstrate that a substance is a neuroprotectant. It is also important to recognize that information generated by use of the rat ischaemia/reperfusion model is not necessarily applicable to true glaucoma. Whatever the limitations of the ischaemia/reperfusion model, it is clearly demonstrated in this study that ACS67 attenuates retinal ganglion cell death.

The present study provides data to support the use of selected sulphur drugs like sulbutiamine, NAC and the H_2S-releasing ACS compounds as neuroprotectants in the treatment of neurodegenerative diseases like glaucoma. However, in the context of treating glaucoma it will prove more challenging to devise delivery systems to target these compounds to the retina. Further rigorous scientific studies looking at the pharmacodynamics and pharmacokinetics of

these compounds need to be undertaken to take it to the next level. For future studies, it would be interesting to determine whether sulbutiamine or NAC has any IOP-lowering characteristics and whether H_2S-releasing sulphur drugs are more efficacious than latanoprost in the management of glaucoma in humans.

Chapter 7

References



Abe K, Kimura H (1996) The possible role of hydrogen sulfide as an endogenous neuromodulator. J Neurosci 16:1066-71

Abu-Amero KK, Morales J, Bosley TM (2006) Mitochondrial abnormalities in patients with primary open-angle glaucoma. Invest Ophthalmol Vis Sci 47:2533-2541

Abele AE, Scholz KP, Scholz WK, Miller RJ (1990) Excitotoxicity induced by enhanced excitatory neurotransmission in cultured hippocampal pyramidal neurons. Neuron 4:413-419

Acuña-Castroviejo D, Martín M, Macías M, et al. (2001) Melatonin, mitochondria, and cellular bioenergetics. J Pineal Res 30:65–74

Adair JC, Knoefel JE, Morgan N (2001) Controlled trial of N-acetylcysteine for patients with probable Alzheimer's disease. Neurology 57:1515-7

Adachi M, Takahashi K, Nishikawa M et al. (1996) High intraocular pressure-induced ischemia and reperfusion injury in the optic nerve and retina in rats. Graefes Arch Clin Exp Ophthalmol 234:445-51

Adom KK, Liu RH (2005) Rapid peroxyl radical scavenging capacity (PSC) assay for assessing both hydrophilic and lipophilic antioxidants. J Agric Food Chem 53:6572-80

Adams JM, Cory S (1998) The Bcl-2 protein family: Arbiters of cell survival. Science 281: 1322-1326

Agardh CD, Gustavsson C, Hagert P et al. (2006) Expression of antioxidant enzymes in rat retinal ischemia followed by reperfusion. Metab-Clin Exp 55:892-898

Ahnelt P, Kolb H (1994) Horizontal cells and cone photoreceptors in human retina: a Golgi-electron microscopic study of spectral connectivity. J Comp Neurol 343: 406-27

Ahmed SA, Gogal RM Jr, Walsh JE (1994) A new rapid and simple non-radioactive assay to monitor and determine the proliferation of lymphocytes: an alternative to [3H] thymidine incorporation assay. J Immunol Methods 170:211-24

Alam J, Stewart D, Touchard C et al. (1991) Nrf2, a Cap'n'Collar transcription factor, regulates induction of the heme oxygenase-1 gene. J Biol Chem 274:26071-26078

Allen JW, Shanker G, Aschner MJW (2001) Methylmercury inhibits the in vitro uptake of the glutathione precursor, cystine, in astrocytes, but not in neurons. Brain Res 894:131–140

Alladina SJ, Song JH, Davidge ST et al. (2005) TRAIL-induced apoptosis in human vascular endothelium is regulated by phosphatidylinositol 3-kinase/Akt through the short form of cellular FLIP and Bcl-2 J. Vasc Res 42:337-47

Alvarado J, Murphy C, Polansky J et al. (1981) Juster R. Age-related changes in trabecular meshwork cellularity. Invest Ophthalmol Vis Sci 21:714–727

Almeida A, Delgado-Esteban M, Bolanos JP et al. (2002) Oxygen and glucose deprivation induces mitochondrial dysfunction and oxidative stress in neurones but not in astrocytes in primary culture. J Neurochem 81: 207-217

Anderson KE, Jackson SP (2003) Class I phosphoinositide 3-kinases. Int J Bioch Cell Biol 35:1028-1033

Ansher SS, Dolan P, Bueding E (1983) Chemoprotective effects of two dithiolthiones and of butylhydroxyanisole against carbon tetrachloride and acetaminophen toxicity, Hepatology 3:932–935

Aouacheria A, Cibiel A, Guillemin Y (2007) Modulating Mitochondria-Mediated Apoptotic Cell Death through Targeting of Bcl-2 Family Proteins. Recent Patents on DNA & Gene Sequences 1: 43-61

Asregadoo ER (1979) Blood levels of thiamine and ascorbic acid in chronic open-angle glaucoma. Ann Ophthalmol 11:1095-1100

Atchison WD, Hare MF (1994) Mechanisms of methylmercury-induced neurotoxicity. FASEB 8:622-629

Ates B, Abraham L, Ercal N (2008) Antioxidant and free radical scavenging properties of Nacetylcysteine amide (NACA) and comparison with N-acetylcysteine (NAC). Free Radic Res 42: 372–377

Atkuri KR, Mantovani JJ, Herzenberg LA et al. (2007) N-Acetylcysteine-a safe antidote for cysteine/glutathione deficiency. Curr Opin Pharmacol 7:355-359

Atlante A, Calissano P, Bobba A et al. (2001) Glutamate neurotoxicity, oxidative stress and mitochondria. FEBS Lett 497: 1-5

Atmaca G (2004) Antioxidant effects of sulfur-containing amino acids. Yonsei Med J 45**:**776–778

Auer M, Pfister LA, Leppert D et al. (2000) Effects of clinically used antioxidants in experimental pneumococcal meningitis. J Infect Dis 182:347-50

Avila MA, Berasain C, Torres L et al. (2000) Reduced mRNA abundance of the main enzymes involved in methionine metabolism in human liver cirrhosis and hepatocellular carcinoma. J Hepatol 33: 907–14

Bao L, Vlcek C, Paces V et al. (1998) Identification and tissue distribution of human cystathionine beta -synthase mRNA isoforms. Arch Biochem Biophys 350: 95-103

Banzet N, Francıois D, Polla BS (1999) Tobacco smoke induces mitochondrial depolarization along with cell death: effects of antioxidants. Redox Rep 4:229-236

Barnett NL, Osborne NN (1995) Prolonged bilateral carotid artery occlusion induces electrophysiological and immunohistochemical changes to the rat retina without causing histological damage. Exp Eye Res 61:83-90

Barnstable CJ, Drager UC (1984) Thy-1 antigen: a ganglion cell specific marker in rodent retina. Neuroscience 11: 847-855

Baffy C, Miyashati I, Williamson JR et al. (1993) Apoptosis induced by withdrawal of interleukin-3 (IL-3) dependent hematopoietic cell line is associated with repartitioning of intracellular calcium and is blocked by enforced Bcl-2 oncogene production. J Biol C 268:6511-6519

Belecky-Adams T, Cook B, Adler R (1996) Correlations between terminal mitosis and differentiated fate of retinal precursor cells in vivo and in vitro: analysis with the “window labeling” technique. Dev Biol 178: 304-315

Belenky MA, Smeraski CA, Provencio I et al. (2003) Melanopsin retinal ganglion cells receive bipolar and amacrine cell synapses. J Comp Neurol. 460:380-93

Berson DM (2003) Strange vision: ganglion cells as circadian photoreceptors. Trends Neurosci.26:314-20

Beswick RA, Dorrance AM, Leite R, Webb RC (2001) NADH/NADPH oxidase and enhanced superoxide production in the mineralocorticoid hypertensive rat. Hypertension 38: 1107-1111

Bettendorff L (1994a) The compartmentation of phosphorylated thiamine derivatives in cultured neuroblastoma cells. Biochim Biophys Acta 1222: 7-14

Bettendorff L, Hennuy B, De Clerck A et al. (1994b) Chloride permeability of rat brain membrane vesicles correlates with thiamine triphosphate content. Brain Res 652:157-60

Bettendorff L, Weekers L, Wins P et al. (1990) Injection of sulbutiamine induces an increase in thiamine triphosphate in rat tissues. Biochem Pharmacol 40:2557-2560

Bernaerts P, Tirelli E (2003) Facilitatory effect of the dopamine D4 receptor agonist PD168,077 on memory consolidation of an inhibitory avoidance learned response in C57BL/6J mice. Behav Brain Res 142: 41–52

Berk M, Copolov DL, Dean O et al. (2008) N-acetyl cysteine for depressive symptoms in bipolar disorder-a double-blind randomized placebo-controlled trial. Biol Psychiatry 64:468-75

Beaver JP, Waring P (1995) A decrease in intracellular glutathione concentration precedes the onset of apoptosis in murine thymocytes. Eur J Cell Biol 68:47–54

Becker B (1979) The decline in aqueous secretion and outflow facility with age. Am J Ophthalmol. 97:1667–1969

Ben-Mahdi MH, Gozin A, Driss F et al. (2000) Anethole dithiolethione regulates oxidant-induced tyrosine kinase activation in endothelial cells. Antioxid Redox Signal. 2:789-99

Ben-Yoseph O, Boxer PA, Ross BD (1996) Assessment of the role of the glutathione and pentose phosphate pathways in the protection of primary cerebrocortical cultures from oxidative stress. J Neurochem 66:2329-37

Benveniste H, Drejer J, Schousboe A, Diemer NH (1984) Elevation of the extracellular concentrations of glutamate and aspartate in rat hippocampus during transient cerebral ischemia monitored by intracerebral microdialysis. J Neurochem 43: 1369-1374

Beauchamp RO Jr, Bus JS, Popp JA et al. (1984) A critical review of the literature on hydrogen sulfide toxicity. Crit Rev Toxicol 13:25–97

Beisner DR, Ch'en IL, Kolla RV et al. (2005) Cutting edge: innate immunity conferred by B cells is regulated by caspase-8. J Immunol. 175:3469-73

Berger NA (1985) Poly(ADP-ribose) in the cellular response to DNA damage. Radiat Res 101: 4-15

Bhatia M, Zhi L, Zhang H et al. (2006) Role of substance P in hydrogen sulfide-induced pulmonary inflammation in mice. Am J Physiol 291**:**896–904

Bhatia M, Wong FL, Fu D et al. (2004) Role of hydrogen sulphide in acute pancreatitis in the mouse and rat. FASEB J 19:623-625

Bhatia M, Sidhapuriwala J, Moochhala SM et al. (2005) Hydrogen sulphide is a mediator of carrageenan-induced hindpaw oedema in the rat. Br J Pharmacol 145:141-144

Bizot JC, Herpin A, Pothion S et al. (2005). Chronic treatment with sulbutiamine improves memory in an object recognition task and reduces some amnesic effects of dizocilpine in a spatial delayed-non-match-to-sample task. Prog Neuropsychopharmacol Biol Psychiatry 29: 928–35

Bliksøen M, Kaljusto ML, Vaage J et al. (2008) Effects of hydrogen sulphide on ischaemia-reperfusion injury and ischaemic preconditioning in the isolated, perfused rat heart. Eur J Cardiothorac Surg 34:344-9

Block F, Schwarz M (1998) The b-wave of the electroretinogram as an index of retinal ischemia. Gen Pharmacol 30:281-87

Boehning D, Snyder SH (2003) Novel neural modulators. Annu Rev Neurosci 26:105-31

Bonfoco E, Krainc D, Ankarcrona M, et al. (1995) Apoptosis and necrosis: two distinct events induced, respectively, by mild and intense insults with N-methyl-D-aspartate or nitric oxide/superoxide in cortical cell cultures. Proc Natl Acad Sci USA. 92:7162–7166

Borgström L, Kågedal B, Paulsen O (1986) Pharmacokinetics of N-acetylcysteine in man. Euro J Clin Pharm 31: 217–222

Bonne C, Muller A, Villain M (1998) Free Radicals in Retinal Ischemia. Gen Pharmacol 30: 275-280

Bonni A, Brunet A, West AE et al. (1999) Cell survival promoted by the Ras-MAPK signaling pathway by transcription-dependent and -independent mechanisms. Science 286: 1358-1362

Boycott BB, Wassle H (1991) Morphological classification of bipolar cells of the primate retina. Eur J Neurosci 3: 1069-1088

Boyd ZS, Kriatchko A, Yang J et al. (2003) Interleukin–10 receptor signaling through STAT–3 regulates the apoptosis of retinal ganglion cells in response to stress. Invest Ophthalmol Vis Sci 44:5206–5211

Boulton M, Dayhaw-Barker P (2001) The role of the retinal pigment epithelium: topographical variation and ageing changes. Eye 15:384-9

Boulton M, Róanowska M, Wess T (2004) Ageing of the retinal pigment epithelium: implications for transplantation. Graefes Arch Clin Exp Ophthalmol. 242:76-84

Boveris A, Chance B (1973) The mitochondrial generation of hydrogen peroxide. General properties and effect of hyperbaric oxygen. Biochem J. 134:707-16

Boveris A, Cadenas E, Stoppani AO (1976) Role of ubiquinone in the mitochondrial generation of hydrogen peroxide. Biochem J. 156:435-44

Boveris A, Navarro A (2008) Brain mitochondrial dysfunction in aging. IUBMB Life.60:308-14

Boehning D, Snyder SH (2003) Novel neural modulators. Annu Rev Neurosco 26:105-31

Bossy-Wetzel E, Newmeyer DD, Green DR (1998) Mitochondrial cytochrome c release in apoptosis occurs upstream of DEVD-specific caspase activation and independently of mitochondrial transmembrane depolarization. Embo J 17: 37-49

Bojes HK, Datta K, Xu J et al. (1997) Bcl-x_L overexpression attenuates glutathione depletion in FL5.12 cells following interleukin-3 withdrawal. Biochem J 325:315-319

Bruce WR, Furrer R, Shangari N et al. (2003) Marginal dietary thiamine deficiency induces the formation of colonic aberrant crypt foci (ACF) in rats. Cancer Lett 202:125-129

Brand MD, Affourtit C, Esteves TC, et al. (2004) Mitochondrial superoxide: production, biological effects, and activation of uncoupling proteins Free Radic Biol Med 37:755-767

Bringmann A, Pannicke T, Grosche J et al. (2006) Müller cells in the healthy and diseased retina. Prog Retin Eye Res 25:397-424

Brandt R, Keston AS (1965) Ssynthesis of diacetyldichlorofluorescin-a stable reagent for fluorometric analysis. Anal Biochem 11: 6-9

Bredesen DE (2007) Key note lecture: toward a mechanistic taxonomy for cell death programs. Stroke. 38:652-60

Bredesen DE (2008) Programmed cell death mechanisms in neurological disease. Curr Mol Med. 8:173-86

Brunet A, Bonni A, Zigmond MJ et al. (1999) Akt promotes cell survival by phosphorylating and inhibiting a forkhead transcription factor. Cell 96: 857-868

Brunet A, Datta SR, Greenberg ME (2001) Transcription-dependent and -independent control of neuronal survival by the PI3K-Akt signaling pathway. Curr Opin Neurobiol 11: 297-305

Bridges CC, Kekuda R, Wang H et al. (2001) Structure, function, and regulation of human cystine/glutamate transporter in retinal pigment epithelial cells. Invest Ophthalmol Vis Sci 42:47–54

Bridges CC, Hu H, Miyauchi S et al. (2004) Induction of cystine–glutamate transporter xc − by human immunodeficiency virus type 1 transactivator protein tat in retinal pigment epithelium. Invest Ophthalmol Vis Sci 45: 2906–2914

Buchi ER (1992) Cell death in the rat retina after a pressure-induced ischaemia-reperfusion insult: an electron microscopic study. I. Ganglion cell layer and inner nuclear layer. Exp Eye Res 55: 605-613

Butterfield DA, Drake J, Pocernich C et al. (2001) Evidence of oxidative damage in Alzheimer's disease brain: central role for amyloid β-peptide Trends in Molecular Medicine 7:548-554

Buetler TM, Gallagher EP, Wang C et al. (1995) Induction of phase I and phase II drug-metabolizing enzyme mRNA, protein, and activity by BHA, Ethoxyquin, and Oltipraz, Toxicol Appl Pharmacol 135: 45-57

Bukovska G, Kery V, Kraus JP (1994) Expression of human cystathionine beta-synthase in Escherichia coli: purification and characterization. Protein Expr Purif 5:442-448

Buttke IM, Sandstrom PA (1994) Oxidative stress as a mediator of apoptosis. Immunol. Today 15: 7-10

Budihardjo I, Oliver H, Lutter M et al. (1999) Biochemical Pathways of Caspase Activation During Apoptosis Annu Rev Cel Dev Biol 15:269-90

Büchi ER (1992) Cell death in the rat retina after a pressure-induced ischaemia-reperfusion insult: An electron microscopic study. I. Ganglion cell layer and inner nuclear layer. Exp Eye Res. 55:605-13

Cacciatore I, Cornacchia C, Pinnen F et al. (2010) Prodrug Approach for Increasing Cellular Glutathione Levels. Molecules 15:1242-1264

Carter WO, Narayanan PK, Robinson JP (1994) Intracellular hydrogen peroxide and superoxide anion detection in endothelial cells. J Leukoc Biol 55:253-258

Casson RJ, Wood JP, Osborne NN (2004) Hypoglycaemia exacerbates ischaemic retinal injury in rats. Br J Ophthalmol. 88:816-20

Cai J, Jones DP (1999) Mitochondrial redox signaling during apoptosis. J Bioenerg Biomembr.31:327–334

Castagné V, Clarke PG (1998) Cooperation between glutathione depletion and protein synthesis inhibition against naturally occurring neuronal death. Neuroscience 86:895-902

Cenni MC, Bonfanti L, Martinou JC et al. (1996) Long-term survival of retinal ganglion cells following optic nerve section in adult Bcl-2 transgenic mice. Eur J Neurosci 8: 1735-1745

Chao DT, Korsmeyer SJ (1998) Bcl-2 Family: Regulators of Cell Death. Annu. Rev. Immunol. 16:395-419

Chan JY, Kwong M (2000) Impaired expression of glutathione synthetic enzyme genes in mice with targeted deletion of the Nrf2 basic-leucine zipper protein. Biochim Biophys Acta. 1517:19-26

Chan-Ling TL (1994) Glial, neuronal and vascular interactions in the mammalian retina. Prog Retin Eye Res 13: 357-389

Chan PH, Fishman RA (1980) Transient formation of superoxide radicals in polyunsaturated fatty acid-induced brain swelling. J Neurochem.35:1004-7

Charles I, Khalyfa A, Kumar DM et al. (2005) Serum deprivation induces apoptotic cell death of transformed rat retinal ganglion cells via mitochondrial signaling pathways. Invest Ophthalmol Vis Sci 46: 1330-1338

Chen PC, Vargas MR, Pani AK et al. (2009) Nrf2-mediated neuroprotection in the MPTP mouse model of Parkinson's disease: Critical role for the astrocyte. Prc Natl Acad Sci 106: 2933-2938

Chen G, Zeng WZ, Yuan PX et al. (1999) The mood-stabilizing agents lithium and valproate robustly increase the levels of the neuroprotective protein bcl-2 in the CNS. J Neurochem 72**:**879-882

Chen G, Rajkowska G, Du F et al. (2000) Enhancement of hippocampal neurogenesis by lithium. J Neurochem 75:1729–1734

Chen G, Yuan P, Li Jet al. (2001) Low Bcl2 levels in frontal cortex in mood disorders and upregulation of Bcl2 by mood-stabilizers. Soc Neurosci Abst 27

Chen RW and Chuang DM (1999) Long term lithium treatment suppresses p53 and Bax expression but increases Bcl-2 expression. A prominent role in neuroprotection against excitotoxicity. J Biol Chem 274: 6039-6042

Chen L, Yang P, Kijlstra A (2002) Distribution, markers, and functions of retinal microglia. Ocul Immunol Inflamm.10:27-39

Chen X, Jhee KH, Kruger WD (2004) Production of the neuromodulator H_2S by cystathionine beta-synthase via the condensation of cysteine and homocysteine. J Biol Chem 279: 52082–6

Cheon EW, Park CH, Kang SS et al. (2003) Change in endothelial nitric oxide synthase in the rat retina following transient ischemia. Neuroreport 14: 329-333

Cetinkaya A, Bulbuloglu E, Kurutas EB et al. (2005) Beneficial Effects of N-Acetylcysteine on Acetic Acid Induced Collitis in Rats. Tohoku J. Exp. Med. 206:131-139

Chung HS, Harris A, Evans DW et al. (1999) Vascular aspects in the pathophysiology of glaucomatous optic neuropathy. Surv Ophthalmol.43*:*S43–S50

Cheung NS, Peng ZF, Chen MJ et al. (2007) Hydrogen sulfide induced neuronal death occurs via glutamate receptor and is associated with calpain activation and lysosomal rupture in mouse primary cortical neurons. Neuropharmacology 53:505-514

Chidlow G, Casson R, Sobrado-Calvo P et al. (2005). Measurement of retinal injury in the rat after optic nerve transection: an RT-PCR Study. Mol Vis. 11:387–96

Charles I, Khalyfa A, Kumar DM et al. (2005) Serum deprivation induces apoptotic cell death of transformed rat retinal ganglion cells via mitochondrial signaling pathways. Invest Ophthalmol Vis Sci. 246:1330-8

Choi DW (1985) Glutamate neurotoxicity in cortical cell-culture is calcium dependent. Neurosci Lett 58: 293-297

Choi DW (1992) Excitotoxic cell-death. J Neurobiol 23: 1261-1276

Christen MO (1995) Anethole dithiolethione: biochemical considerations.Methods Enzymol. 252:316-323

Christen S, Schaper M, Lykkesfeldt J et al. (2001) Oxidative stress in brain during experimental bacterial meningitis: differential effects of alpha-phenyl-tert-butyl nitrone and N-acetylcysteine treatment.Free Radic Biol Med. 31:754-62

Collin M, Anuar FBM, Murch O et al. (2005). Inhibition of endogenous hydrogen sulfide formation reduces the organ injury caused by endotoxemia. Br J Pharmacol 146: 498–505

Cook JA, Mitchell JB (1995) Measurement of thiols in cell populations from tumor and normal tissue. Methods Enzymol 251:203-12

Cook JA, Mitchell JB (1995) Measurement of thiols in cell populations from tumor and normal tissue. Methods Enzymol 251:203-212

Cossarizza A, Franceschi C, Monti D et al. (1995) Protective effect of *N*-acetylcysteine in tumor necrosis factor-alpha-induced apoptosis in U937 cells: the role of mitochondria, Exp Cell Res 220:1232–240

Coyle JI, Puttfarcken P (1993) Oxidative stress, glutamate, and neurodegenerative disorders. Science 262: 689-695

Cohen CM, Sun XM, Snowden R et al. (1992) Key morphological features of apoptosis may recur in the absence of internucleosomal DNA fragmentation. Biochem. J 286:331-334

Cory S, Huang DC, Adams JM (2003) The Bcl-2 family: roles in cell survival and oncogenesis. Oncogene 22: 8590-8607

Cryns V, Yuan JY (1998) Proteases to die for. Genes Dev 12: 1551-1570

Cui Q, Harvey AR (1995) At least two mechanisms are involved in the death of retinal ganglion cells following target ablation in neonatal rats. J Neurosci 15: 8143-8155

Cui Q, So KF, Yip HK (1998) Major biological effects of neurotrophic factors on retinal ganglion cells in mammals. Biol. Signals Recept 7: 220-226

Curcio CA, Allen KA (1990) Topography of ganglion cells in human retina. J Comp Neurol 300:5-25

Czerski L, Nunez G (2004) Apoptosome formation and caspase activation: is it different in the heart? J Mol Cell Cardiol 37:643-52

Danbolt NC (2001) Glutamate uptake. Prog Neurobiol 65:1–105

Datta SR, Dudek H, Tao X et al. (1997) Akt phosphorylation of BAD couples survival signals to the cell-intrinsic death machinery. Cell 91: 231-241

Davies MH, Blacker AM, Schnell RC (1987) Dithiolthione-induced alterations in hepatic glutathione and related enzymes in male mice. Biochem Pharmacol 36:568-570

Dawson VL, Dawson TM (2004) Deadly conversations: nuclear-mitochondrial cross-talk. J Bioenerg Biomembr 36:287-94

Decaudin D, Geley S, Hirsch T et al. (1997) Bcl-2 and Bcl-XL antagonize the mitochondrial dysfunction preceding nuclear apoptosis induced by chemotherapeutic agents. Cancer Res 57:62-7

Dello Russo C, Tringali G, Ragazzoni E et al. (2000) Evidence that hydrogen sulphide can modulate hypothalamo-pituitary-adrenal axis function: in vitro and in vivo studies in the rat. J Neuroendocrinol 12:225-3

De Vries N, De Flora S (1993) *N*-Acetyl-l-Cysteine. J. Cell. Biochem. 53:270-277

Deneke SM (2000) Thiol-based antioxidants. Curr Top Cell Regul. 36:151-80

De Flora S, Izzotti A, D'Agostini F et al. (2001) Mechanisms of N-acetylcysteine in the prevention of DNA damage and cancer, with special reference to smoking-related end-points. Carcinogenesis 22:999-1013

Degterev A, Huang Z, Boyce M et al. (2005) Chemical inhibitor of nonapoptotic cell death with therapeutic potential for ischemic brain injury. Nat Chem Biol. 1:112-9

Deng HX, Hentati A, Tainer JA et al. (1993) Amyotrophic lateral sclerosis and structural defects in Cu.Zn superoxide dismutase. Science 261:1047-1051

De Murcia G, De Murcia JM (1994) Poly(ADP-ribose) polymerase: a molecular nick-sensor. Trends Biochem Sci. 19: 172-176

Dilger RN, Baker DH (2007) Oral N-acetyl-L-cysteine is a safe and effective precursor of cysteine. J Anim Sci. 85:1712-8

Dillin A, Hsu AL, Arantes-Oliveira N, et al. (2002) Rates of behavior and aging specified by mitochondrial function during development. Science. 298:2398-2401

Dmitriev DG, Gamidov SI, Permiakova OV (2005) Clinical efficacy of the drug enerion in the treatment of patients with psychogenic (functional) erectile dysfunction. Urologiia 1 :32-35

Douzenis A, Michopoulos I, Lykouras L (2006) Sulbutiamine, an 'innocent' over the counter drug, interferes with therapeutic outcome of bipolar disorder. World J Biol Psychiatry 7:183-185

Doeller JE, Isbell TS, Benavides G et al. (2005) Polarographic measurement of hydrogen sulfide production and consumption by mammalian tissues. Anal Biochem 341:40-51

Doronicheva N, Yasui H, Sakurai H (2007) Chemical Structure-Dependent Differential Effects of Flavonoids on the Catalase Activity as Evaluated by a Chemiluminescent Method Biol. Pharm Bull 30: 213-217

Dreyer EB, Zurakowski D, Schumer RA et al. (1996) Elevated glutamate levels in the vitreous body of humans and monkeys with glaucoma. Arch Ophthal 114:299-305

Drukarch B, Jongenelen CAM, Schepens E et al. (1996) Glutathione is involved in the granular storage of dopamine in rat PCI2 pheochromocytoma cells: implications for the pathogenesis of Parkinson's disease. J Neurosci 16:6038-6045

Dudek H, Datta SR, Franke TF et al. (1997) Regulation of Neuronal Survival by the Serine-Threonine Protein Kinase Akt. Sci 275:661-5

Du J, Yan H, Tang C (2003) Endogenous H_2S is involved in the development of spontaneous hypertension. Beijing Da Xue Xue Bao 35:102

Du KY, Montminy M (1998) CREB is a regulatory target for the protein kinase Akt/PKB. J Biol Chem 273: 32377-32379

Dun Y, Mysona B, Van Ells T et al. (2006) Expression of the cystine-glutamate exchanger (xc-) in retinal ganglion cells and regulation by nitric oxide and oxidative stress. Cell Tissue Res. 324:189-202

Dyer MA, Cepko CL (2001) Regulating proliferation during retinal development. Nat Rev Neurosci 2:333-42

Edwards RB, Adler AJ, Dev S et al. (1992) Synthesis of retinoic acid from retinol by cultured rabbit Müller cells. Exp Eye Res 54:481-90

Ellis RE, Yuan Y, Horvitz HR (1991) Mechanisms and functions of cell death. Annu Rev Cell Biol 7:663-98

Elrod JW, Calvert JW, Morrison J et al. (2007) Hydrogen sulfide attenuates myocardial ischemia-reperfusion injury by preservation of mitochondrial function. Proc Natl Acad Sci. 104:15560-5

Emonet N**,** Leccia MT**,** Favier A et al. (1997) Thiols and selenium: protective effect on human skin fibroblasts exposed to UVA radiation. J Photochem Photobiol B. 40:84-90

Eto K, Kimura H (2002) A novel enhancing mechanism for hydrogen sulfide-producing activity of cystathionine beta-synthase. J Biol Chem 277: 42680-5

Eskes R, Antonsson B, Osen-Sand A et al. (1998) Bax-induced cytochrome c release from mitochondria is independent of the permeability transition pore but highly dependent on Mg^{2+} ions. J Cell Biol 143: 217-224

Eto K, Asada T, Arima K et al. (2002) Brain hydrogen sulfide is severely decreased in Alzheimer's disease. Biochem Biophys Res Commun 293: 148-58

Eto K, Ogasawara M, Umemura K et al. (2002) Hydrogen Sulfide Is Produced in Response to Neuronal Excitation. J Neuro 22:3386-3391

Eto K, Kimura H (2002) The production of hydrogen sulfide is regulated by testosterone and *S*-adenosyl-*L*-methionine in mouse brain. J Neurochem 83: 80-6

Erickson PF, Maxwell IH, Su LJ et al. (1990) Sequence of cDNA for rat cystathionine gamma-lyase and comparison of deduced amino acid sequence with related Escherichia coli enzymes. Biochem J 269:335-340

Euler T, Wassle H (1998) Different contributions of GABA(A) and GABA(C) receptors to rod and cone bipolar cells in a rat retinal slice preparation. J Neurophysiol 79: 1384-1395

Fadok VA, Cathelineau AD, Daleke DL et al. (2001) Loss of phospholipid asymmetry and surface exposure of phosphatidylserine is required for phagocytosis of apoptotic cells by macrophages and fibroblasts, J. Biol. Chem. 276**:**1071–1077

Famiglietti E, Kolb H (1976) Structural basis for ON-and OFF-center responses in retinal ganglion cells. Science 194: 193-195

Ferreira IL, Duarte CB, Carvalho AP (1996) Ca^{2+} influx through glutamate receptor-associated channels in retina cells correlates with neuronal cell death. Eur J Pharmacol 302: 153-162

Ferreira SM, Lerner SF, Brunzini R et al. (2004) Oxidative stress markers in aqueous humor of glaucoma patients. Am J Ophthalmol.137:62–69

Fernandes RS, Cotter TG (1994) Apoptosis and necrosis: intracellular levels of glutathione influence mode of cell death. Biochem. Pharmacol. 48:675-681

Fernández-Durango R, Fernández-Martínez A, García-Feijoo J et al. (2008) Expression of nitrotyrosine and oxidative consequences in the trabecular meshwork of patients with primary open-angle glaucoma. Invest Ophthalmol Vis Sci. 49:2506-11

Ferrari R , Ceconi C, Curello S et al. (1991) Oxygen free radicals and myocardial damage: Protective role of thiol-containing agents Am J Med. 91:95-105

Fishkin N, Yefidoff R, Gollipalli DR et al. (2005) On the mechanism of isomerization of all-trans-retinol esters to 11-cis-retinol in retinal pigment epithelial cells: 11-fluoro-all- trans-retinol as substrate/inhibitor in the visual cycle. Bioorg Med Chem 13: 5189-5194

Fields RD, Lancaster MV (1993) Dual-attribute continuous monitoring of cell proliferation/cytotoxicity. Am Biotechnol Lab 1:48-50

Field AG (2003) Parametric Statistics, In: How to Design and Report Experiments. Sage Publications Ltd: Chapter 6

Feiner L, Piltz-Seymour JR (2003) Collaborative Initial Glaucoma Treatment Study. Collaborative Initial Glaucoma Treatment Study: a summary of results to date. Curr Opin Ophthalmol 14*:*106-111

Fiers W, Beyaert R, Declercq W, Vandenabeele P (1999) More than one way to die: apoptosis, necrosis and reactive oxygen damage. Oncogene 18: 7719-7730

Finkel T, Holbrook NJ (2000) Oxidants, oxidative stress and the biology of ageing. Nature 408:239-247

Fishkin N, Yefidoff R, Gollipalli DR et al. (2005) On the mechanism of isomerization of all-trans-retinol esters to 11-cis-retinol in retinal pigment epithelial cells: 11-fluoro-all-trans-retinol as substrate/inhibitor in the visual cycle. Bioorg Med Chem. 13:5189-94

Fiskum G (2000) Mitochondrial participation in ischemic and traumatic neural cell death. J Neurotrauma 17: 843-855

Flammer J, Haefliger IO, Orgül S et al. (1999) Vascular dysregulation: a principal risk factor for glaucoma damage? J Glaucoma. 8*:*212-219

Flammer J, Orgul S (1998) Optic nerve blood-flow abnormalities in glaucoma. Prog Retin Eye Res 17:267-89

Floresco SB, Phillips AG (2001) Delay-dependent modulation of memory retrieval by infusion of a dopamine D1 agonist into the rat medial prefrontal cortex. Behav Neurosci 115 :934–9

Foresti R, Clark JE, Green CJ et al. (1997) Thiol compounds interact with nitric oxide in regulating heme oxygenase-1 induction in endothelial cells. Involvement of superoxide and peroxynitrite anions. J Biol Chem. 272:18411-7.

Foster RG (1998). Shedding light on the biological clock. Neuron. 20:829-32

Foster PJ, Buhrmann R, Quigley HA et al., (2002) The definition and classification of glaucoma in prevalence surveys. Br J Ophthalmol 86:238-242

Fox JH, Barber DS, Singh B et al. (2004) Cystamine increases L-cysteine levels in Huntington's disease transgenic mouse brain and in a PC12 model of polyglutamine aggregation. J Neurochem. 91:413-22

Fournier AE, Beer J, Arregui CO et al. (1997) Brain-derived neurotrophic factor modulates GAP-43 but not t alpha 1 expression in injured retinal ganglion cells of adult rats. J Neurosci Res 47: 561-572

Fohlmeister JF, Miller RF (1997) Mechanisms by which cell geometry controls repetitive impulse firing in retinal ganglion cells. J. Neurophysiol. 78, 1948-1964

Frishman LJ, Steinberg RH (1989) Light-evoked increases in [K+]o in proximal portion of the dark-adapted cat retina. J Neurophysiol 61: 1233-1243

Fridovich I (1989) Superoxide dismutases. An adaptation to a paramagnetic gas. J Biol Chem. 264:7761-4

Furne J, Springfield J, Koenig T et al. (2001) Oxidation of hydrogen sulfide and methanethiol to thiosulfate by rat tissues: a specialized function of the colonic mucosa. Biochem Pharmacol 62: 255–9

Fu Y, Zheng S, Lu SC et al. (2008) Epigallocatechin-3-gallate Inhibits Growth of Activated Hepatic Stellate Cells by Enhancing the Capacity of Glutathione Synthesis. Mol Pharmaco. 73:1465-73

Galante JM, Mortenson MM, Schlieman MG et al. (2004) Bold Targeting NF-kB/BCL-2 pathway increases apoptotic susceptibility to chemotherapy in pancreatic cancer. J Surg Res 121:306-307

Galluzzi L, Kepp O, Kroemer G (2009) RIP kinases initiate programmed necrosis.J Mol Cell Biol.1:8-10

Germain M, Affar EB, D'Amours D et al. (1999) Cleavage of automodified poly(ADP-ribose) polymerase during apoptosis-Evidence for involvement of caspase-7. J Biol Chem 274: 28379-28384

Geier DA, Geier MR (2006) A clinical and laboratory evaluation of methionine cycle-transsulfuration and androgen pathway markers in children with autistic disorders. Horm Res. 66:182-8

Geng B, Yang J, Qi Y et al. (2004) H_2S generated by heart in rat and its effects on cardiac function. Biochem. Biophys Res Commun 313:362-368

Gehlbach PL, Purple RL (1994) A paired-comparison of 2 models of experimental retinal ischemia. Curr Eye Res 13: 597-602

Gerding H (1998) Ocular complications and a new surgical approach to lens dislocation in homocystinuria due to cystathionine-beta-synthetase deficiency. Eur J Pediatr.157:S94-101

Gherghel D, Griffiths HR, Hilton EJ et al. (2005) Systemic Reduction in Glutathione Levels Occurs in Patients with Primary Open-Angle Glaucoma. Invest Ophthalmol Vis Sci 46:877-883

Ghosh S, May MJ, Kopp EB (1998) NF-kB and REL proteins: evolutionarily conserved mediators of immune responses. Annu. Rev. Immunol. 16:225-260

Gilgun-Sherki Y, Melamed E, Offen D (2001) Oxidative stress induced-neurodegenerative diseases: the need for antioxidants that penetrate the blood brain barrier. Neuropharmacology 40:959-975

Gilgun-Sherki Y, Rosenbaum Z, Melamed E, Offen D (2002) Antioxidant therapy in acute central nervous system injury: current state. Pharmacol Rev 54:271-284

Giustarini D, Del Soldato P, Sparatore A (2010) Modulation of thiol homeostasis induced by H_2S-releasing aspirin. Free Radic Biol Med. 48:1263-72

Gioda CR, Barreto TDO, Prímola-Gomes TN et al. (2010) Cardiac oxidative stress is involved in heart failure induced by thiamine deprivation in rats Am J Physiol Heart Circ Physiol 298: 2039–2045

Globus MY, Busto R, Dietrich WD et al. (1988) Effect of ischemia on the in vivo release of striatal dopamine, glutamate, and gamma-aminobutyric acid studied by intracerebral microdialysis. J Neurochem 51: 1455-1464

Godbout JP, Berg BM, Kelley KW et al. (2004) alpha-Tocopherol reduces lipopolysaccharide-induced peroxide radical formation and interleukin-6 secretion in primary murine microglia and in brain. J Neuroimmunol.149:101-9

Gordon MO, Beiser JA, Brandt JD et al. (2002) The Ocular Hypertension Treatment Study: baseline factors that predict the onset of primary open-angle glaucoma. Arch Ophthalmol.120:714–720

Goldstein IM, Ostwald P, Roth S (1996) Nitric oxide: A review of its role in retinal function and disease. Vision Res 36: 2979-2994

Griffith OW, Meister A (1979): Potent and specific-inhibition of glutathione synthesis by buthionine sulfoxime (S-normal-butyl homocysteine sulfoxime). J Biol Chem 254:7558-7560

Groenendyk J, Michalak M (2005) Endoplasmic reticulum quality control and apoptosis. Acta. Biochim. Pol. 52:381-95

Griffiths SW, Cooney CL (2002) Relationship between protein structure and methionine oxidation in recombinant human alpha 1-antitrypsin. Biochemistry *41*: 6245-6252

Graham DG, Tiffany SM, Bell WR Jr et al. (1978) Autoxidation versus covalent binding of quinones as the mechanism of toxicity of dopamine, 6-hydroxydopamine, and related compounds toward C1300 neuroblastoma cells in vitro. Mol Pharmacol. 14:644-53.

Greenwald RA (1990) Superoxide dismutase and catalase as therapeutic agents for human diseases a critical review Free Radical Biology and Medicine 8:201-209

Grandjean E, Berthet P, Ruffmann R et al. (2000). Efficacy of oral long-term -acetylcysteine in chronic bronchopulmonary disease: A meta-analysis of published double-blind, placebo-controlled clinical trials. Clinical Therapeutics 22:209-21

Grütter MG (2000) Caspases: key players in programmed cell death. Curr Opin Struct Biol. 10:649-55

Guo L, Salt TE, Maass A et al. (2006) Assessment of neuroprotective effects of glutamate modulation on glaucoma-related retinal ganglion cell apoptosis in vivo. Invest Ophthalmol Vis Sci. 47:626-33

Guistarini D, Dalle-Donne I, Lorenzini S et al. (2006) Age-Related Influence on Thiol, Disulfide, and Protein-Mixed Disulfide Levels in Human Plasma. J Gerontol 61:1030-1038

Guidotti TL (1996) Hydrogen sulphide. Occup Med (Lond) 46:367–71

Guerin CJ, Anderson DH, Fisher SK (1990) Changes in intermediate filament immunolabeling occur in response to retinal-detachment and reattachment in primates. Invest Ophthalmol Vis Sci 31: 1474-1482

Gukasyan HJ, Kannan R, Lee VH et al. (2003) Regulation of L–cystine transport and intracellular GSH level by a nitric oxide donor in primary cultured rabbit conjunctival epithelial cell layers. Invest Ophthalmol Vis Sci 44:1202–1210

Gutteridge JM, Richmond R, Halliwell B (1979) Inhibition of the iron-catalysed formation of hydroxyl radicals from superoxide and of lipid peroxidation by desferrioxamine. Biochem J. 184:469-72

Halterman MW, Miller CC, Federoff HJ (1999) Hypoxia-inducible factor-1 alpha mediates hypoxia-induced delayed neuronal death that involves p53. J Neurosci 19: 6818-6824

Han Y, Qin J, Bu DF et al. (2006) Gammaaminobutyric acid B receptor regulates the expression of hydrogen sulfide/cystathionine-beta-synthase system in recurrent febrile seizures. Zhongguo Dang Dai Er Ke Za Zhi 2006; 8: 141–3

Halliwell B (1992) Reactive oxygen species and the central nervous system. J Neurochem. 59:1609-1623

Halliwell B (1996a) Oxidative stress, nutrition and health. Experimental strategies for optimization of nutritional antioxidant intake in humans. Free Radic Res. 25:57-74

Halliwell B (1996b) Antioxidants in human health and disease. Annu Rev Nutr.16:33-50

Hartline HK (1938) The response of single optic nerve fibers of the vertebrate eye to illumination of the retina. American Journal of Physiology. 121:400-415

Harris SL, Levine AJ (2005) The p53 pathway: positive and negative feedback loops Oncogene. 24(17):2899-908

Harris EW, Ganong AH, Cotman CW (1984) Long-term potentiation in the hippocampus involves activation of *N*-methyl-*D*-aspartate receptors. Brain Res 323: 132–7

Harris SG, Padilla J, Koumas L et al. (2002) Prostaglandins as modulators of immunity. Trends Immunol 23: 144-150

Hajnóczky G, Hoek JB (2007) Cell signaling: mitochondrial longevity pathways. Science.315:607-609

Hanitzsch R, Lichtenberger T, Mattig WU (1996) The influence of MgCl2 and APB on the light-induced potassium changes and the ERG b-wave of the isolated superfused rat retina. Vision Res 36: 499-507

Hauschildt S (1975) The influence of thiamine deficiency on porphyrin synthesis and porphyrin proteins. J Nutr Sci Vitaminol (Tokyo). 21:403-10

Haviv R, Lindenboim L, Li HG et al. (1997) Need for caspases in apoptosis of trophic factor-deprived PC12 cells. J Neurosci Res 50: 69-80

Hayashi A, Koroma BM, Imai K, de Juan E (1996) Increase of protein tyrosine phosphorylation in rat retina after ischemia-reperfusion injury. Invest Ophthalmol Vis Sci 37: 2146-2156

Hayes JD, Strange RC (1995) Invited commentary potential contribution of the glutathione-s-transferase supergene family to resistance to oxidative stress. Free Radic Res 22: 193-207

Heussler VT, Fernandez PC, Machado J et al. (1999) *N*-Acetylcysteine blocks apoptosis induced by *N*-alpha-tosyl-l-phenylalanine chloromethyl ketone in transformed T-cells, Cell Death Differ 6**:**342–350

Heinecke JW, Rosen H, Suzuki LA et al. (1987) The role of sulfur-containing amino acids in superoxide production and modification of low density lipoprotein by arterial smooth muscle cells. J Biol Chem. 262:10098-103

Heijl A, Leske MC, Bengtsson B et al. (2002) Reduction of intraocular pressure and glaucoma progression-Results from the early manifest glaucoma trial. *Arch Ophthalmol* 120(10): 1268-1279

Held KD, Sylvester FC, Hopcia KL et al. (1996) Role of Fenton chemistry in thiol-induced toxicity and apoptosis. Radiat Res.145:542-53

Hennet I, Richter C, Peterhans E (1993) Tumour necrosis factor-alpha induces superoxide anion production in mitochondria of L929 cells. Biochern. J. 289:587-592

He Y, Leung KW, Zhang YH et al. (2008) Mitochondrial complex I defect induces ROS release and degeneration in trabecular meshwork cells of POAG patients: protection by antioxidants. Invest Ophthalmol Vis Sci.49:1447-58

Hernandez MR (2000) The optic nerve head in glaucoma: role of astrocytes in tissue remodeling. Prog Retin Eye Res. 19:297-321

Hitomi J, Christofferson DE, Ng A et al. (2008) Identification of a molecular signaling network that regulates a cellular necrotic cell death pathway. Cell. 135:1311-23

Hockenbery DM, Oltvai ZN, Yin X-M et al (1993) Bcl-2 functions in an antioxidant pathway to prevent apoptosis. Cell 75:241-251

Hosoki R, Matsuki N, Kimura H (1997) The possible role of hydrogen sulfide as an endogenous smooth muscle relaxant in synergy with nitric oxide. Biochem. Biophys. Res. Commun. 237:527-531

Hoffmann U, Fischereder M, Krüger B et al. (2004) The Value of N-Acetylcysteine in the Prevention of Radiocontrast Agent-Induced Nephropathy Seems Questionable. J Am Soc Nephrol.15:407-10

Ho YS, Lee HM, Mou TC et al. (1997) Suppression of nitric oxide-induced apoptosis by N-acetyl-l-cysteine through modulation of glutathione, Bcl-2, and bax protein levels. Mol. Carcinog. 19:101–113

Ho PI, Collins SC, Dhitavat S, et al. (2001) Homocysteine potentiates β-amyloid neurotoxicity: role of oxidative stress Journal of Neurochemistry 78:249-253

Hosoya K, Tomi M, Ohtsuki S et al. (2002) Enhancement of L–cystine transport activity
and its relation to xCT gene induction at the blood–brain barrier by diethyl maleate treatment. J Pharmacol Exp Ther 302:225–231

Hou ST, MacManus JP (2002) Molecular mechanisms of cerebral ischemia-induced neuronal death in "International Review of Cytology: a Survey of Cell Biology" Eds Kwang W. Jeon. Academic Press Inc, San Diego: 93-148

Hong SJ, Dawson TM, Dawson VL (2004) Nuclear and mitochondrial conversations in cell death: PARP-1 and AIF signaling. Trends Pharmacol Sci.25:259-64.

Hu FL, Gu Z, Kozich V et al. (1993) Molecular basis of cystathionine beta-synthase deficiency in pyridoxine responsive and nonresponsive homocystinuria. Hum Mol Genet 2:1857–60

Hu LF, Lu M, Tiong CX et al. (2010) Neuroprotective effects of hydrogen sulfide on Parkinson's disease rat models. Aging Cell.9:135-46

Hyoudou K, Nishikawa M, Kobayashi Y et al. (2007) Analysis of in vivo nuclear factor-kappaB activation during liver inflammation in mice: prevention by catalase delivery.Mol Pharmacol. 71:446-53

Ichinohe A, Kanaumi T, Takashima S et al. (2005) Cystathionine beta-synthase is enriched in the brains of Down's patients. Biochem Biophys Res Commun 338:1547–50

Iino M, Ozawa S, Tsuzuki K (1990) Permeation of calcium through excitatory amino-acid receptor channels in cultured rat hippocampal-neurons. J Physiol 424: 151-165

Ikonomidou C, Kaindl AM (2001) Neuronal death and oxidative stress in the developing brain. Antioxid Redox Signal. 14:1535-50

Irschick EU, Sgonc R, Bock G et al. (2004) Retinal pigment epithelial phagocytosis and metabolism differ from those of macrophages. Ophthalmic Res 36: 200-210

Islam KN, Kayanoki Y, Kaneto H et al. (1997) TGF-β1 triggers oxidative modifications and enhances apoptosis in HIT cells through accumulation of reactive oxygen species by suppression of catalase and glutathione peroxidase, Free Radical Biol. Med 22:1007-1017

Issels RD, Nagele A, Eckert KG et al. (1998) Promotion of cystine uptake and its utilization for glutathione biosynthesis induced by cysteamine and N-acetylcysteine. Biochem Pharmacol.37:881-8.

Ishii T, Itoh K, Takahashi S et al. (2000) Transcription factor Nrf2 coordinately regulates a group of oxidative stress-inducible genes in macrophages. J Biol Chem. 275:16023-9

Ishige K, Tanaka M, Arakawa M et al. (2005). Distinct nuclear factor-kB/Rel proteins have opposing modulatory effects in glutamate-induced cell death in HT22 cells. Neurochemistry International 47:545–555

Izzotti A, Sacca SC, Cartiglia C et al. (2003) Oxidative deoxyribonucleic acid damage in the eyes of glaucoma patients. Am J Med 114: 638–646

Izzotti A, Bagnis A, Saccà SC (2006) The role of oxidative stress in glaucoma. Mutat Res. 612:105-14

Jacobson. MD, Raff MC (1995) Programmed cell death and Bcl-2 protection in very low oxygen. Nature 374:814-816

Jacobson MD (1997) Apoptosis: Bcl-2-related proteins get connected. Curr Biol 7: R277-R281

Jacob C, Anwar A, Burkholz T et al. (2008) Perspective on recent developments on sulfur-containing agents and hydrogen sulfide signaling. Planta Med 74:1580-1592

Jain A, Mårtensson J, Stole E et al. (1991) Glutathione deficiency leads to mitochondrial damage in brain. Proc Natl Acad Sci U S A. 88:1913-7

James AM, Murphy MP (2002) How mitochondrial damage affects cell function. J Biomed Sci 9: 475-487

Jha S, Calvert JW, Duranski MR et al. (2008) Hydrogen sulfide attenuates hepatic ischemia-reperfusion injury: role of antioxidant and antiapoptotic signaling. Am J Physiol Heart Circ Physiol.295:801-6

Ji D, Li GY, Osborne NN (2008). Nicotinamide attenuates retinal ischemia and light insults to neurones. Neurochem Int. 52:786-98

Johnson JE, Barde YA, Schwab M et al. (1986) Brain-derived neurotrophic factor supports the survival of cultured rat retinal ganglion-cells. J Neurosci 6: 3031-3038

Jókay I, Kelemenics K, Gyuris A et al. (1998) S-methylthio-cysteine and cystamine are potent stimulators of thiol production and glutathione synthesis. Life Sci. 62:PL27-33

Joza N, Susin SA, Daugas E et al. (2001) Essential role of the mitochondrial apoptosis-inducing factor in programmed cell death. Nature 410: 549-554

Jurgensmeier JM, Xie ZH, Deveraux Q et al. (1998) Bax directly induces release of cytochrome c from isolated mitochondria. Proc Natl Acad Sci U S A 95: 4997-5002

Jung SH, Kim BJ, Lee EH et al. (2010) Isoquercitrin is the most effective antioxidant in the plant Thuja orientalis and able to counteract oxidative-induced damage to a transformed cell line (RGC-5 cells). Neurochem Int. 57:713-21.

Kanter MZ (2006) Comparison of oral and i.v. Acetylcysteine in the treatment of acetaminophen poisoning. Am J Health Syst Pharm. 63:1821-7

Kasielski M, Nowak D (2001) Long-term administration of N-acetylcysteine decreases hydrogen peroxide exhalation in subjects with chronic obstructive pulmonary disease Respiratory Medicine 95: 448-56

Kaur C, Foulds WS, Ling E-A (2008) Hypoxia-ischemia and retinal ganglion cell damage. Clin Ophthalmol 2: 879-889

Kahn MG, Giblin FJ, Epstein DL (1983) Glutathione in calf trabecular meshwork and its relation to aqueous humor outflow facility. Invest Ophthalmol Vis Sci. 24:1283-1287

Kahns AH, Bundgaard H (1990) Prodrugs as drug delivery systems. Synthesis and chemical and enzymatic hydrolysis kinetics of various mono- and diester prodrugs of N-acetylcysteine. Int. J.Pharm. 62:193-205

Kalloniatis M, Fletcher EL (1993) Immunocytochemical localization of the amino acid neurotransmitters in the chicken retina. J Comp Neurol 336: 174-193

Kanamori A, Naka M, Fukuda M et al. (2009) Latanoprost protects rat retinal ganglion cells from apoptosis in vitro and in vivo. Exp Eye Res. 88:535-41

Kane LP, Shapiro VS, Stokoe D, Weiss A (1999) Induction of NF-kappa B by the Akt PKB kinase. Curr Biol 9: 601-604

Karwoski CJ, Proenza LM (1977) Hyperpolarizing on/off-responses in mudpuppy retina. Vision Res 17(1): 152-153

Katai N, Yoshimura N (1999) Apoptotic retinal neuronal death by ischemia-reperfusion is executed by two distinct caspase family proteases. Invest Ophthalmol Vis Sci. 40:2697-705

Kato S, Negishi K., Mawatari K, Kuo CH (1992) A mechanism for glutamate toxicity in the C6 glioma cells involving inhibition of cystine uptake leading to glutathione depletion. Neuroscience 48**:** 903–914

Kato S, Ishita S, Sugawara K et al. (1993) Cystine/glutamate antiporter expression in retinal Müller glial cells: implications for DL–alpha–aminoadipate toxicity. Neuroscience 57:473-482

Kanwar SS, Nehru B (2007) Modulatory effects of N-acetylcysteine on cerebral cortex and cerebellum regions of ageing rat brain Nutr Hosp. 22:95-100

Kaufmann SH, Desnoyers S, Ottaviano Y et al. (1993) Specific proteolytic cleavage of poly(ADP-ribose) polymerase: an early marker of chemotherapy-induced apoptosis. Cancer Res 53: 3976-3985

Kerr JF, Wyllie AH, Currie AR (1972) Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics. Br.J. Cancer 26:239-257

Kerrigan-Baumrind LA, Quigley HA, Pease ME et al. (2000) Number of ganglion cells in glaucoma eyes compared with threshold visual field tests in the same persons. Invest Ophthalmol Vis Sci 41: 741-748

Kischkel FC, Hellbardt S, Behrmann I (1995) Cytotoxicity-dependent APO-1 (Fas/CD95)-associated proteins form a death-inducing signaling complex (DISC) with the receptor. EMBO J. 14:5579-5588

Kim SJ, Kim HG, Oh CD et al. (2002) p38 kinase-dependent and -independent Inhibition of protein kinase C zeta and -alpha regulates nitric oxide-induced apoptosis and dedifferentiation of articular chondrocytes. J Biol Chem. 277:30375-81.

Kim R, Emi M, Tanabe K (2005) Role of mitochondria as the gardens of cell death. Cancer Chemother. Pharmacol.21:1-9

Kim R (2005) Unknotting the roles of Bcl-2 and Bcl-xL in cell death. Biochem Biophys Res Commun 333:336-43

Kimura Y, Kimura H (2004) Hydrogen sulfide protects neurons from oxidative stress. FASEB J 18:1165-7

Kimura H (2000) Hydrogen sulfide induces cyclic AMP and modulates the NMDA receptor. Biochem Biophys Res Commun 267:129-33

Kimura H, Nagai Y, Umemura K et al. (2005) Physiological roles of hydrogen sulfide: synaptic modulation, neuroprotection, and smooth muscle relaxation. Antioxid Redox Signal.7:795-803

Kimura Y, Dargusch R, Schubert D et a.l (2006): Hydrogen sulphide protects HT22 neuronal cells from oxidative stress. Antioxid. Redox Signal 8:661-670

Kimura Y, Kimura H (2004) Hydrogen sulfide protects neurons from oxidative stress. FASEB J 18:1165-1167

Kimura H (2007) Hydrogen sulfide enhances reducing activity in neurons: neurotrophic role of H2S in the brain? Antioxid Redox Signal. 9:2035-41

Kischkel FC, Hellbardt S, Behrmann I et al. (1995) Cytotoxicity-dependent APO-1 (Fas/CD95)-associated proteins form a death-inducing signaling complex (DISC) with the receptor. EMBO J. 14:5579-88

Khanna S, Sen CK, Roy S et al. (1998) Protective effects of anethole dithiolethione against oxidative stress-induced cytotoxicity in human Jurkat T cells, Biochem Pharmacol. 56**:**61-69

Klein JA, Longo-Guess CM, Rossmann MP et al. (2002) The harlequin mouse mutation downregulates apoptosis-inducing factor. Nature 419: 367-74

Klusha VE, Kimenis AA, Petersone IO et al. (1976) A mucolytic agent-Acetylcysteine Pharm. Chem J. 10:1281-1283

Kluck RM, Bossy-Wetzel E, Green DR et al. (1997) The release of cytochrome c from mitochondria: A primary site for Bcl-2 regulation of apoptosis. Science 275: 1132-1136

Klocker N, Cellerino A, Bahr M (1998) Free radical scavenging and inhibition of nitric oxide synthase potentiates the neurotrophic effects of brain-derived neurotrophic factor on axotomized retinal ganglion cells in vivo. J Neurosci 18: 1038-1046

Kono Y, Fridovich I., (1982) Superoxide radical inhibits catalase. The Journal of Biological Chemistry, 257:5751-5754

Ko ML, Hu DN, Ritch R et al. (2000) The combined effect of brain-derived neurotrophic factor and a free radical scavenger in experimental glaucoma. Invest Ophthalmol Vis Sci 41: 2967-2971

Ko ML, Hu DN, Ritch R et al. (2001) Patterns of retinal ganglion cell survival after brain-derived neurotrophic factor administration in hypertensive eyes of rats. Neurosci Lett 30: 139-142

Korteum K, Lynette K, Geiger LK et al. (2000) Differential Susceptibility of Retinal Ganglion Cells to Reactive Oxygen Species Invest. Ophthalmol. Vis. Sci. 41:3176-3182

Kombian SB, Reiffenstein RJ, Colmers WF (1993) The actions of hydrogen sulfide on dorsal raphe serotonergic neurons in vitro. J Neurophysiol.70:81-96

Kolb H (1979) Inner plexiform layer in the retina of the cat-electron-microscopic observations. J Neurocytol 8: 295-329

Kolb H (1994) The architecture of functional neural circuits in the vertebrate retina. The Proctor Lecture. Invest Ophthalmol Vis Sci 35: 2385-2404

Kolb H, Linberg KA, Fisher SK (1992) Neurons of the human retina: a Golgi study. J Comp Neurol 318: 147-187

Kolb H, West RW (1977) Synaptic connections of the interplexiform cell in the retina of the cat. J Neurocytol 6: 155-170

Koopman G, Reutelingsperger CP, Kuijten GA (1994) Annexin V for flow cytometric detection of phosphatidylserine expression on B cells undergoing apoptosis. Blood.84:1415-20

Koriyama Y, Ohno M, Kimura T et al. (2009) Neuroprotective effects of 5-S-GAD against oxidative stress-induced apoptosis in RGC-5 cells. Brain Res 1296: 187-195

Krieger C, Duchen MR (2002) Mitochondria, Ca2+ and neurodegenerative disease. Eur J Pharmacol 447:177 - 188

Kremlev SG, Roberts RL, Palmer C (2004) Differential expression of chemokines and chemokine receptors during microglial activation and inhibition. J Neuroimmunol.149:1-9

Kroemer, C (1995) The pharmacology of T cell apoptosis. Adv Immunol. 58:211-296

Kroemer G, Dallaporta B, Resche-Rigon M (1998) The mitochondrial death/life regulator in apoptosis and necrosis. Annu Rev Physiol.60:619-642

Kroemer G., Galluzzi L, Brenner C (2007) Mitochondrial Membrane Permeabilization in Cell Death. Physiol Rev 87: 99–163:2007

Kroemer G, Reed JC (2000) Mitochondrial control of cell death. Nat Med. 6:513-9

Kruman I, Guo Q, Mattson MP (1998) Calcium and reactive oxygen species mediate staurosporine-induced mitochondrial dysfunction and apoptosis in PC12 cells. J Neurosci Res 51: 293-308

Krishnamoorthy RR, Agarwal P, Prasanna G et al. (2001) Characterization of a transformed rat retinal ganglion cell line. Brain Res Mol Brain Res 86:1-12

Kudo H, Nakazawa T, Shimura M et al. (2006) Neuroprotective effect of latanoprost on rat retinal ganglion cells. Graefes Arch Clin Exp Ophthalmol. 244:1003-9

Kujoth GC, Hiona A, Pugh TD, et al. (2005) Mitochondrial DNA mutations, oxidative stress, and apoptosis in mammalian aging. Science. 309:481–484

Laemmli (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227:680–85

Lafon-Cazal M, Pietri S, Culcasi M et al. (1993) NMDA-dependent superoxide production and neurotoxicity. Nature 364:535–537

Laird MD, Wakade C, Alleyne CH Jr et al. (2008) Hemin-induced necroptosis involves glutathione depletion in mouse astrocytes.Free Radic Biol Med. 45:1103-14

Lane N (2006) Mitochondrial disease: powerhouse of disease. Nature. 440:600–602

Lascaratos G, Ji D, Wood JP, Osborne NN (2007) Visible light affects mitochondrial function and induces neuronal death in retinal cell cultures. Vision Res 47: 1191-1201

Lautier D, Lagueux J, Thibodeau J et al. (1993) Molecular and biochemical features of poly (ADP-ribose) metabolism. Mol Cell Biochem 122: 171-193

Lee JM, Li J, Johnson DA, Stein TD, et al. (2005) Nrf2, a multi-organ protector? FASEB J.19:1061-6

Levkovitch-Verbin H, Harris-Cerruti C, Groner Y et al. (2000) RGC death in mice after optic nerve crush injury: oxidative stress and neuroprotection. Invest Ophthalmol Vis Sci 41: 4169-4174

LeBel CP, Ischiropoulos H, Bondy SC (1992) Evaluation of the probe 2′,7′-dichlorofluorescin as an indicator of reactive oxygen species formation and oxidative stress, Chem. Res. Toxicol. 5:227–231

Lemasters JJ, Qian T, Bradham CA, et al. (1999) Mitochondrial dysfunction in the pathogenesis of necrotic and apoptotic cell death. J Bioenerg Biomembr.31:305–319

Levin ED, Bettegowda C, Weaver T (1998) Nicotine-dizocilpine interactions and working and reference memory performance of rats in the radial-arm maze. Pharmacol Biochem Behav 61: 335–40

Levin LA, Gordon LK (2002) Retinal ganglion cell disorders: types and treatments. Prog Retina Eye Res 21:465–484

LeVine SM (1992) The role of reactive oxygen species in the pathogenesis of multiple sclerosis. Medical Hypotheses 39:271-274

Levonen AL, Lapatto R, Saksela M et al. (2000) Human cystathionine gamma-lyase: developmental and in vitro expression of two isoforms. Biochem J 347: 291–295

Lewerenz J, Maher P, Methner A (2011) Regulation of xCT expression and system [Formula: see text] function in neuronal cells. Amino Acids.

Leske MC, Connell AMS, Wu SY et al. (1995) Risk-factors for open-angle glaucoma-the Barbados eye study. Arch Ophthalmol 113: 918-924

Lefer DJ (2007) A new gaseous signaling molecule emerges: cardioprotective role of hydrogen sulphide. Prc Natl Acad Sci 104:17907-17908

Li P, Nijhawan D, Budihardjo I et al. (1997) Cytochrome c and dATP-dependent formation of Apaf-1/caspase-9 complex initiates an apoptotic protease cascade. Cell. 91:479-89

Li L, Bhatia M, Zhu, Y et al. (2005) Hydrogen sulfide is a novel mediator of lipopolysaccharide-induced inflammation in the mouse. FASEB J. 19:1196-1198

Li GY, Osborne NN (2008) Oxidative-induced apoptosis to an immortalized ganglion cell line is caspase independent but involves the activation of poly(ADP-ribose)polymerase and apoptosis-inducing factor. Brain Res. 1188:35-43

Li L, Rossoni G, Sparatore A et al. (2007) Anti-inflammatory and gastrointestinal effects of a novel diclofenac derivative. Free Radic Biol Med 42:706-719

Li L, Moore PK (2007) An overview of the biological significance of endogenous gases: new roles for old molecules. Biochemical Society Transactions 35: 1138-1141

Lim JH, Park HS, Choi JK et al. (2007)Isoorientin induces Nrf2 pathway-driven antioxidant response through phosphatidylinositol 3-kinase signaling. Arch Pharm Res. 30:1590-8

Lin MT, Beal MF (2006) Mitochondrial dysfunction and oxidative stress in neurodegenerative diseases. Nature. 443:787–795

Lindahl T, Satoh MS, Poirier GG (1995) Post-translational modification of poly(ADP-ribose) polymerase induced by DNA strand breaks. Trends Biochem Sci 20: 405-411

Li LY, Luo X, Wang X (2001) Endonuclease G is an apoptotic DNase when released from mitochondria Nature 412:95-99

Liu M, Pelling JC, Ju J et al. (1998) Antioxidant action via p53-mediated apoptosis. Cancer Res. 58:1723–1729

Loewen N, Fautsch MP, Peretz M, et al. (2001) Genetic modification of human trabecular meshwork with lentiviral vectors. Hum Gene Ther.12:2109–2119

Lu M, Hu LF, Hu G et al., (2008) Hydrogen sulfide protects astrocytes against H(2)O(2)-induced neural injury via enhancing glutamate uptake. Free Radic Biol Med.45:1705-13

Lu YB, Franze K, Seifert G et al. (2006) Viscoelastic properties of individual glial cells and neurons in the CNS. Proc Natl Acad Sci U S A. 103:17759-17764

Lutjen-Drecoll E (2005) Morphological changes in glaucomatous eyes and the role of TGFβ2 for the pathogenesis of the disease. Exp Eye Res. 81:1–4

Lütjen-Drecoll E, Rittig M, Rauterberg J et al. (1989) Immunomicroscopical study of type VI collagen in the trabecular meshwork of normal and glaucomatous eyes. Exp Eye Res.48:139–147

Maher P, Hanneken A (2005). The molecular basis of oxidative stress-induced cell death in an immortalized retinal ganglion cell line. Invest Ophthalmol Vis Sci 46:749-757

Makar TK, Nedergaard M, Preuss A et al. (1994) Vitamin E, ascorbate, glutathione, glutathione disulfide, and enzymes of glutathione metabolism in cultures of chick astrocytes and neurons: evidence that astrocytes play an important role in antioxidative processes in the brain. J Neurochem 62: 45-53

Mandel SA, Avramovich-Tirosh Y, Reznichenko L et al. (2005) Multifunctional activities of green tea catechins in neuroprotection. Modulation of cell survival genes, iron-dependent oxidative stress and PKC signaling pathway. Neurosignals.14:46-60

Manji HK, Moore GJ, Chen G (2000) Lithium up-regulates the cytoprotective protein Bcl-2 in the CNS in vivo: a role for neurotrophic and neuroprotective effects in manic depressive illness. J. Clin. Psychiatry 61:82–96

Manji HK, Moore GJ, Rajkowska G et al. (2000) Neuroplasticity and cellular resilience in mood disorders. Mol. Psychiatry 5:578–593

Manji HK, Moore GJ, Chen G. (1999) Lithium at 50: have the neuroprotective effects of this unique cation been overlooked?. Biol. Psychiatry 46:929–940

Mansuy D, Sassi A, Dansette PM et al. (1986) A new potent inhibitor of lipid peroxidation in vitro and in vivo, the hepatoprotective drug anysyldithiolthione. Biochem. Biophys. Res. Commun. 135:1015-1021

Martínez M, Ferrándiz ML, Díez A, Miquel J (1995) Depletion of cytosolic GSH decreases the ATP levels and viability of synaptosomes from aged mice but not from young mice. Mech Ageing Dev. 84:77-81

Martinou JC, Duboisdauphin M, Staple JK et al. (1994) Overexpression of Bcl-2 in transgenic mice protects neurons from naturally occurring cell death and experimental ischemia. Neuron 13: 1017-1030

Malorni W, Rivabene R, Santini MT et al. (1993) N-acetylcysteine inhibits apoptosis and decreases viral particles in HIV-chronically infected U937 cells. FEBS Lett. 327:75-8

Markesbury WR (1997) Oxidative stress hypothesis in Alzheimer's disease. Free Rad. Biol. Med. 23:134-137

Martin KR, Quigley HA, Zack DJ et al. (2003) Gene therapy with brain-derived neurotrophic factor as a protection: Retinal ganglion cells in a rat glaucoma model. Invest Ophthalmol Vis Sci 44: 4357-4365

Martin DP, Schmidt RE, Distefano PS et al. (1988) Inhibitors of protein synthesis and RNA synthesis prevent neuronal death caused by nerve growth factor deprivation. J Cell Biol 106: 829-844

Martin SJ, Reutelingsperger CP, McGahon AJ et al. (1995) Early redistribution of plasma membrane phosphatidylserine is a general feature of apoptosis regardless of the initiating stimulus: inhibition by overexpression of Bcl-2 and Abl. J Exp Med.182:1545-56

Mariani AP (1983) Giant bistratified bipolar cells in monkey retina. The Anatomical Record 206: 215-220

Maslim J, Webster M, Stone J (1986) Stages in the structural differentiation of retinal ganglion cells. J Comp Neurol. 254:382-402

Masdehors P, Glaisner S, Maciorowski Z, et al. (2000) Ubiquitin-dependent protein processing controls radiation-induced apoptosis through the N-end rule pathway. Exp Cell Res 257: 48-57

Matsuda T, Cooper JR (1981) Thiamine as an integral component of brain synaptosomal membranes. Proc Natl Acad Sci 78: 5886–9

Matsuura S, Ikegaya Y, Yamada MK et al. (2002) Endothelin downregulates the glutamate transporter GLAST in cAMP-differentiated astrocytes in vitro. Glia 37: 178-182

Makarchikov AF, Lakaye B, Gulyai IE., et al. (2003) Thiamine triphosphate and thiamine triphosphatase activities: from bacteria to mammals. Cell Mol Life Sci 60: 1477:88

McConkey DJ, Nicotera P, Orrenius S (1994) Signalling and chromatin fragmentation in thymocyte apoptosis. Immunol Rev 142:343-6

McBean GJ (2002) Cerebral cystine uptake: a tale of two transporters. Trends Pharmacol Sci 23:299-302

McLellan LI, Lewis AD, Hall DJ et al. (1995) Uptake and distribution of N-acetylcysteine in mice: tissue-specific effects on glutathione concentrations.Carcinogenesis. 16:2099-106

Merry DE, Korsmeyer SJ (1997) Bcl-2 gene family in the nervous system. Annu Rev Neurosci 20(245-267)

Meister A, Anderson ME (1983) Glutathione. Annu Rev Biochem. 52:711-60

Meister A., Anderson ME, Hwang O (1986) Intracellular Cysteine and glutathione delivery systems. J Am Col Nutr S 137-151

Melena J, Osborne NN (2003) Metipranolol attenuates lipid peroxidation in rat brain: a comparative study with other antiglaucoma drugs. Graefes Arch Clin Exp Ophthalmol 241: 827-833

Miner SE, Dzavik V, Nguyen-Ho P et al. (2004) N-acetylcysteine reduces contrast-associated nephropathy but not clinical events during long-term follow-up. Am Heart J. 148:690-5

Mitchell RS (2000) Retrograde axonal transport of BDNF in retinal ganglion cells is blocked by acute IOP elevation in rats. Invest Ophthalmol Vis Sci 41: 3460-3466

Micheau J, Durkin TP, Destrade C et al. (1985) Chronic administration of sulbutiamine improves long term memory formation in mice: possible cholinergic mediation. Pharmacol Biochem Behav 23:195-198

Miceli MV, Liles MR, Newsome DA (1994) Evaluation of oxidative processes in human pigment epithelial cells associated with retinal outer segment phagocytosis. Exp Cell Res. 214:242-9

Miller RF, Dowling JE (1970) Intracellular responses of the Müller (glial) cells of mudpuppy retina: their relation to b-wave of the electroretinogram. J Neurophysiol 33: 323-341

Moore GJ, Bebchuk JM, Hasanat K et al. (2002) Prostaglandins as modulators of immunity. Trends Immunol 23: 144-150

Moore GJ, Bebchuk JM, Hasanat K et al. (2000) Lithium increases *N*-acetyl-aspartate in the human brain: in vivo evidence in support of bcl-2's neurotrophic effects?. Biol. Psychiatry 48**:**1-8

Moore PK, Bhatia M, Moochhala S (2003) Hydrogen sulfide: from the smell of the past to the mediator of the future? Trends Pharmacol Sci 24:609-611

Mok YY, Atan MS, Yoke PC. (2004) Role of hydrogen sulphide in haemorrhagic shock in the rat: protective effect of inhibitors of hydrogen sulphide biosynthesis. Br. J. Pharmacol. 143:881-889

Morito N, Yoh K, Itoh K et al. (2003) Nrf2 regulates the sensitivity of death receptor signals by affecting intracellular glutathione levels. Oncogene 22:9275–9281

Moreno MC, Campanelli J, Sande P et al. (2004) Retinal oxidative stress induced by high intraocular pressure. Free Radic Biol Med. 2004 Sep 15:37:803-12

Mohanty JG, Jaffe JS, Schulman ES et al. (1997) A Highly Sensitive Fluoroscent Micro-Assay of H2O2 Release from Activated Human Leukocytes Using a Dihydroxyphenoxazine Derivative. J Immunol Methods 202: 133-141

Moon EY (2008) Serum deprivation enhances apoptotic cell death by increasing mitochondrial enzyme activity. Biomol Ther 16: 1-8

Mueller S, Riedel DH, Stremmel W (1997) Determination of Catalase Activity at Physiological H_2O_2 Concentrations. Anal Biochem 245: 55–60

Muzaffar S, Jeremy JY, Sparatore A et al (2008) H_2S donating sildenafil (ACS6) inhibits superoxide formation and gp91phox expression in arterial endothelial cells: role of protein kinases A and G. Br J Pharmacol 155:984-994

Murphy TH, Miyamoto M, Sastre A et al. (1989) Glutamate toxicity in a neuronal cell line involves inhibition of cystine transport leading to oxidative stress. Neuron 2:1547–1558

Murphy TH, Schnaar RL, Coyle JT (1990) Immature cortical neurons are uniquely sensitive to glutamate toxicity by inhibition of cystine uptake. FASEB J 4:1624 –1633

Nagai Y, Tsugane M, Oka J et al. (2004) Hydrogen sulfide induces calcium waves in astrocytes. FASEB J 18: 557-9

Nakajima Y, Tsuruma K, Shimazawa M et al. (2009) Comparison of bee products based on assays of antioxidant capacities. BMC Complement Altern Med. 9:4

Nash MS, Osborne NN (1999) Assessment of Thy-1 mRNA levels as an index of retinal ganglion cell damage. Invest Ophthalmol Vis Sci. 40:1293–98

Nanetti L, Taffi R, Vignini A et al. (2007) Reactive oxygen species plasmatic levels in ischemic stroke Mol Cell Biochem 303:19-25

Newman E, Odette L (1984) Model of electroretinogram b-wave generation: a test of the K^+ hypothesis. J Neurophysiol 51:164-182

Nicotera P, Orrenius S (1992) Ca^{2+} and cell death. Ann N Y Acad Sci 648: 17-27

Nishiyama T, Nishukawa S, Hiroshi et al. (2000) Müller cells in the preconditioned retinal ischemic injury rat. Tohoku J Exp Med.191:221-32

Nickells RW (1996) Retinal ganglion cell death in glaucoma: The how, the why, and the maybe. J Glaucoma 5: 345-356

Nicholson DW, Thornberry NA (1997) Caspases: killer proteases. Trends Biochem Sci 22: 299-306

Nordberg J, Arnér ES (2001) Reactive oxygen species, antioxidants, and the mammalian thioredoxin system. Free Radic Biol Med. 31:1287-312

Nucci C, Tartaglione R, Rombola L et al. (2005) Neurochemical evidence to implicate elevated glutamate in the mechanisms of high intraocular pressure (IOP)-induced retinal ganglion cell death in rat. Neurotoxicology 26: 935-941

Ohkawa H, Ohishi N, Yagi K (1979) Assay for lipid peroxides in animal-tissues by thiobarbituric acid reaction. Anal Biochem 95: 351-358

Ollat H, Laurent B, Bakchine S et al. (2007) Effects of the association of sulbutiamine with an acetylcholinesterase inhibitor in early stage and moderate Alzheimer disease. Encephale 33:211-215

Olney JW (1969) Brain lesions obesity and other disturbances in mice treated with monosodium glutamate. Science 164:719-721

Olney JW, Ho OL (1970) Brain damage in infant mice following oral intake of glutamate, aspartate or cysteine. Nature 227: 609-611

Olson KR (2009) Is hydrogen sulfide a circulating "gasotransmitter" in vertebrate blood? Biochim Biophys Acta 1787:856-63

O'Neill EC, Mackey DA, Connell PP et al. (2009) The optic nerve head in hereditary optic neuropathies. Nat Rev Neurol 5:277-87

Osborne NN, Larsen AK (1996) Antigens associated with specific retinal cells are affected by ischaemia caused by raised intraocular pressure: effect of glutamate antagonists. Neurochem Int. 29:263–70

Osborne NN, Chidlow G, Nash MS et al. (1999a) The potential of neuroprotection in glaucoma treatment. Curr Opin Ophthalmol 10: 82-92

Osborne NN, DeSantis L, Bae JH et al. (1999b) Topically applied betaxolol attenuates NMDA-induced toxicity to ganglion cells and the effects of ischaemia to the retina. Exp Eye Res 69: 331-342

Osborne NN, Safa R, Nash MS (1999c) Photoreceptors are preferentially affected in the rat retina following permanent occlusion of the carotid arteries. Vision Res 39: 3995-4002

Osborne NN, Ugarte M, Chao M et al. (1999d) Neuroprotection in relation to retinal ischemia and relevance to glaucoma. Surv Ophthalmol 43: S102-S128

Osborne NN, Wood JP, Chidlow G, et al. (1999e) Ganglion cell death in glaucoma: what do we really know? Br J Ophthalmol 83: 980-986

Osborne NN, Melena J, Chidlow G et al. (2001). A hypothesis to explain ganglion cell death caused by vascular insults at the optic nerve head: possible implication for the treatment of glaucoma.Br J Ophthalmol. 85:1252-59

Osborne NN, Casson RJ, Wood JP et al. (2004) Retinal ischemia: mechanisms of damage and potential therapeutic strategies. Prog Retin Eye Res. 23:91-147

Osborne NN, Lascaratos G, Bron AJ et al. (2006) A hypothesis to suggest that light is a risk factor in glaucoma and the mitochondrial optic neuropathies. Br J Ophthalmol. 90:237-41

Osborne NN(2010b).Mitochondria: Their role in ganglion cell death and survival in primary open angle glaucoma. Exp Eye Res. 90:750-7

Osborne NN, Ji D, Abdul Majid AS et al. (2010b) ACS67, a hydrogen sulphide-releasing derivative of latanoprost acid, attenuates retinal ischemia and oxidative stress to RGC-5 cells in culture. Invest Ophthalmol Vis Sci 51: 284-294

Owuor ED, Kong AN (2002) Antioxidants and oxidants regulated signal transduction pathways, Biochem Pharmacol 64: 765-770

Pache M, Flammer J (2006). A sick eye in a sick body? Systemic findings in patients with primary open-angle glaucoma. Surv Ophthalmol.51:179-212

Packer L, Tritschler HJ, Wessel K (1997)Neuroprotection by the metabolic antioxidant alpha-lipoic acid. Free Radic Biol Med. 22:359-78

Palacin M, Estevez R, Bertran J et al. (1998) Molecular biology of mammalian plasma membrane amino acid transporters. Physiol Rev 78:969–1054

Pandey S, Lopez C, Jammu A (2003) Oxidative stress and activation of proteasome protease during serum deprivation-induced apoptosis in rat hepatoma cells; inhibition of cell death by melatonin. Apoptosis 8: 497-508

Parada-Turska J, Turski WA (1990) Excitatory amino acid antagonists and memory: effect of drugs acting at N-methyl-D-aspartate receptors in learning and memory tasks. Neuropharmacology. 29:1111-6

Patel M, Day BJ, Crapo JD et al. (1996) Requirement for superoxide in excitotoxic cell death. Neuron 16:345–355.

Perovic S, Pialoglou P, Schroder HC et al. (1996) Flupirtine increases the levels of glutathione and Bcl-2 in hNT (human Ntera/D1) neurons: mode of action of the drug-mediated anti-apoptotic effect. Eur. J. Pharmacol. 317:157–164

Perry HE, Shannon MW (1998) Efficacy of oral versus intravenous N-acetylcysteine in acetaminophen overdose: results of an open-label, clinical trial. J Pediatr. 132:149-52

Perry MJ, Lawson SN, Robertson J (1991) Neurofilament immunoreactivity in populations of rat primary afferent neurons - a quantitative study of phosphorylated and nonphosphorylated subunits. J Neurocytol 20: 746-758

Pease ME, McKinnon SJ, Quigley HA et al. (2000) Obstructed axonal transport of BDNF and its receptor TrkB in experimental glaucoma. Invest Ophthalmol Vis Sci 41: 764-774

Perrino E, Cappelletti G, Tazzari V et al. (2008) New sulfurated derivatives of valproic acid with enhanced histone deacetylase inhibitory activity, Bioorg. Med. Chem. Lett**.** 18 1893-1987

Perrino E, Uliva C, Lanzi C et al. (2009) New prostaglandin derivative for glaucoma treatment. Bio Med Chem Let 19:1639-1642

Persa C, Osmotherly K, Chao-Wei CK et al. (2006) The distribution of cystathionine beta-synthase (CBS) in the eye: implication of the presence of a trans-sulfuration pathway for oxidative stress defense. Exp Eye Res 83:817–823

Peterson WM, Miller SS (1995) Identification and functional characterization of a dual GABA taurine transporter in the bullfrog retinal pigment epithelium. J Gen Physiol 106: 1089-1122

Peinado-Ramon P, Salvador M, Villegas-Perez MP et al. (1996) Effects of axotomy and intraocular administration of NT-4, NT-3, and brain-derived neurotrophic factor on the survival of adult rat retinal ganglion cells. A quantitative in vivo study. Invest Ophthalmol Vis Sci 37: 489-500

Philpott KL, McCarthy MJ, Klippel A, Rubin LL (1997) Activated phosphatidylinositol 3-kinase and Akt kinase promote survival of superior cervical neurons. J Cell Biol 139: 809-815

Piantadosi CA, Zhang J (1996) Mitochondrial generation of reactive oxygen species after brain ischemia in the rat. Stroke 27327-331

Portari GV, Marchini JS, Vannucchi H et al. (2008) Antioxidant effect of thiamine on acutely alcoholized rats and lack of efficacy using thiamine or glucose to reduce blood alcohol content. Basic Clin Pharmacol Toxicol 103:482-486

Polyak K, Xia Y, Zweier JL et al. (1997) Vogelstein B.A model for p53-induced apoptosis. Nature. 389:300-5

Potau JM, Canals M, Costa J et al. (2000). Ultrastructural characteristics of glaucomatous trabecular meshwork. Arch Soc Esp Oftalmol.75*:*21–28

Pong WW, Stouracova R, Frank N et al. (2007) Comparative localization of cystathionine beta-synthase and cystathionine gamma-lyase in retina: differences between amphibians and mammals. J Comp Neurol 505:158–165

Powers SK, Jackson MJ (2008) Exercise-Induced Oxidative Stress: Cellular Mechanisms and Impact on Muscle Force Production *Physiol Rev* 88:1243-1276

Pow DV (2001) Visualising the activity of the cystine–glutamate antiporter in glial cells using antibodies to aminoadipic acid, a selectively transported substrate. Glia 34:27–38

Polster BM, Basanez G., Etxebarria A (2005) Calpain I induces cleavage and release of apoptosis-inducing factor from isolated mitochondria. J Biol Chem 280: 6447-6454

Putnam CD, Arvai AS, Bourne Y, Tainer JA (2000) Active and inhibited human catalase structures: ligand and NADPH binding and catalytic mechanism. J Molec Biol 296:295-309

Puma C, Baudoin C, Bizot JC (1998).Effects of intraseptal infusions of N-methyl-D-aspartate receptor ligands on memory in an object recognition task in rats. Neurosci Lett 244: 97–100

Purves D, Augustine GJ, Fitzpatrick D, et al. (2001) Neuroscience. 2nd edition. Sunderland (MA): Sinauer Associates; 2001.

Pussinen R, Sirviö J (1999). Effects of D-cycloserine, a positive modulator of N-methyl-D-aspartate receptors, and ST 587, a putative alpha-1 adrenergic agonist, individually and in combination, on the non-delayed and delayed foraging behaviour of rats assessed in the radial arm maze. J Psychopharmacol (Oxford) 13 (2): 171–9

Quigley HA (1993) Open-Angle Glaucoma N Engl J Med 328:1097-1106

Quigley HA (1995) Ganglion-cell death in glaucoma-pathology recapitulates ontogeny. Aust N Z J Ophthalmol 23: 85-91

Quigley HA (1996) Number of people with glaucoma worldwide. Br J Ophthalmol.80:389–393

Quigley HA, Vitale S (1997) Models of open-angle glaucoma prevalence and incidence in the United States. Invest Ophthalmol Vis Sci 38: 83-91

Quigley HA (1999) Neuronal death in glaucoma. Prog Retin Eye Res 18:39-57

Quigley HA, Broman AT (2006) The number of people with glaucoma worldwide in 2010 and 2020. *Br J Ophthalmol* 90(3): 262-267

Qu K, Chen CP, Halliwell B et al (2006) Hydrogen sulfide is a mediator of cerebral ischemic damage. Stroke 37:889-893

Qu K, Lee SW, Bian JS, Low CM et al. (1998) Hydrogen sulfide: neurochemistry and neurobiology. Neurochem Int. 52:155-65

Ransom BR (1992) Glial modulation of neuronal excitability mediated by extracellular pH: a hypothesis. Prog Brain Res 94: 37-46

Rapaport DH, Rakic P, La Vail MM (1996) Spatiotemporal gradients of cell genesis in the primate retina. Perspect Dev Neurobiol 3: 147-159

Raps SP, Lai JC, Hertz L, Cooper AJ (1989) Glutathione is present in high concentrations in cultured astrocytes but not in cultured neurons. *Brain Res* 493:398-401

Reichelt W, Stabel BJ, Pannicke T et al. (1997) The glutathione level of retinal Muller glial cells is dependent on the high affinity sodium-dependent uptake of glutamate. Neuroscience 77:1213–1224

Reyes O, Sosa I, Kuffler DP (2006) Neuroprotection of adult human neurons against ischemia by hypothermia and alkalinization. P R Health Sci J.25:43-50

Resnikoff S, Pascolini D, Etya'ale D et al. (2004) Global data on visual impairment in the year 2002. Bull World Health Organ 82:844–851

Robin LN, Kalloniatis M (1992) Interrelationship between retinal ischaemic damage and turnover and metabolism of putative amino acid neurotransmitters, glutamate and GABA. *Doc Ophthalmol* 80: 273-300

Roebuck BD, Curphey TJ, Li Y et al. (2003) Evaluation of the cancer chemopreventive potency of dithiolethione analogs of oltipraz. Carcinogenesis. 24:1919-28

Rosenberg P (2004) Mitochondrial dysfunction and heart disease. Mitochondrion. 4:621-8

Rosen DR, Siddique I, Patterson D et al. (1993) Mutations in Cu/Zn superoxide dismutase gene are associated with familial amyotrophic lateral sclerosis. Nature 362:59-62

Rothstein JD (1996) Excitotoxicity and neurodegeneration in amyotrophic lateral sclerosis. Clin. Neurosci. 3:348-359

Rothstein JD, Dykes-Hoberg M, Pardo CA et al., (1996) Knockout of glutamate transporters reveals a major role for astroglial transport in excitotoxicity and clearance of glutamate. Neuron 16:675-686

Rothman SM, Samaie M (1985) Physiology of excitatory synaptic transmission in cultures of dissociated rat hippocampus. J Neurophysiol 54:701-713

Rossoni G, Sparatore A, Tazzari V et al. (2008) The hydrogen sulphide-releasing derivative of diclofenac protects against ischaemia-reperfusion injury in the isolated rabbit heart. Br J Pharmacol. 153:100-9

Roh YJ, Moon C, Kim SY et al. (2007) Glutathione depletion induces differential apoptosis in cells of mouse retina, in vivo. Neurosci Lett. 7:266-70

Sagara JI, Miura K, Bannai S (1993) Maintenance of neuronal glutathione by glial cells. J Neurochem 61:1672-1676

Salvesen GS, Dixit VM (1997) Caspases: Intracellular signaling by proteolysis. Cell 91: 443-446

Sandstrom PA, Mannie MD, Buttke TM (1994) Inhibition of activation-induced death in T cell hybridomas by thiol antioxidants: oxidative stress as a mediator of apoptosis. J. Leukoc. Biol. 55:221–226

Santos AM, Calvente R, Tassi M et al. (2008) Embryonic and postnatal development of microglial cells in the mouse retina. J Comp Neurol. 506:224-39

Sasaki H, Sato H, Kuriyama–Matsumura K et al. (2002) Electrophile response element–mediated induction of the cystine/ glutamate exchange transporter gene expression. J Biol Chem 277:44765–44771

Sato H, Kuriyama–Matsumura K, Hashimoto T et al. (2001) Effect of oxygen on induction of the cystine transporter by bacterial lipopolysaccharide in mouse peritoneal macrophages. J Biol Chem 276: 10407–10412

Scaffidi C, Fulda S, Srinivasan A et al. (1998) Two CD95 (APO-1/Fas) signaling pathways EMBO Journal 17:1675 - 1687

Schnitzer J (1988) Astrocytes in the guinea pig, horse, and monkey retina: their occurrence coincides with the presence of blood vessels. Glia.1:74-89

Schwartz M (2003) Neurodegeneration and neuroprotection in glaucoma: Development of a therapeutic neuroprotective vaccine: the Friedenwald Lecture. Invest Ophthalmol Vis Sci 44: 1407-1411

Schulze-Osthoff K, Krammer PH, Drhge W (1994) Divergent signalling via APO-1/Fas and the TNF receptor, two homologous molecules involved in physiological cell death. EMBOJ. 13:4587-4596

Schulze-Osthoff K, Beyaert R, Vandevoorde V (1993) Depletion of the mitochondrial electrone transport abrogates the cytotoxic and gene-inductive effects of TNF. EMBOJ. 12:3095-3104

Schraermeyer U, Heimann K (1999) Current understanding on the role of retinal pigment epithelium and its pigmentation. Pigment Cell Res 12: 219-236

Schiller PH (1992) The ON and OFF channels of the visual system. Trends Neurosci. 15:86-92

Schiller PH (1996) On the specificity of neurons and visual areas. Behav Brain Res 76: 21-35

Sedel F, Challe G, Mayer JM et al (2008) Thiamine responsive pyruvate dehydrogenase deficiency in an adult with peripheral neuropathy and optic neuropathy. J Neurol Neurosurg Psychiatry 79:846-847

Sen CK, Traber KE, Packer L (1996) Inhibition of NF-kappaB activation in human T-cell lines by anetholdithiolthione. Biochem. Biophys Res Commun 218, 148-153

Sen CK (1998) Redox signaling and the emerging therapeutic potential of thiol antioxidants. Biochem. Pharmacol. 55: 1747-1758

Sen CK, Packer L (2000) Thiol homeostasis and supplements in physical exercise. Am J Clin Nutr.72:653S-69S

Seiler T, Wollensak J (1985) The resistance of the trabecular meshwork to aqueous humor outflow. Graefes Arch Clin Exp Ophthalmol. 223:88–91

Setsukinai K, Urano Y, Kakinuma K et al. (2003) Development of novel fluorescence probes that can reliably detect reactive oxygen species and distinguish specific species. J Biol Chem. 2003 Jan 31;278(5):3170-5

Shaw G, Weber K (1981) The distribution of the neurofilament triplet proteins within individual neurons. Exp Cell Res 136: 119-125

Shangari N, Mehta R, O'Brien PJ (2007) Hepatocyte susceptibility to glyoxal is dependent on cell thiamin content. Chem Biol Interact 165:146-154

Shah SN (2003) Adjuvant role of vitamin B analogue (sulbutiamine) with anti-infective treatment in infection associated asthenia. J Assoc Physicians India 51:891-895

Shah ZA, Li RC, Thimmulappa RK et al. (2007) Role of reactive oxygen species in modulation of Nrf2 following ischemic reperfusion injury.Neuroscience. 147:53-9

Shanker G, Allen JW, Mutkus LA et al. (2001) The uptake of cysteine in cultured primary astrocytes and neurons. Brain Res 902:156–163

Shibuki H, Katai N, Yodoi J et al. (2000) Lipid peroxidation and peroxynitrite in retinal ischemia-reperfusion injury. Invest Ophthalmol Vis Sci 41: 3607-3614

Shimizu S, Eguehi Y, Kosaka H et al. (1995) Prevention of hypoxia-induced cell death by Bcl-2 and Bcl-xL. Nature 374, 811-813

Shin BH, Choi SH, Cho EY, et al. (2004) Thiamine Attenuates Hypoxia-induced Cell Death in Cultured Neonatal Rat Cardiomyocytes Mol. Cells. 18:133-140

Shiraishi H, Okamoto H, Yoshimura A (2006) ER stress-induced apoptosis and caspase-12 activation occurs downstream of mitochondrial apoptosis involving Apaf-1. Journal of Cell Science 119:3958-3966

Shimazawa M, Nakajima Y, Mashima Y et al. (2009) Docosahexaenoic acid (DHA) has neuroprotective effects against oxidative stress in retinal ganglion cells. Brain Res 1251:269-275

Shao JL, Wan XH, Chen Y et al. (2011) H_2S protects hippocampal neurons from anoxia-reoxygenation through cAMP-mediated PI3K/Akt/p70S6K cell-survival signaling pathways. J Mol Neurosci. 43:453-60

Sidhapuriwala J, Li L, Sparatore A (2007) Effect of S-diclofenac, a novel hydrogen sulfide releasing derivative, on carrageenan-induced hindpaw oedema formation in the rat. Eur J Pharmacol. 569:149-54

Sinclair AJ, Bayer AJ, Johnston JO et al. (1998) Altered Plasma Antioxidant Status in Subjects with Alzheimer's Disease and Vascular Dementia. Int. J. Geriat. Psychiatry 13:840-845

Sivarajah A, McDonald MC, Thiemermann C (2006) The production of hydrogen sulfide limits myocardial ischemia and reperfusion injury and contributes to the cardioprotective effects of preconditioning with endotoxin, but not ischemia in the rat. Shock 26:154-61

Siesjo BK (1992) Pathophysiology and treatment of focal cerebral ischemia. Part II: Mechanisms of damage and treatment. J Neurosurg 77: 337-354

Simonian NA, Coyle JT (1996) Oxidative stress in neurodegenerative diseases. Annu Rev Pharmacol Toxicol 36:83-116

Singh RP, Sharad S, Kapur S (2004) Free Radicals and Oxidative Stress in Neurodegenerative Diseases: Relevance of Dietary Antioxidants JIACM 5: 218-25

Siram N, Kalayasaran S, Sudhandrian G (2008) Enhancement of Antioxidant Defense System by Epigallocatechin-3-gallate during Bleomycin Induced Experimental Pulmonary Fibrosis. Biol Pharm Bull.31:1306-11

Snow RL, Robson JA (1994) Ganglion cell neurogenesis, migration and early differentiation in the chick retina. Neuroscience 58: 399-409

Sparatore A, Perrino E, Tazzari V et al. (2009) Pharmacological profile of a novel H(2)S-releasing aspirin. Free Radic Biol Med 46:586-592

Stipanuk MH, Dominy JE, Lee J-I et al. (2006) Mammalian Cysteine Metabolism: New Insights into Regulation of Cysteine Metabolism J. Nutr. 136:1652-1659

Staal FH, Anderson MI, Staal CE et al. (1994) Redox regulation of signal transduction: tyrosine phosphorylation and calcium influx. Proc. Nat. Acad. Sci. 91:3619-3622

Stipanuk MH, Beck PW (1982) Characterization of the enzyme capacity for cysteine desulphhydration in liver and kidney of the rat. Biochem J 206:267-277

Stefansson E, The impact of ocular blood flow in glaucoma. Prog Retin Eye Res. 21:359–93

Stjernschantz J, Ocklind A, Wentzel P et al. (2000) Latanoprost-induced increase of tyrosinase transcription in iridial melanocytes. Acta Ophthalmol Scand. 78:618-22

Strack PR, West Frey M, Rizzo CJ et al. (1996) Apoptosis mediated by HIV protease is preceded by cleavage of Bcl-2. Proc. Natl. Acad. Sci. 93:9571–9576

Stadtman ER (1992) Protein oxidation and aging. Science 257: 1220-1224S

Stone J, Dreher Z (1987) Relationship between astrocytes, ganglion cells and vasculature of the retina. J Comp Neurol. 255:35-49

Sucher NJ, Lipton SA, Dreyer EB (1997) Molecular basis of glutamate toxicity in retinal ganglion cells. Vis Res 37:3483–3493

Susin SA, Lorenzo HK, Zamzami N et al. (1999) Molecular characterization of mitochondrial apoptosis-inducing factor. Nature 397(6718): 441-6

Sutton HC, Winterbourn CC (1989) On the participation of higher oxidation-states of iron and copper in fenton reactions. Free Radic Biol Med 6: 53-60

Suzuki S, Kumanomido T, Nagata E et al. (1997) Optic neuropathy from thiamine deficiency. Intern Med 36:532

Sun D, Jiang Y, Zheng S et al. (1994) Separate metabolic pathways leading to DNA fragmentation and apoptotic chromatin condensation. J. Exp. Med. 179:559-568

Sun M-H, Pang J-H P, Chen S-L et al. (2010) Retinal Protection from Acute Glaucoma-Induced Ischemia-Reperfusion Injury through Pharmacologic Induction of Heme Oxygenase-1 IOVS 51:4798-4808

Sury MD, Frese-Schaper M, Mühlemann MK (2006) Evidence that N-acetylcysteine inhibits TNF-alpha-induced cerebrovascular endothelin-1 upregulation via inhibition of mitogen- and stress-activated protein kinase.Free Radic Biol Med. 41:1372-83

Szabo ME, Droy-Lefaix MT, Doly M et al. (1991) Ischemia and reperfusion-induced histologic changes in the rat retina. Demonstration of a free radical-mediated mechanism. Invest. Ophthalmol. Vis. Sci. 32:1471-1478

Szabo ME, Droy-Lefaix MT, Doly M (1992) Ischaemia- and reperfusion-induced Na+, K+, Ca2+ and Mg2+ shifts in rat retina: effects of two free radical scavengers, SOD and EGB 761. Exp Eye Res. 55:39-45

Szabó C (2007) Hydrogen sulphide and its therapeutic potential. Nat Rev Drug Discov 6: 917-35

Tabner BJ, Turnbull S, El-Aganf et al. (2001) Production of Reactive Oxygen Species from Aggregating Proteins Implicated in Alzheimer's Disease, Parkinson's Disease and Other Neurodegenerative Disease. Current Topics in Medicinal Chemistry 6:507-517

Talley AK, Dewhurst S, Perry SW et al. (1995) Tumor necrosis factor alpha-induced apoptosis in human neuronal cells: protection by the antioxidant N-acetylcysteine and the genes bcl-2 and crmA Mol. Cell. Biol., 5:2359-2366

Tang XQ, Yang CT, Chen J et al. (2008) Effect of hydrogen sulphide on beta-amyloid-induced damage in PC12 cells. Clin Exp Pharmacol Physiol.35:180-6

Tempestini A, Schiavone N, Papucci L et al. (2003) The mechanisms of apoptosis in biology and medicine: a new focus for ophthalmology. Eur J Ophthalmol 13: S11-S18

Tezel G, Wax MB (2000a) Increased production of tumor necrosis factor-alpha by glial cells exposed to simulated ischemia or elevated hydrostatic pressure induces apoptosis in cocultured retinal ganglion cells. J Neurosci 20: 8693-8700

Tezel G, Wax MB (2000b) The mechanisms of hsp27 antibody-mediated apoptosis in retinal neuronal cells. J Neurosci 20: 3552-3562

Tezel G, Wax MB (2003) Glial modulation of retinal ganglion cell death in glaucoma. J Glaucoma 12: 63-68

Tezel G, Wax MB (2004a) Hypoxia-inducible factor 1 alpha in the glaucomatous retina and optic nerve head. Arch Ophthalmol 122: 1348-1356

Tezel G, Yang XJ (2004) Caspase-independent component of retinal ganglion cell death, in vitro. Invest Ophthalmol Vis Sci 45: 4049-4059

Tezel G, Yang XJ, Yang JJ, Wax MB (2004) Role of tumor necrosis factor receptor-1 in the death of retinal ganglion cells following optic nerve crush injury in mice. Brain Res 996: 202-212

Tezel G (2006) Oxidative stress in glaucomatous neurodegeneration: Mechanisms and consequences. Progr Retin Eye Res 25: 490-513

Tepel M, Giet MD, Schwarzfeld C (2000) Prevention of Radiographic-Contrast-Agent–Induced Reductions In Renal Function by Acetylcysteine. N Engl J Med. 343:180-184

Tewari M, Quan LT, Orourke K et al. (1995) Yama/CPP32 beta, a mammalian homolog of CED-3, is a CrmA-inhibitable protease that cleaves the death substrate poly(ADP-ribose) polymerase. Cell 81: 801-809

Tielsch JM, Katz J, Singh K et al. (1991) A population-based evaluation of glaucoma screening: the Baltimore eye survey. Am J Epidemiol 134: 1102-1110

Tomi M, Hosoya K, Takanaga H et al. (2002) Induction of xCT Gene Expression and L-CystineTransport Activity by Diethyl Maleate at the Inner Blood–Retinal Barrier Invest Ophthalmol Vis Sci. 43:774-9

Tomi M, Funaki T, Abukawa H et al. (2003) Expression and regulation of L–cystine transporter, system xc − , in the newly developed rat retinal Muller cell line (TR–MUL). Glia 43:208–217

Towbin H, Staehelin T, Gordon J (1979) Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets-procedure and some applications. Proc Natl Acad Sci U S A 76: 4350-4354

Toole JF, Malinow MR, Chambless LE et al. (2004) Lowering homocysteine in patients with ischemic stroke to prevent recurrent stroke, myocardial infarction, and death: the Vitamin Intervention for Stroke Prevention (VISP) randomized controlled trial. JAMA 291:565-75

Trump BF, Berezesky IK (1995) Calcium-mediated cell injury and cell death. FASEB J 9:219-228

Troy CM, Shelanski ML (1994) Down-regulation of copper/zinc superoxide dismutase causes apoptotic death in PC12 neuronal cells. Proc. Nail. Acad. Sci. 91:6384-6387

Troy CM, Rabacchi SA, Hohl JB et al. (2001) Death in the balance: Alternative participation of the caspase-2 and -9 pathways in neuronal death induced by nerve growth factor deprivation. *J Neurosci* 21(14): 5007-5016

Tripathi RC (1977) Pathologic anatomy of the outflow pathway of aqueous humor in chronic simple glaucoma. Exp Eye Res. 25*:*403–407

Tredger JM (2000) N-acetylcysteine: not simply a glutathione precursor. Transplantation. 69:703-4

Tsujimoto Y, Hashizume H, Yamazaki M (1993) Superoxide radical scavenging activity of phenolic-compounds. Int J Biochem 25: 491-494

Tsai JC (2008) Influencing ocular blood flow in glaucoma patients: the cardiovascular system and healthy lifestyle choices. Can J Ophthalmol.43:347-50

Tyagi N, Moshal KS, Sen U et al. (2009) H_2S protects against methionine-induced oxidative stress in brain endothelial cells. Antioxid Redox.Signal 11:25–33

Tzekov R, Arden GB (1999) The electroretinogram in diabetic retinopathy. Surv Ophthalmol 44: 53-60

Unoki K, Lavail MM (1994) Protection of the rat retina from ischemic-injury by brain-derived neurotrophic factor, ciliary neurotrophic factor, and basic fibroblast growth-factor. Invest Ophthalmol Vis Sci 35: 907-915

Uren RT, Dewson G, Bonzon C et al. (2004) Mitochondrial release of pro-apoptotic proteins: electrostatic interactions can hold cytochrome c but not Smac/DIABLO to mitochondrial membranes. J Biol Chem. 280:2266-74

Vaux DL, Haecker C, Strasser A (1994) An evolutionary perspective on apoptosis. Cell 76:777-78 1

Volvert ML, Seyen S, Piette M et al. (2008).Benfotiamine, a synthetic S-acyl thiamine derivative, has different mechanisms of action and a different pharmacological profile than lipid-soluble thiamine disulfide derivatives. BMC Pharmaco 8: 10

Van Reeth O (1999) Pharmacologic and therapeutic features of sulbutiamine. Drugs Today (Barc) 35:187-192

Vannucci RC (1990) Experimental biology of cerebral hypoxia-ischemia-relation to perinatal brain-damage. Pediatr Res 27: 317-326

Vannucci RC (1992) Cerebral carbohydrate and energy-metabolism in perinatal hypoxic-ischemic brain-damage. Brain Pathol 2: 229-234

Vetrugno M, Cantatore F, Ruggeri G (2008) Primary open angle glaucoma: an overview on medical therapy. Prog Brain Res.173:181-93

Vivanco I, Sawyers CL (2002) The phosphatidylinositol 3-Kinase–AKT pathway in human cancer. Nat Rev Cancer. 2:489-501

Vitorica J, Machado A, Satrústegui J (1984) Age-dependent variations in peroxide-utilizing enzymes from rat brain mitochondria and cytoplasm. J Neurochem. 42:351-6

Von Bartheld CS (1998) Neurotrophins in the developing and regenerating visual system. Histol Histopathol 13(2): 437-459

Wakabayashi N, Itoh K, Wakabayashi J et al. (2003) Keap1-null mutation leads to postnatal lethality due to constitutive Nrf2 activation. Nat Genet. 35:238-45

Walker PD, Brokering KL, Theobald JC (2003) Fenoldopam and N-acetylcysteine for the prevention of radiographic contrast material-induced nephropathy: a review. Pharmacotherapy. 23:1617-26

Wang K, Yin XM, Chao DT et al. (1996) BID: a novel BH3 domain-only death agonist. Genes Dev. 10:2859-69

Wang R (2002) Two's company, three's a crowd: can H_2S be the third endogenous gaseous transmitter? FASEB J 16:1792–8

Wang JJL**,** Hua Z**,** Fentress HM et al. (2000) JNK1 is inactivated during thiamine deficiency-induced apoptosis in human neuroblastoma cells The Journal of Nutritional Biochemistry 11, 208-215

Wang L, Dong J, Cull G et al. (2003) Varicosities of intraretinal ganglion cell axons in human and nonhuman primates. Invest Ophthalmol Vis Sci. 44:2-9

Wang CT, Chang HH, Hsiao CH et al. (2009) The effects of green tea (–)-epigallocatechin-3-gallate on reactive oxygen species in 3T3-L1 preadipocytes and adipocytes depend on the glutathione and 67 kDa laminin receptor pathways. Mol Nutr Food Res. 53:349-60

Watson RWG, Rotstein OD, Nathens AB et al. (1996*)* Thiol-mediated redox regulation of neutrophil apoptosis. Surgery 120:150–158

Wässle H, Boycott BB (1991) Functional architecture of the mammalian retina. Physiol Rev.71:447-80

Wassle H, Yamashita M, Greferath U (1991) The rod bipolar cell of the mammalian retina. Vis Neurosci 7: 99-112

Watase K, Hashimoto K, Kano M et al. (1998) Motor discoordination and increased susceptibility to cerebellar injury in GLAST mutant mice. Eur J Neurosci 10: 976-988

Wax MB, Tezel G (2002) Neurobiology of glaucomatous optic neuropathy-Diverse cellular events in neurodegeneration and neuroprotection. Mol Neurobiol 26: 45-55

Wax MB, Tezel G, Yang J et al. (2008) Induced autoimmunity to heat shock proteins elicits glaucomatous loss of retinal ganglion cell neurons via activated T-cell-derived Fas-ligand. J Neurosci 28: 12085-12096

Waxman SG, Black JA, Ransom BR et al. (1994) Anoxic injury of rat optic nerve-ultrastructural evidence for coupling between Na^+ influx and Ca^{2+}-mediated injury in myelinated CNS axons. Brain Res 644: 197-204

Whiteman M, Cheung NS, Zhu YZ et al. (2005) Hydrogen sulphide: a novel inhibitor of hypochlorous acidmediated oxidative damage in the brain? Biochem Biophys Res Commun 326: 794–8

Wilk S Teaching resources. Apoptosis. Sci. STKE. 2005(285):tr16.

Williamson JM, Boettcher B, Meister A (1982) Intracellular cysteine delivery system that protects against toxicity by promoting glutathione synthesis. Proc. Nat. Aced. Sci. 79:6246-6249

Winterbourn CC (1995) Toxicity of iron and hydrogen peroxide: The Fenton reaction. Toxicol Lett 82:969-974

Whitmore AV, Libby RT, John SW (2005) Glaucoma: thinking in new ways-a role for autonomous axonal self-destruction and other compartmentalised processes? Prog Retin Eye Res 24:639-662

Weinreb RN, Khaw PT (2004) Primary open-angle glaucoma. Lancet 22: 1711-1720

Wood PL, Khan MA, Moskal JR (2007) Cellular thiol pools are responsible for sequestration of cytotoxic reactive aldehydes: central role of free cysteine and cysteamine. Brain Res 16:158-163

Wolz P, Krieglstein J (1996) Neuroprotective effects of alpha-lipoic acid and its enantiomers demonstrated in rodent models of focal cerebral ischemia. Neuropharmacology. 35:369-75

Wong PT, Qu K, Chimon GN et al. (2006) High Plasma cysteine level may indicate poor clinical outcome in patients with acute stroke: possible involvement of hydrogen sulphide. J Neuropatholo Exp Neurol 5:109-115

Wood JP, Lascaratos G, Bron AJ et al. (2008) The influence of visible light exposure on cultured RGC-5 cells. Mol Vis 14:334-344

Wostyn P, Audenaert K, De Deyn PP (2009) Alzheimer's disease and glaucoma: is there a causal relationship? Br J Ophthalmol. 93:1557-9

Xia ZG, Dickens M, Raingeaud J et al. (1995) Opposing effects of ERK and JNK-p38 MAP kinases on apoptosis. Science 270: 1326-1331

Xiang MQ, Zhou H, Nathans J (1996) Molecular biology of retinal ganglion cells. Proc Natl Acad Sci U S A 93: 596-601

Xu BJ, Goulding EH, Zang KL et al. (2003) Brain-derived neurotrophic factor regulates energy balance downstream of melanocortin-4 receptor. Nat Neurosci 6: 736-742

Yang J, Liu XS, Bhalla K et al. (1997) Prevention of apoptosis by Bcl-2: Release of cytochrome c from mitochondria blocked. Science 275: 1129-1132

Yang J, Tezel G, Patil RV et al. (2001) Serum autoantibody against glutathione-S-transferase in patients with glaucoma. Invest Ophthalmol Vis Sci. 42:1273–1276

Yang Y, Zhao S, Song JG (2004) Caspase-dependent apoptosis and -independent poly(ADP-ribose) polymerase cleavage induced by transforming growth factor beta 1. Int J Biochem Cell Biol 36: 223-234

Yabuki M, Kariya S, Ishisaka R et al. (1999) Resistance to nitric oxide-mediated apoptosis in the HL-60 variant cells is associated with increased activities of Cu,Zn-superoxide dismutases and catalase, Free Radical Biol. Med 26,325–332

Yildirim O, Ateçs NA, Ercan B, et al. (2005) Role of oxidative stress enzymes in open angle glaucoma. Eye. 19:580–583

Yin ST, Tang ML, Su L et al. (2008) Effects of Epigallocatechin-3-gallate on lead-induced oxidative damage. Toxicology 249:45-54

York RD, Molliver DC, Grewal SS et al. (2000) Role of phosphoinositide 3-kinase and endocytosis in nerve growth factor-induced extracellular signal-regulated kinase activation via Ras and Rap1. Mol Cell Biol 20: 8069-8083

Yoles E, Schwartz M (1998) Degeneration of spared axons following partial white matter lesion: implications for optic nerve neuropathies. Exp Neurol 153: 1–7

Yuan JY, Yankner BA (2000) Apoptosis in the nervous system. Nature 407: 802-809

Yu SW, Wang HM, Poitras MF et al. (2002) Mediation of poly(ADP-ribose) polymerase-1-dependent cell death by apoptosis-inducing factor. Science 297:259-263

Yuste VJ, Sanchez-Lopez I, Sole C et al. (2005) The contribution of apoptosis-inducing factor, caspase-activated DNase, and inhibitor of caspase-activated DNase to the nuclear phenotype and DNA degradation during apoptosis. J Biol Chem 280: 35670-35683

Zafarullah M, Li WQ, Sylvester J, Ahmad M (2003) Molecular mechanisms of N-acetylcysteine actions. Cell Mol Life Sci. 60:6-20

Zamzanii N, Marchetti P, Castedo M et al. (1995) Sequential reduction of mitochondriat transmembrane potential amidgeneration of reactive oxygen species iii early programmed cell death. J. Exp. Med. 182:367-377

Zanardi RC, Brancaleone V, Distrutti E et al. (2006) Hydrogen sulfide is an endogenous modulator of leukocyte-mediated inflammation, FASEB J 20**:**1–8

Zanardo RC, Brancaleone V, Distrutti E et al., (2006) Hydrogen sulfide is an endogenous modulator of leukocyte-mediated inflammation. FASEB J. 20:2118-2120

Zanon-Moreno V, Marco-Ventura P, Lleo-Perez A et al. (2008) Oxidative stress in primary open-angle glaucoma. J Glaucoma 17: 263-268

Zeevalk GD, Nicklas WJ (1990) Chemically induced hypoglycemia and anoxia: relationship to glutamate receptor-mediated toxicity in retina. J Pharmacol Exp Ther 253(3): 1285-1292

Zhao W, Ndisang JF, Wang R (2003) Modulation of endogenous production of H_2S in rat tissues. Can J Physiol Pharmacol 81:848–53

Zhou JY, Prognon P (2006) Raw material enzymatic activity determination: a specific case for validation and comparison of analytical methods—the example of superoxide dismutase (SOD), J. Pharm. Biomed. Anal. 40:1143–1148

Zhou W, Rangaswamy N, Ktonas P et al. (2007) Oscillatory potentials of the slow-sequence multifocal ERG in primates extracted using the Matching Pursuit method. Vision Res. 47:2021-36

Zhang X, Chen X, Song H et al. (2005) Activation of the Nrf2/antioxidant response pathway increases IL-8 expression**.** Eur J Immunol. 35:3258-67

Zhang H, Bhatia M (2008) Hydrogen sulfide: a novel mediator of leukocyte activation. Immunopharmacol Immunotoxicol.30:631-45

Zhang B, Safa R, Rusciano D et al. (2007) Epigallocatechin gallate, an active ingredient from green tea, attenuates damaging influences to the retina caused by ischemia/reperfusion. Brain Res. 23:40-53

Zheng Z, Lee JE, Yenari MA (2003) Stroke: Molecular mechanisms and potential targets for treatment. Curr Mol Med 3: 361-372

Zhou M, Diwu Z, Panchuk-Voloshina N et al. (1997) A Stable Nonfluorescent Derivative of Resorufin for the Fluorometric Determination of Trace Hydrogen Peroxide: Applications in Detecting the Activity of Phagocyte NADPH Oxidase and Other Oxidases. Anal Biochem 253: 162-168

Zhao W, Ndisang JF, Wang R. (2003) Modulation of endogenous production of H2S in rat tissues. Can J Physiol Pharmacol. 81:848-53

Zhou XH, Li F, Kong L et al. (2005) Involvement of inflammation, degradation, and apoptosis in a mouse model of glaucoma. J Biol Chem 280: 31240-31248

Zhuge J, Cederbaum AI (2006) Serum deprivation-induced HepG2 cell death is potentiated by CYP2E1. Free Radic Biol Med 40: 63-74

Zou H, Henzel WJ, Liu XS et al. (1997) Apaf-1, a human protein homologous to C-elegans CED-4, participates in cytochrome c-dependent activation of caspase-3. Cell 90: 405-413