Arginine transport and other determinants of nitric oxide production in human septic shock

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Abstract

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The arterial vasodilation seen in human septic shock is conventionally attributed to increased nitric oxide (NO) production, primarily by extrapolation of animal and cellular studies. Little is known of the cellular source of NO in human septic shock. Other mediators, such as carbon monoxide (CO), may modulate NO production, and could also directly contribute to vasodilation.

This study has examined the NO and CO synthetic pathways in peripheral blood mononuclear cells and mesenteric arterial smooth muscle from patients with septic shock, and from non-septic controls.

Peripheral blood mononuclear cells from septic patients had increased NO production, though this was perhaps more modest than expected. The transport of arginine, the substrate for NO synthase, into these cells was increased; this was due to an increase in the activity of one transporter system, y^+. mRNA for a protein encoding y^+ activity, CAT2B, was increased in these cells. However, mRNA and protein for inducible and endothelial NO synthase was decreased in sepsis, while inducible heme oxygenase (the enzyme responsible for CO production) mRNA and protein was increased.

NO production in arterial smooth muscle from septic patients was reduced, as was mRNA for inducible and endothelial NO synthase, and the arginine transporter CAT1. There was no increase in inducible NO synthase protein, though there were small increases in endothelial NO synthase protein and NO synthase activity. In contrast, both mRNA and protein for inducible heme oxygenase were increased.

These results challenge the assumption that NO is central to the pathogenesis of human sepsis. Negative feedback systems for NO production have been demonstrated in cell models. These may be relatively more important in human sepsis. In addition to forming one of these feedback systems, it may be that CO, more than NO, is responsible for the hypotension observed in these patients.

Contents

Abstract
Contents 1
Acknowledgements 4
Financial support and awards 5
Declaration 6
Abbreviations 7
Ethical approval for studies on human subjects 10

1. Introduction

1.1 Background 13
Vasodilatory shock 13
Nitric oxide 14
The cellular origin of nitric oxide in sepsis:
a. endothelium? 17
b. macrophages? 19
c. circulating immune cells? 21
d. vascular smooth muscle cells? 23
e. adventitia? 24
Strategies to reduce nitric oxide production in septic shock 27
Reducing the upregulated component of nitric oxide in sepsis:
a. Selective inhibition of iNOS activity? 29
b. Reduction of the availability of BH_4 co-factor? 30
c. Non-specific inhibition of protein synthesis? 31
d. Reduction in the amount of 'recycled' intracellular arginine? 31
e. Limiting arginine transport? 32
Plasma membrane arginine transport 34
Vasodilation by mechanisms other than nitric oxide 35
Carbon monoxide as a link between sepsis and vasodilation in septic shock 37
1.2 Aims 49

2. Preparation of patient samples

2.1 Diagnostic criteria for severe sepsis and septic shock 52
2.2 Preparation of the peripheral blood mononuclear cell fraction from patient blood samples 56
2.3 Preparation of isolated arterial smooth muscle from patient bowel samples 59

3. Nitric oxide production: Diaminofluorescein

3.1 Quantitation of nitric oxide production using diaminofluorescein: Method 65
3.2 Nitric oxide production by peripheral blood mononuclear cells 72
3.3 Nitric oxide production by arterial smooth muscle 76

4. Nitric oxide synthase activity: ^{3}H arginine to ^{3}H citrulline conversion

4.1 Quantitation of nitric oxide synthase activity by rate of ^{3}H arginine to ^{3}H citrulline conversion: Method 80
4.2 Nitric oxide synthase activity in peripheral blood mononuclear cells 85
4.3 Nitric oxide synthase activity in arterial smooth muscle 88

5. Arginine transporter function: Labelled amino acid uptake

5.1 Characterisation of arginine transport through y^{+} and $y^{+}L$ in peripheral blood mononuclear cells: Method 93
5.2 Arginine transport through y^{+} and $y^{+}L$ in peripheral blood mononuclear cells 105

6. Arginine transporter and nitric oxide synthase mRNA quantity: Real-time reverse transcription polymerase chain reaction

6.1 Real-time reverse transcription polymerase chain reaction: Method 110
6.2 Nitric oxide synthase and arginine transporter mRNA in peripheral blood mononuclear cells 128
6.3 Nitric oxide synthase and arginine transporter mRNA in arterial smooth muscle 134

7. Arginine transporter and nitric oxide synthase protein quantity: Western blotting

7.1 Western blot: Method 140
7.2 Nitric oxide synthase protein in peripheral blood mononuclear cells 147
7.3 Nitric oxide synthase protein in arterial smooth muscle 150

8. Heme oxygenase mRNA and protein quantity: Real-time reverse transcription polymerase chain reaction and Western blotting

8.1 Real-time reverse transcription polymerase chain reaction and Western blotting for inducible heme oxygenase mRNA and protein: Method 156
8.2 Inducible heme oxygenase mRNA and protein in peripheral blood mononuclear cells and arterial smooth muscle 159

9. Location of nitric oxide synthase and heme oxygenase protein in vessel wall: Immunohistochemistry

9.1 Localisation of nitric oxide synthase and heme oxygnase in arterial vessel wall by immunocytochemistry: Method 164

9.2 Location of nitric oxide synthase and heme oxygenase in arterial vessel wall 166

10. Summary, discussion and conclusion

10.1 Summary of Results 172

10.2 Discussion 173

10.3 Conclusion and potential therapeutic implications 187

11. Future Work

11.1 Future Work 190

Appendices

I Patient data and detailed experimental results for each subject 195

II Reagent suppliers and details of solutions 197

III Quantitation of protein concentration: method 200

IV Investigation of a potential modulator of y^+ function: Resveratrol 202

V Attempts to develop antibodies recognising human proteins with y^+ activity 207

VI Attempted functional study of heme oxygenase activity: Rate of bilirubin formation measured by spectrophotometry 215

VII Publications 223

References 252

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Declaration

The work described here was supervised by Dr C.A.R. Boyd of the Department of Human Anatomy and Genetics and Dr J.D. Young of the Nuffield Department of Anaesthetics, University of Oxford. Dr Young and Dr Boyd first conceived the idea of investigating arginine transport as a means of modulating nitric oxide production in sepsis. It was they who suggested investigation of the molecular changes underlying preliminary evidence of abnormalities of arginine transport in blood cells from patients with sepsis.

Dr J.L. Millo of the Nuffield Department of Anaesthetics has undertaken part of the burden of collecting vascular smooth muscle from patients at the John Radcliffe Hospital. I have collaborated in developing the protocol for confocal and routine immunohistochemistry of vascular smooth muscle with Dr Millo.

The studies of heme oxygenase and nitric oxide synthase are entirely my own design.

Other than the results obtained in collaboration with Dr Millo, all of the experiments presented in this thesis are my own work.

Michael Reade
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Abbreviations

18S	The smaller of the two subunits comprising ribosomal RNA
20-HETE	20-Hydroxy-(5Z,8Z,11Z,14Z)-eicosatetraenoic acid
ACh	acetylcholine
AEBSF	4-(2-aminoethyl) benzenesulfonyl fluoride hydrochloride, a protease inhibitor
ASM	arterial smooth muscle
BH_4	tetrahydrobiopterin dihydrochloride, a cofactor for NOS and other enzymes
BME	β mercaptoethanol, a reducing agent
BSA	bovine serum albumin
CAT1, 2, 4	cationic amino acid transporters, types 1, 2 and 4 (the proteins known to have y^+ amino acid transport activity in human cells)
CAT2A, 2B	Post transcriptional variants of the CAT2 gene product, distinguished by mRNA sequence and transporter function
CD98	heavy chain of the y^+L heterodimeric amino acid transporter protein family (as well as forming a component of other amino acid transporters, in addition to many non-transport related functions)
CHAPS	3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate, a detergent
CO	carbon monoxide
CPM	counts per minute (a measure of the radioactivity of a substance when dissolved in scintillation fluid)
C_t	cycle number above which the amount of PCR product exceeds a threshold, in real-time PCR
DAF-2	4, 5 diaminofluorescein, a nitric oxide sensitive dye
DAF-2 DA	4, 5 diaminofluorescein diacetate, the plasma membrane permeable form of DAF-2
DMSO	dimethyl sulfoxide, a solvent
DPM	disintegrations per minute (the rate of radioactive decay of a substance, which is calculated from the measured CPM value)
DTT	1, 4-Dithio-DL-threitol, a reducing agent
EDTA	ethylene diamine tetraacetic acid, a chelator of calcium and hence an anticoagulant
EGTA	ethylene glycol-bis(2-aminoethylether)-N,N,N',N'-tetraacetic acid, a chelator of calcium and thus an inhibitor of calcium dependent enzymes
eNOS	endothelial (constitutive) isoform of nitric oxide synthase, elsewhere known as NOS III

FACS	fluorescence activated cell sorting
FAD	flavine adenine dinucleotide, an enzyme cofactor
FAM	carboxyfluorescein, an oligonucleotide-linked reporter dye used in the TaqMan real-time PCR assay
FMN	riboflavin 5' monophosphate, an enzyme cofactor
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
H&E	haematoxylin and eosin, stains for cell nuclei and cytoplasm
HO-1	heme oxygenase isoform 1 (the inducible form)
HO-2	heme oxygenase isoform 2 (a constitutive form)
HO-3	heme oxygenase isoform 3 (a constitutive form)
IFNγ	interferon gamma, a proinflammatory cytokine
iNOS	inducible isoform of nitric oxide synthase, elsewhere known as NOS II
IL-1	interleukin 1, a pro-inflammatory cytokine
IL-6	interleukin 6, a pro-inflammatory cytokine
IL-10	interleukin 10, an anti-inflammatory cytokine
L-NAME	N_{ω}-Nitro-L-arginine methyl ester, an inhibitor of eNOS and iNOS
L-NMMA	N_{ω}-Methyl-L-arginine acetate, an inhibitor of plasma membrane arginine transport, eNOS and iNOS
LPS	lipopolysaccharide, a constituent of the cell wall of gram-negative bacteria
mRNA	messenger RNA
NADH	nicotinamide adenine dinucleotide (reduced form), an enzyme cofactor
NADPH	nicotinamide adenine dinucleotide phosphate (reduced form), an enzyme cofactor
NO	nitric oxide
nNOS	neuronal (constitutive) isoform of nitric oxide synthase, elsewhere known as NOS I
PBMC	peripheral blood mononuclear cell
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PMA	phorbol 12-myristate 13-acetate
ROX	carboxy-x-rhodamine (the passive reference dye in the TaqMan real-time PCR reaction)
RT	reverse transcription (of RNA into cDNA)
SDS	sodium dodecyl sulphate, a detergent
SEITU	2-ethyl-2-thiopseudourea, an inhibitor of all nitric oxide synthases
SWG	standard wire gauge
TAMRA	carboxytetramethlyrhodamine (the quenching dye in the TaqMan real-time PCR reaction)
TBS	tris buffered saline
TBS-T	tris buffered saline with 0.1% Tween 20
TGF B	transforming growth factor B, an anti-inflammatory cytokine
TNFα	tumour necrosis factor alpha, a pro-inflammatory cytokine
TRITC	tetramethylrhodamine isothiocyanate, a fluorescent dye
UNG	uracil-N-glycosylase

VIC	Applied Biosystems proprietary reporter dye, with a similar function but different spectra to FAM
VSM	vascular smooth muscle (ie. relating to both arterial and venous smooth muscle, in contrast to ASM)
y^+	A membrane transport system defined by kinetic criteria: transports cationic amino acids. electrogenic low affinity and high capacity (compared to y^+L). (see table 1.1e)
y^+L	A membrane transport system defined by kinetic criteria: transports cationic amino acids regardless of sodium concentration, and neutral amino acids only when sodium is present; not electrogenic high affinity and low capacity (compared to y^+) (see table 1.1e)
y^+LAT1 and 2	Alternate light chains of the y^+L heterodimeric protein (which is formed by combination of a light chain with CD98)

Ethical approval for studies on human subjects

All of the experiments described in this thesis were performed on human biological material.

Volunteer healthy laboratory staff acted as controls for the studies of peripheral blood mononuclear cells; verbal consent for blood sampling was obtained after an explanation of the experiments proposed.

Peripheral blood mononuclear cells were collected from patients on the intensive care unit at the John Radcliffe Hospital. In meeting the study inclusion criteria these patients were by definition critically ill. Patients in such a state are universally acknowledged as being often incapable of providing informed consent for participation in research studies (Lemaire *et al.* 1997). Where possible, the assent of a relative was obtained; however, this was occasionally not possible within the window of opportunity defined by the study protocol. In these cases the Standing Orders of the Intensive Care Unit at the John Radcliffe Hospital (which have the approval of the Central Oxfordshire Research Ethics Committee) were followed: up to 10% of the volume of blood drawn for clinical purposes may be used solely for research, without the need for consent by the patient or assent of the relative. This is in accordance with the official policy of the European Society of Intensive Care Medicine (Lemaire *et al.* 1997).

Arterial smooth muscle was collected from surgical material sent for pathological analysis. No extra material was removed for the purposes of this study. The biological specimen removed at surgery is routinely discarded after the necessary pathology tests have been performed. The small portions of tissue used for these studies were destroyed in the experimental process; no tissue has been retained other than the minute quantities on histology slides. As such this study was formally exempted from the need to obtain informed consent from the patients involved, and approved by the Chairman of the Central Oxfordshire Ethics Committee in a letter dated 6 March 2000.

No genetic or personal information has been retained by which a patient could subsequently be identified.

Chapter 1:

Introduction

1.1 Background

Shock due to overwhelming infection might be considered relatively simple to treat. Most organisms responsible for septic shock are initially sensitive to a large number of antibiotics. Vascular tone and cardiac output are usually easily manipulated using infusions of vasopressors and inotropes. Why then is septic shock the leading cause of death in non-coronary intensive care units (Society of Critical Care Medicine, 2002), with a mortality rate of around 50% (Friedman *et al.* 1998)?

It is now clear that systemic infection initiates a generalised inflammatory response, with multiple positive feedback loops stimulating ever increasing production of pro-inflammatory cytokines and effector molecules. It is this inflammatory response rather than the direct effect of the micro-organism which is responsible for the high mortality in sepsis. The very large number of cytokines involved probably explains the disappointing results of clinical trials blocking the actions of individual mediators (as reviewed by Zeni *et al.* (1997)).

Vasodilatory shock

While there are many deleterious cellular effects of systemic inflammation, of greatest importance is relaxation of vascular smooth muscle. Vasodilation reduces organ

perfusion pressure, precipitating hypoxia, which increases inflammation even further. Vasodilatory shock is characterised by increased endogenous plasma catecholamine levels (Benedict & Rose 1992) and activation of the renin-angiotensin system (Cumming *et al.* 1988). The fundamental problem must therefore be failure of the vascular smooth muscle to contract. This also accounts for the eventual ineffectiveness of pharmacological vasopressors in septic shock. A recent review highlighted three mechanisms as possible causes of this vascular hyporeactivity: activation of ATP sensitive potassium channels in the plasma membrane of vascular smooth muscle; deficiency of the hormone vasopressin; and activation of the inducible form of nitric oxide synthase (iNOS) (Landry & Oliver 2001). In passing this review noted the activation of guanylate cyclase by carbon monoxide poisoning as another example of non-septic vasodilatory shock. The relative importance of each of these possible mechanisms is not known. While they are potential therapeutic targets, the roles of ATP sensitive potassium channels and vasopressin in the pathogenesis of septic shock will not be discussed further in this thesis. Nitric oxide, almost since the discovery of its biological significance, has been considered central to the pathogenesis of septic shock. The majority of research into effector mechanisms in septic shock concentrates on nitric oxide, and similarly nitric oxide is the focus of much of this thesis.

Nitric Oxide

Nitric oxide (NO) was first isolated in 1772 by Joseph Priestley and, though its mechanism of action was not understood at the time, was used (in the form of various

NO-liberating compounds such as nitroglycerine) to treat ischaemic cardiac pain from around 1890. However, the biological role of nitric oxide was only properly appreciated when it was demonstrated by Ignarro (Ignarro *et al.* 1987) and Palmer, Ferridge and Moncada (Palmer *et al.* 1987) to be the endothelial derived relaxing factor discovered in a series of experiments by Furchgott in 1980 (Furchgott & Zawadzki 1980).

There is much to suggest that overproduction of nitric oxide is central to vasodilation in sepsis. Whole body NO production is dramatically increased in rats (Hecker *et al.* 1995) (Kitajima *et al.* 1995) (Sakemi *et al.* 1998), mice (Soeters *et al.* 2002) (Harbrecht *et al.* 1994) (Krecic-Shepard *et al.* 1999), pigs (Soeters *et al.* 2002), and sheep (Allman *et al.* 1996) given endotoxin. Whole body NO production is also increased in human septic shock (Barthlen *et al.* 1994) (Groeneveld *et al.* 1996) (Krafte-Jacobs *et al.* 1997) (Ochoa *et al.* 1991) (Shi *et al.* 1993). Studies of nitric oxide metabolites in clinical human sepsis have been criticised for inadequately controlling for the confounding effect of altered renal function in sepsis. However, when the elevation of nitric oxide metabolites due solely to renal failure is removed by analysis of covariance, the same conclusion is reached (MacKenzie *et al.* 2001).

The levels of NO metabolites in human sepsis are less than in septic animal models (Pastor & Suter 1998), though there is some variability in the studies on which this conclusion is based (table 1.1a). The higher level of NO metabolites in endotoxaemic animals suggests they are more resistant to the pathological effects of NO than humans. Humans have perhaps also evolved counter-regulatory controls which limit

NO production. Conversely, the lower levels of nitric oxide might suggest the pathogenesis of septic shock in humans also involves other mechanisms.

Animal sepsis		
Harbrecht *et al.* 1994	Mouse LPS model	804 ± 84
Krecic-Shepard *et al.* 1999	Mouse Bacteria + LPS model	146 ± 18
Hecker *et al.* 1995	Rat LPS model	Approx. 160
Kitajima *et al.* 1995	Rat LPS model	304 ± 11
Sakemi *et al.* 1998	Rat LPS model	430
Allman *et al.* 1998	Sheep LPS model	13.8
Human sepsis		
Evans *et al.* 1993		124
Barthlen *et al.* 1994		72.1
Groeneveld *et al.* 1996		52 ± 16
Krafte-Jacobs *et al.*, 1997		81.9 ± 18.2
MacKenzie *et al.* 2001		66.6

Table 1.1a

Nitric oxide metabolite levels (nitrate + nitrite, umol/L, ± standard error, not corrected for renal function) in clinical human sepsis and animal models of disease.

That inhibitors of nitric oxide synthase increase blood pressure in various models of sepsis is often used as evidence for the central role of nitric oxide in septic shock. Certainly, inhibitors such as L-NMMA and L-NAME increase the blood pressure of animals (Robertson *et al.* 1994) (Julou-Schaeffer *et al.* 1990) and patients (Petros *et al.* 1991) (Petros *et al.* 1994) (Lin *et al.* 1994) (Schilling *et al.* 1993) with symptoms of septic shock. However it is incorrect to use this alone to implicate nitric oxide in the pathogenesis of the disease. Nitric oxide is a vasodilator produced continuously by endothelial cells, certainly in health and presumably also in sepsis. Removal of this vasodilator must increase blood pressure; this does not indicate that NO was being *overproduced* in sepsis, just that it was produced in a physiologically significant quantity. Only if the NOS inhibitors increase blood pressure more than in healthy

controls could their effect be taken as evidence of extra NO production in sepsis. One study in fact showed the opposite effect. Study of a porcine model of sepsis found that, as expected, lipopolysaccharide reduced mean arterial pressure (MAP) from 68 to 53 mmHg, and that L-NAME returned the MAP to 63 mmHg. However, L-NAME raised the MAP of a control animal even more, from 68 to 98 mmHg, indicating that the role of NO in controlling vascular tone had if anything decreased with lipopolysaccharide (Robertson *et al.* 1994). Mesenteric arteries from patients with septic shock were hyporesponsive to noradrenaline, and this hyporesponsiveness was reversed by L-NAME (Tsuneyoshi *et al.* 1996). However, the effect of L-NAME on the control vessels was not reported, which as explained invalidates that study's conclusion that nitric oxide was responsible for the observed hyporesponsiveness. There are, however, some in vitro studies in which control vessels are unaffected by inhibitors of nitric oxide synthase, while the response of vessels from septic patients is returned to normal (Stoclet *et al.* 1999). Thus some in vitro inhibitor experiments do lend weight to the theory that nitric oxide is central to the pathogenesis of sepsis, even though the conclusions of many in vivo NO inhibitor studies are not justified.

The cellular origin of nitric oxide in sepsis:

a. endothelium?

The cellular origin of nitric oxide in septic shock is not known. The view for many years was that vasoactive nitric oxide is produced mainly by endothelial cells. While certainly true in healthy vessels, many studies suggest this may not be so in sepsis. Endothelium from rats made septic by caecal ligation and puncture produced less

nitric oxide, and had less eNOS protein, than endothelial cells from healthy controls (Zhou *et al.* 1997). In a separate study the thoracic aortae from these rats showed less endothelium dependent relaxation to ACh (Wang *et al.* 1996), confirming the functional significance of this finding. Downregulation of endothelial nitric oxide production may indeed be integral to the pathogenesis of septic shock: transgenic mice overexpressing eNOS were more resistant to intraperitoneal lipopolysaccharide, with reduced inflammatory adhesion molecule expression and less leukocyte infiltration into tissues than control mice (Yamashita *et al.* 2000).

There are also studies of human endothelial tissue. Human aortic endothelial cells in culture with a mixture of cytokines and LPS decreased their expression of eNOS mRNA, while no iNOS mRNA was detected (MacNaul & Hutchinson 1993). However the results of some other studies of human endothelial cells are confusing. Human umbilical vein endothelial (HUVEC) cells isolated and cultured in vitro showed reduced eNOS protein (consistent with the studies just mentioned), but paradoxically had increased eNOS activity (measured by calcium dependent arginine to citrulline conversion, and by chemiluminescent detection of nitric oxide). This was explained by the observed increase in the cofactor BH_4 in these cells, which could have increased the activity of the remaining eNOS (Rosenkranz-Weiss *et al.* 1994). A similar study of isolated HUVEC cells also showed increased nitric oxide production (Irie *et al.* 1997).

It would seem that while both human and animal endothelial cells have reduced eNOS mRNA and protein expression, endothelium from animals with clinical sepsis makes less nitric oxide, while human endothelial cells activated in vitro make more. This

may be simply the result of species differences, perhaps in the regulation of the necessary cofactors for eNOS activity. However, the animal cells had been 'activated' whilst in close proximity to their underlying arterial smooth muscle, while the human cells had been exposed to LPS and cytokines in isolation. It is possible that the animal arterial smooth muscle produced a substance which acted on the endothelium to reduce not only its eNOS content but activity as well. This hypothesis will be explored in more detail in subsequent sections which consider the negative feedback effects of carbon monoxide and nitric oxide itself on NO production.

Endothelial tissue is difficult to obtain in sufficient quantities to study in isolation. In that it appears endothelium is not the source of extra NO in sepsis, this study has only examined endothelium from patients with septic shock where it is present in immunocytochemistry sections of vessel wall.

The cellular origin of nitric oxide in sepsis:

b. macrophages?

In addition to endothelial cells, the other conventionally recognised sources of nitric oxide are cells of the inflammatory system: circulating mononuclear cells (peripheral blood mononuclear cells (PBMCs)) (including T cells, B cells and monocytes); neutrophils; and tissue macrophages. Immune cells are thought to synthesise nitric oxide as a means of killing micro-organisms (reviewed by MacMicking *et al.* (1997)). Rat and mouse macrophages in particular seem to produce large quantities of nitric oxide in response to a variety of stimuli, making them a reliable cell in which to study the mechanisms of NO upregulation and its effects – as shown by the large number of

studies listed in table 1.1b. Whether human macrophages can also upregulate their nitric oxide production was for a long time the source of debate. Human macrophages cultured in vitro with a very wide variety of stimuli (all of which were sufficient to activate murine macrophages) could not be stimulated to produce nitric oxide or to upregulate their NOS activity (Schneemann *et al.* 1993). However it is now clear that, as shown in table 1.1b, human inflammation in vivo does upregulate nitric oxide in tissue macrophages. The extra factors required for this (which seem to not be required in rat and mouse cells) have not yet been identified. Nitric oxide produced by tissue macrophages is likely to affect the indices of whole body NO production described above, though whether it has the same haemodynamic significance as NO produced in the vessel wall is unclear. It is not unreasonable to expect that at least some of this extra NO will be produced by macrophages in sufficiently close proximity to the vascular smooth muscle cells to affect vascular tone.

Tissue macrophages in the vessel wall have been examined in immunocytochemistry studies. However, isolation of sufficient quantities of tissue macrophages from patients with systemic sepsis is difficult. Macrophages infiltrating localised infected areas may not be representative of other body macrophages, and can be difficult to distinguish from bacteria and necrotic cells. Circulating immune cells are a much more readily available means of studying cells of the inflammatory system.

The cellular origin of nitric oxide in sepsis:

c. circulating immune cells?

Unlike the results from studies of macrophages, abnormalities of nitric oxide production in circulating immune cells appear much less certain. Virtually all reported studies of circulating immune cells use human tissue. Animal circulating immune cells presumably produce less reliable results than macrophages, and are thus less attractive as an experimental model. One study showed bovine PBMCs responded to a mixture of proinflammatory cytokines with an increase in nitrite production and iNOS mRNA (Goff *et al.* 1996). However, in vitro studies of human circulating immune cells show conflicting results. Paralleling the result of the human in vitro macrophage study described above, human PBMCs did not show increased mRNA for iNOS when incubated with LPS, whereas murine macrophages studied under the same conditions (by the same investigators) did (Chesrown *et al.* 1994). Yet in another study, human PBMCs stimulated in vitro with lipopolysaccharide and interferon gamma (IFNγ) did show an increase in iNOS mRNA and protein. Surprisingly, though, their production of nitrite/nitrate and their NOS activity (assayed by the rate of conversion of arginine to citrulline) was strikingly lower than that of mouse macrophages, and not significantly affected by LPS or IFNγ (Weinberg *et al.* 1995). Further confusing the picture, PBMCs from a patient with abdominal gas gangrene expressed new iNOS activity (measured by rate of calcium independent arginine to citrulline conversion) when incubated with live group B streptococcus or E. faecalis, but not following exposure to a variety of other bacteria (Annane *et al.* 2000). Whether this effect was due to some factor induced by the patient's clinical

condition is not known, as the experiment was not also performed using PBMCs from healthy subjects.

Prior to the experiments described in this thesis, nitric oxide production by PBMCs from patients with clinical sepsis had not been reported. However, two studies of other human clinical diseases demonstrate that PBMCs do at least have the capacity to upregulate nitric oxide using iNOS. iNOS protein was present in PBMCs from children living in an area where malaria is endemic, and the metabolites of NO in the serum of these children was related to their disease severity (Anstey *et al.* 1996). PBMCs from patients with rheumatoid arthritis had increased NOS activity and increased iNOS antigen content compared to those from normal subjects (St Clair *et al.* 1996). It would seem, therefore, that PBMCs can upregulate NO production by iNOS. Whether they do so in sepsis is not yet known.

The other main class of circulating immune cell is the neutrophil. Neutrophils from patients with sepsis have a greater NOS activity (assayed by the rate of conversion of arginine to citrulline) than do cells from healthy volunteers (Goode *et al.* 1995). The number of patients with detectable neutrophil iNOS protein is greatest in those diagnosed with sepsis (100%), somewhat less in those with a systemic inflammatory response to a stimulus other than infection (70%), and least in postoperative patients making an uneventful recovery (18%) (Tsukahara *et al.* 1998). Neutrophils sequestered at the site of an infection also show increased NOS activity, iNOS mRNA and protein (Wheeler *et al.* 1997). Neutrophils are thus likely to be an important source of nitric oxide in the early phases of septic shock; however, as the infection becomes more prolonged the circulating immune cell population increasingly

becomes dominated by PBMCs. In that this subacute period is when the bulk of sepsis mortality occurs, it would seem logical to further investigate PBMC responses to sepsis. PBMCs from patients with septic shock have been studied in detail in this thesis.

The cellular origin of nitric oxide in sepsis:

d. vascular smooth muscle cells?

Until recently vascular smooth muscle was ignored as a potential source of nitric oxide; indeed, many studies of vessel wall NO production did not attempt to distinguish the contribution of the endothelium from that of the media. However, there is growing indirect evidence that vascular smooth muscle can manufacture its own nitric oxide – which if true is of obvious potential haemodynamic significance. The evidence for NO production by vascular smooth muscle cells is summarised in table 1.1c. The only studies of vessels taken from patients with clinical sepsis do implicate NO in the reduced contractility (compared to appropriate controls) observed in mesenteric (Stoclet *et al.* 1999) and omental (Tsuneyoshi *et al.* 1996) arteries. L-NAME (a NOS inhibitor) increased the contractile responsiveness of septic omental arteries while having no effect on healthy controls, though confusingly the response of septic arteries to noradrenaline in the absence of L-NAME was no different to control (Stoclet *et al.* 1999). Unfortunately the effect of L-NAME on control omental arterial tissue was not studied by Tsuneyoshi et al., so their observation that L-NAME and methylene blue (which binds NO) restored contractile responsiveness to noradrenaline in septic vessels must be interpreted with caution.

These studies of human vascular smooth muscle in sepsis involved tissue from a very small number of patients: neither study was sufficient to allow the identification of statistically significant differences. Other than qualitatively demonstrating increased iNOS in the media of vessels from patient with sepsis (Stoclet *et al.* 1999), these studies were entirely functional, and did not investigate the molecular basis of the hyporesponsiveness observed. Vascular smooth muscle from patients with septic shock has been studied in detail in this thesis, at a functional and molecular level, using tissue from sufficient numbers of patients to allow a statistical appreciation of the validity of the conclusions drawn.

The cellular origin of nitric oxide in sepsis:

e. adventitia?

The other tissue type of potential interest in septic shock is the vascular adventitia. Rat aorta incubated in vitro with LPS was divided into medial and adventitial layers: the adventitia produced 3.5x as much nitrite as the media in each aortic segment. Arginine relaxed LPS-activated endothelium-denuded aortic rings, but when the adventitia was removed this effect disappeared, suggesting the only functionally significant quantities of nitric oxide were derived from the adventitia and not the media. The noradrenaline EC_{50} of LPS treated whole aorta was significantly greater than that of the media alone, again suggesting the adventitia was the principle source of nitric oxide (Kleschyov *et al.* 1998). The media and adventitia of rat aortas treated either in vitro or in vivo with LPS both showed increased iNOS protein; however this increase was greater in the adventitia. Adventitial NO production was also greater

than that of the media after both in vitro and in vivo exposure to LPS (Kleschyov *et al.* 2000). Adventitia of rat aorta also contains nNOS mRNA and protein (Schwarz *et al.* 1999), though the functional significance of this is unclear.

The adventitial layer of blood vessels contains a multitude of cell types, including adipocytes, fibroblasts, and vasa vasorum in the larger vessels. It was the fibroblasts and macrophages which contained the iNOS identified in both the in vitro and in vivo LPS experiments mentioned above (Kleschyov *et al.* 2000). How relevant this is to human disease is unknown. The only study to specifically address this used human omental arteries from patients with sepsis, and found iNOS in the adventitia, but in lesser amounts than in the media of these vessels (Stoclet *et al.* 1999).

Quantification of changes in the adventitia of blood vessels is difficult because adventitia tends to be friable tissue, which is difficult to isolate intact. Additionally, adventitia is present in different amounts in vessels of the same size, making comparisons between patients difficult. In that the nitric oxide producing cells in the adventitia are most likely to be infiltrated inflammatory cells, this thesis has concentrated on the PBMCs described above, rather than look in detail at the adventitia. However, adventitia is present in the immunocytochemistry sections of vessel wall, allowing some comment to be made about the response of this tissue in septic shock.

In summary, it seems the most likely sources for the increased nitric oxide produced in human septic shock are the vascular smooth muscle cells, cells within the adventitia (probably infiltrated macrophages), and circulating immune cells. To date, though,

none of these cell types have been convincingly shown to produce increased nitric oxide in clinical human septic shock; nor have their molecular changes in sepsis been characterised. It is these cells which are the focus of the studies in this thesis.

Strategies to reduce nitric oxide production in septic shock

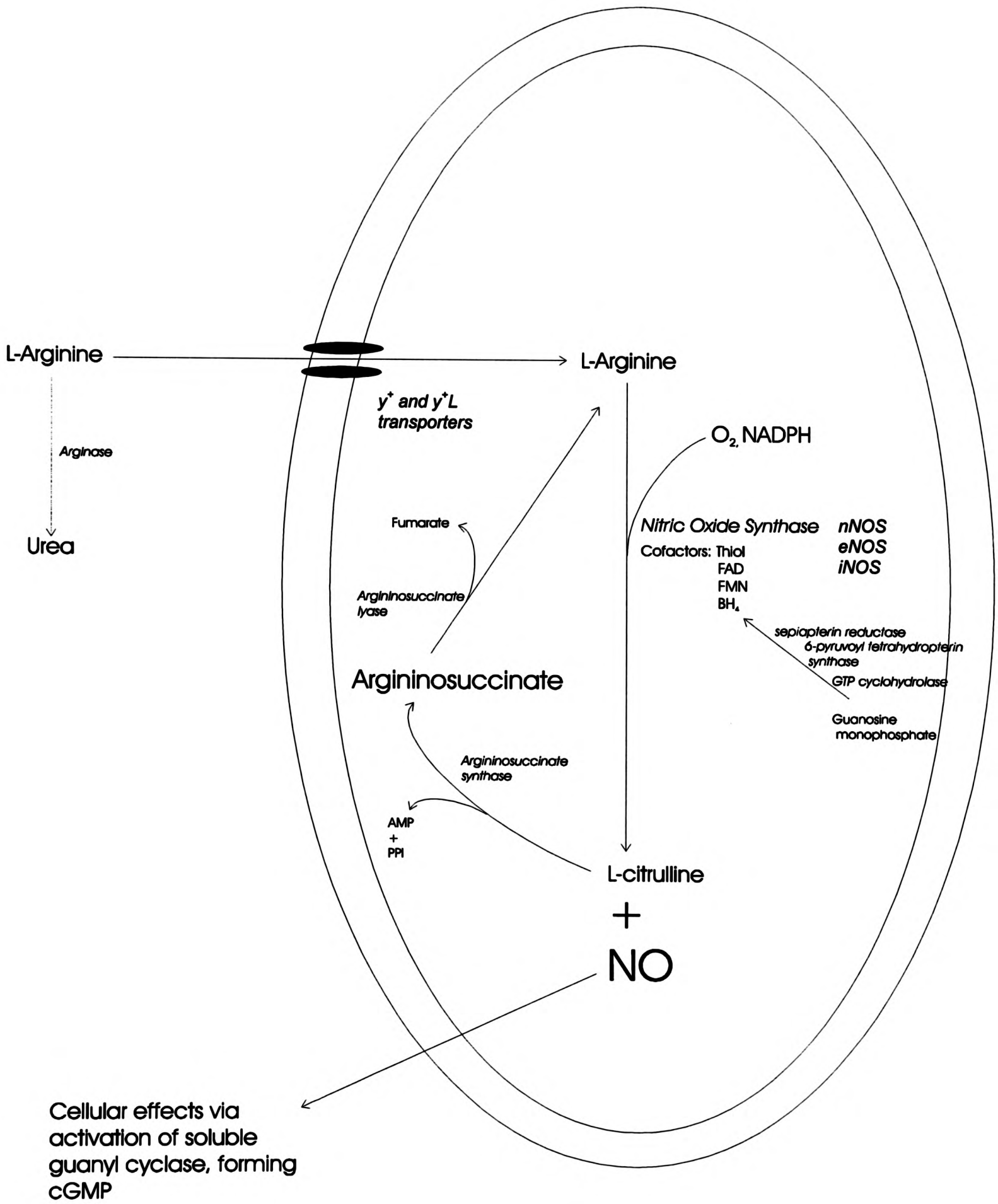


Figure 1.1a
The nitric oxide biosynthetic pathway

The current treatment of septic shock involves using sympathomimetic agents to restore vascular tone and cardiac output. As noted above, vessels become progressively resistant to such vasoconstrictors in sepsis, and the increasing doses required begin to cause their own deleterious effects: increased myocardial oxygen consumption; a shift from anabolic to catabolic metabolism; coagulopahy; acute renal failure; microvascular ischaemia; and a further upregulation of the inflammatory response. A better strategy might be to target the biological link between infection and vasodilation. The evidence presented above suggests nitric oxide may be that link.

The only known potent inhibitors of the nitric oxide biosynthetic pathway act non-specifically on all the nitric oxide synthase enzymes. There are numerous case reports showing such drugs (for example, N_ω-Nitro-L-arginine methyl ester (L-NAME) and N_ω-Methyl-L-arginine acetate (L-NMMA)) can restore blood pressure to normal. These undoubtedly saved some patients' lives where conventional treatments had failed (Petros *et al.* 1991) (Schilling *et al.* 1993). The only clinical trial of NOS inhibition was halted prematurely because of increased mortality in the treatment group. Presumably a small amount of nitric oxide is required to prevent vascular tone from increasing above a critical level which precipitates microvascular ischaemia.

It would appear, then, what is needed is a drug which inhibits only the production of extra nitric oxide precipitated by sepsis, while leaving the basal production intact.

Reducing the upregulated component of nitric oxide production in sepsis

Potential strategies to selectively reduce the upregulated activity of the nitric oxide synthetic pathway include directly inhibiting the activity of iNOS, reducing the amount of an iNOS cofactor, preventing overall inflammation-stimulated protein synthesis, inhibiting the 'recycling' of intracellular citrulline to arginine, or reducing arginine transport. Each of these possibilities will be dealt with in turn.

Reducing the upregulated component of nitric oxide production in sepsis:

a. Selective inhibition of iNOS activity?

Selective inhibitors of iNOS have been developed, and tested with some success in animal models. For example, L-canavanine dramatically reduced the mortality of septic rats, whereas L-NAME killed them (Liaudet *et al.* 1998). Whether this approach will work in humans is unknown. Rats produce more NO in sepsis than humans (table 1.1a), so the therapeutic index of a nitric oxide inhibitor is likely to be larger in rats. If a relatively larger dose of inhibitor is needed in humans, the iNOS selectivity may be reduced. There is even controversy over whether iNOS is important in human sepsis (Bhagat *et al.* 1996) (Bhagat *et al.* 1999). iNOS is constitutively expressed in some human cells (Buchwalow *et al.* 2002) and may well serve just as indispensable a function as eNOS. Notwithstanding these concerns, the bulk of pharmaceutical research aimed at modifying nitric oxide production in sepsis is directed at iNOS.

Reducing the upregulated component of nitric oxide production in sepsis:

b. Reduction of the availability of BH_4 co-factor?

BH_4 is an essential co-factor for all of the NOS enzymes. Exogenous BH_4 directly increased the activity of NOS in human umbilical vein endothelial cells (as measured by rate of conversion of arginine to citrulline) (Rosenkranz-Weiss *et al.* 1994). Interestingly this effect was greater in control cells than those stimulated with cytokines, suggesting activation had already increased BH_4 levels in these cells. The authors went on to confirm this explanation. An increase in the mRNA for the enzyme which makes BH_4, GTP cyclohydrolase, was the only abnormality which could explain the hyporeactivity of human veins produced by cytokines infused in vivo (Bhagat *et al.* 1999). The effect is not limited to blood vessels: cultured rat ventricular myocytes stimulated with cytokines to produce more nitric oxide simultaneously increased their GTP cyclohydrolase mRNA (Simmons *et al.* 1996). Reducing BH_4 might not abolish all NO production: basal NO production by mouse ileum was normal in a BH_4 deficient mouse, but the increase in NO production and cGMP activation in response to endotixin was markedly attenuated in tissue from these animals (Rolfe *et al.* 1997).

It would seem that limiting the production of BH_4 would be an ideal target in the quest to reduce NO production. However, BH_4 is a ubiquitous cofactor in many biochemical pathways, particularly the synthesis of neurotransmitters. There is nothing which distinguishes the enzyme making BH_4 in vascular cells from that in other cells, so it would be difficult to block this selectively. There is a strong

suspicion that reducing the overall production of BH_4 will have adverse effects in the organism as a whole (Werner-Felmayer *et al.* 1992).

Reducing the upregulated component of nitric oxide production in sepsis:

c. ***Non specific inhibition of protein synthesis?***

Some studies show that reducing the synthesis of cyclo-oxygenase products (using dexamethasone or non-steroidal cyclo-oxygenase inhibitors) before septic changes occur is protective in animal models of sepsis, possibly by preventing upregulated arginine transport and nitric oxide production (Clark 1998). This suggests that these cytokines are amongst the first steps on the pathway which eventually leads to increased production of nitric oxide. However, once the cyclooxygenase products are generated, the subsequent events can no longer be stopped by then inhibiting the cyclooxygenase pathway (Clark 1998). Recommending that the population take cyclooxygenase inhibitors or steroids in case they become septic is obviously impractical.

Reducing the upregulated component of nitric oxide production in sepsis:

d. ***Reduction in the amount of 'recycled' intracellular arginine?***

In addition to membrane arginine transport, cells can supply substrate to NOS by recycling citrulline. This recycling pathway is upregulated in a number of models of sepsis. Macrophages from rats treated with LPS convert 3 times as much citrulline to arginine as do healthy controls (Wu & Brosnan 1992). A variety of tissue homogenates from similarly treated rats had more argininosuccinate synthetase and

argininosuccinate lyase mRNA (Nagasaki *et al.* 1996) (Tabuchi *et al.* 2000), though interestingly this did not seem to be reflected at the protein level (Tabuchi *et al.* 2000). Still, the evidence for upregulation of this pathway in experimental sepsis is overwhelming (as reviewed by Mori & Gotoh (2000)). This may represent a potential site of modulation of NO production. However, only about 20% of arginine derived citrulline can be recycled in either normal or LPS stimulated cells (Wu & Brosnan 1992), which suggests inhibition of this pathway is unlikely to therapeutically alter cellular nitric oxide production to the required extent.

Reducing the upregulated component of nitric oxide production in sepsis:

e. Limiting arginine transport?

The arginine concentration in both normal and septic cells is high enough to saturate both iNOS and eNOS enzymes. Despite this, the availability of arginine from the extracellular space is rate limiting to the production of NO. Supplemental arginine given to healthy volunteers reduced their blood pressure (Hishikawa *et al.* 1992), and when given to sheep reduced systemic vascular resistance and increased their mesenteric blood flow (Allman *et al.* 1996). L-lysine competes with arginine for membrane transport, and cells placed in solutions with high lysine concentrations make less nitric oxide, in spite of theoretically sufficient concentrations of intracellular arginine (Closs *et al.* 2000). This is termed the arginine paradox. A number of possible explanations have been proposed (reviewed by Kurz & Harrison (1997)). One suggestion rests on the recent observation that NOS enzymes (certainly eNOS) are not truly cytoplasmic, but in fact exist in membrane bound caveloae. They

take their arginine not from the cytoplasm, but rather directly from the extracellular space (McDonald *et al.* 1997). Thus it is the extracellular concentration of arginine which is important, and hence the transport of arginine across the cell membrane is critical. Sequestration of arginine in functional or structural intracellular pools, available to constitutive NOS but not to iNOS, has also been suggested (Closs *et al.* 2000). In any case, reduction of plasma membrane arginine transport could be reasonably expected to substantially reduce nitric oxide production.

An experiment of nature has provided the best evidence to date that limiting membrane arginine transport reduces nitric oxide production to a functionally significant extent. Patients with lysinuric protein intolerance lack one of the main membrane arginine transport proteins (y^+LAT1; see below). Such patients have a reduced systemic nitric oxide production (in health; no such patient has been studied when septic). They were also found to have reduced endothelium dependent vasodilation of the brachial artery (Kamada *et al.* 2001). The authors of the study concluded this was due to reduced gastrointestinal arginine absorption; however, the defect must also have been present at the level of cells in the vasculature (Reade *et al.* 2001), implying the effects seen were due to reduced cell membrane arginine transport.

There is abundant evidence that arginine transport is increased in cells stimulated to produce nitric oxide, as summarised in table 1.1d. Furthermore, inhibition of arginine transport does decrease nitric oxide production in these cells. Nitric oxide production by rat cardiac myocytes stimulated with IFNγ and IL-1 decreased by 70% in the presence of a high concentration of lysine, a competitive inhibitor of arginine

transport (Simmons *et al.* 1996). Similar results were found using activated murine macrophages, whose nitric oxide production was decreased by another competitive inhibitor of arginine transport, ornithine (Kiemer & Vollmar 2001). The same was not true in an in vivo model of human sepsis (Allman *et al.* 1998), but this may have been because the experiment was concluded prematurely, prior to the upregulation of arginine transport (Clark 1998).

Plasma membrane arginine transport

Arginine transporters were originally classified according to their kinetic properties – for example, their interactions with other types of amino acids and the dependence of their function on the presence of sodium and on membrane potential. A number of proteins with similar kinetic functions have subsequently been identified. The properties of the known plasma membrane arginine transporters are summarised in table 1.1e.

Previous work in our laboratory has firmly established that in healthy peripheral blood mononuclear cells, the majority of arginine uptake is through the y^+L transporter (Clark 1998). The other transporter known to exist in functionally significant amounts in PBMCs is the y^+ system; however, this represents a small fraction of the total arginine transport in PBMCs from healthy subjects. In contrast, arginine transport in vascular smooth muscle is thought to have a much more substantial y^+ component, at least in the rat cells studied to date (Hattori *et al.* 1999) (Durante *et al.* 1995). Changes in plasma arginine transport in various models of sepsis have been

extensively studied in animal models. Most studies of the molecular basis of arginine transport in models of sepsis have found an increase in y^+ function and CAT2B mRNA. There is however some variability depending on tissue, species and stimulus (table 1.1d). For example, cytokine activated rat aortic smooth muscle cells had arginine transporters kinetically more similar to CAT1 (Durante *et al.* 1995); in the same cells CAT1 mRNA underwent the more long lasting change when stimulated with angiotensin II (Low & Grigor 1995); and it was CAT2A rather than CAT2B mRNA which was upregulated in skeletal muscle after surgical trauma (Low & Grigor 1995). To date there have been no reports on the changes in arginine transporter expression or function in any human cells from patients with clinical septic shock.

The potential therapeutic implications for septic shock, if indeed CAT2B is primarily responsible for the required increase in arginine delivery to iNOS in human sepsis, were highlighted by a recent study of CAT2B knockout mice. Macrophages from these mice, when activated using a mixture of cytokines, produced 92% less nitric oxide and had a 95% reduction in arginine uptake compared to marcophages from similarly stimulated wild-type mice. No adverse consequences of CAT2B deletion were noted in the animals (Nicholson *et al.* 2001).

Vasodilation by mechanisms other than nitric oxide

Notwithstanding occasional negative studies, the above paragraphs have summarised the convincing evidence for the upregulation of nitric oxide in septic shock. Certainly

nitric oxide is a powerful vasodilator, but is it the main factor responsible for hypotension in sepsis? Some studies suggest this might not be the case.

Thoracic aortae from rats made septic by caecal ligation and puncture were hyporesponsive to noradrenaline, but this was not reversed by inhibitors of either iNOS or all NOS enzymes (Vromen *et al.* 1996). iNOS inhibition did not affect the mortality of these animals, in contrast to an LPS mouse model, where selective iNOS inhibition reduced mortality (Liaudet *et al.* 1998). Perhaps clinical sepsis is fundamentally different to experimental infusion of LPS.

Conclusions drawn from experiments on iNOS knockout mice are conflicting. iNOS knockout mice have no increase in their plasma nitriate/nitrite levels when LPS is injected intraperitoneally, but have no survival advantage over wild-type mice (Laubach *et al.* 1995). Similar mice challenged with live bacteria and LPS also had no eventual survival advantage (MacMicking *et al.* 1995). In contrast, iNOS knockout mice with bacterial sepsis (after caecal ligation and puncture) treated with antibiotics and fluids did fare better than wild-type mice (Hollenberg *et al.* 2000). Perhaps there are both NO-dependent and NO-independent mechanisms of vasodilation in sepsis. Which is more important may depend on the species and the nature of the septic insult.

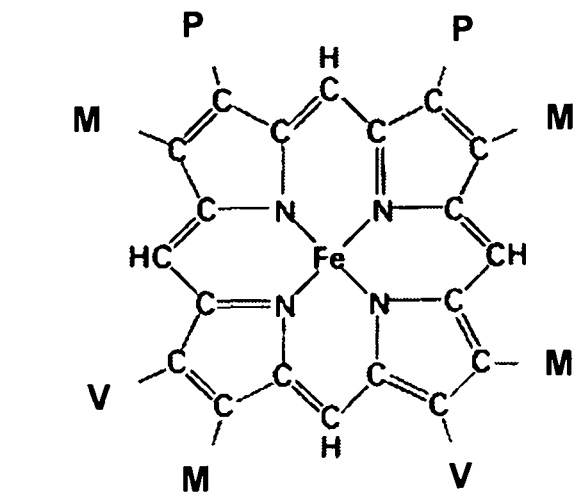
Carbon monoxide as a link between sepsis and vasodilation in septic shock

A possible alternative mediator linking sepsis to shock is carbon monoxide. Carbon monoxide is a vasodilator which activates guanylyl cyclase in a manner similar to nitric oxide, albeit with 100 times less potency (Snyder & Baranano 2001). It also opens calcium sensitive potassium channels (Kaide *et al.* 2001) (Zhang *et al.* 2001a), and decreases synthesis of vasoconstrictors such as endothelin and 20-HETE (Zhang *et al.* 2001b). Constitutive production of carbon monoxide has a role in the physiological control of vascular tone in rat small arteries and arterioles (Kozma *et al.* 1999).

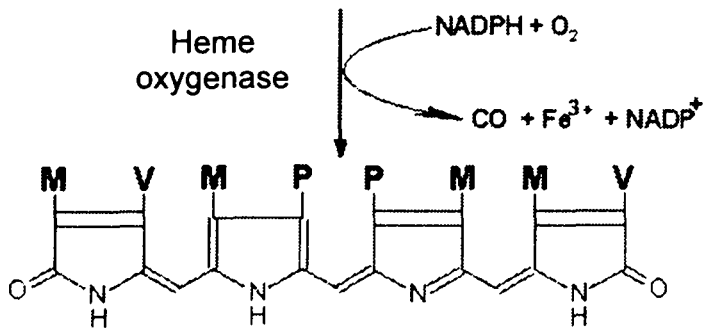
Carbon monoxide is formed by heme oxygenase as part of the catabolic pathway for heme (figure 1.1b). There are three known forms of heme oxygenase: constitutive HO-2 and 3, and inducible HO-1. The amount and activity of HO-1 are increased in conditions causing oxidative stress such as clinical sepsis or LPS exposure (Yet *et al.* 1997) (Otani *et al.* 2000) (Camhi *et al.* 1995) (Suzuki *et al.* 2000) (Tsukiji *et al.* 2000) (Pellacani *et al.* 1998). The exhaled breath of critically ill patients contains increased concentrations of carbon monoxide (Scharte *et al.* 2000), and their plasma carboxyhaemoglobin (Moncure *et al.* 1999) and carbon monoxide (Shi *et al.* 2000) concentrations are also increased.

HO-1 expression in sepsis is thought to have evolved as a protective response, as another product of the reaction, bilirubin, is an antioxidant which can prevent tissue damage. In producing this helpful antioxidant, however, it may be that the negative effects of vasodilation come to dominate, leading to a net detrimental response when

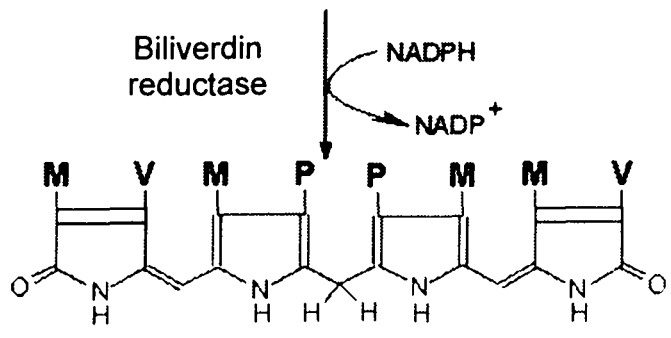
heme oxygenase activity is very high. Descending aortae of rats in which HO-1 had been induced were hyporesponsive to phenylephrine; this hyporesponsiveness was reversed with the heme oxygenase inhibitor tin protoporphrin IX (Gaine *et al.* 1999). Similar conclusions were drawn from an in vivo study: inhibition of HO-1 with zinc protoporphyrin IX restored arterial pressure in rats given LPS (Yet *et al.* 1997). At least some HO activity appears essential, however, as the mortality of rats made septic by caecal ligation and puncture (which caused increased HO-1 mRNA expression) was greater if treated with a heme oxygenase inhibitor (Downard *et al.* 1997). It may be that a small amount of HO activity is helpful, but a large amount detrimental. This is obviously a comparable situation to that of nitric oxide in sepsis.



Heme



Biliverdin



Bilirubin

Figure 1.1b
The heme degradation pathway
M = methyl; -CH_3
V = vinyl; -$CH{=}CH_2$
P = propionic acid; -CH_2-CH_2-COO^-

In spite of the obvious possibility that overproduction of carbon monoxide contributes to the hypotension of human septic shock, essentially nothing is currently known about HO-1 in human tissue, either in vitro or in clinical sepsis.

Rat

In vitro activation

(Chou *et al.* 1998) Rat alveolar macrophages in primary culture with LPS and IFNγ for 24 h displayed significant increases in nitric oxide synthase activity measured by nitrite production and rate of arginine to citrulline conversion.

(Hammermann *et al.* 2000) Rat alveloar macrophages in primary culture with LPS and IFNγ showed a concentration- and time-dependent increase in NO synthesis (measured by nitrite accumulation) and mRNA expression for iNOS. This required the gene promoter NF_KB.

(Wu & Brosnan 1992) Rat peritoneal macrophages in primary culture with LPS had 3x the NOS activity (as measured by rate of arginine to citrulline conversion) of controls. could make arginine from citrulline.

(Chesrown *et al.* 1994) Rat peritoneal macrophages in primary culture with LPS have increased iNOS mRNA.

In vivo activation

(Kobzik *et al.* 1993) Macrophages in sections of lung from rats given LPS in vivo showed strong induction of iNOS, while eNOS expression was low and constant regardless of LPS treatment.

Mouse

In vitro activation

(Caivano 1998) RAW 264.7 macrophages with LPS + IFNγ show increased iNOS protein amount and nitrite production.

(Iyengar *et al.* 1987) RAW 264.7 macrophages activated with LPS and IFNγ synthesised increased amounts of nitrite and nitrate.

(Mori *et al.* 1998) iNOS mRNA and protein were increased when RAW264.7 cells where exposed to LPS.

(Kojima *et al.* 1998) Unstimulated RAW264.7 macrophages produce detectable amounts of nitric oxide (using the NO sensitive dye DAF), but stimulation with LPS and IFNγ increases this NO production over 3x.

(Nakatsubo *et al.* 1998) RAW264.7 cells show a LPS-dose dependent increase in nitric oxide production (measured by DAF fluorescence), which is reduced by L-NMMA in a dose dependent manner.

(Bianchi *et al.* 1995) RAW 264.7 macrophages activated with LPS and IFNγ show increased nitrite production.

(Bogle *et al.* 1992) J774 macrophages cultured with LPS and IFNγ show increased nitrite production.

(Chesrown *et al.* 1994) Both RAW264.7 and J774 macrophages cultured with LPS show increased iNOS mRNA.

(Closs *et al.* 2000) J774 macrophages incubated with LPS showed increased iNOS mRNA.

(Colton *et al.* 2001) NO production is increased in RAW264.7 cells activated with LPS or polyinosinic:polycytidylic acid.

(Kakuda *et al.* 1999) J774 macrophages stimulated with IFNγ and LPS have a 25x increase in iNOS mRNA and increased NO production.

(Shibazaki *et al.* 1996) Rat peritoneal macrophages in primary culture with LPS produced more nitrite than control cells.

Human

Clinical disease

(Kobzik *et al.* 1993) Human alveolar macrophages in areas of chronic inflammation contained iNOS protein identified by immunocytochemistry.

(Nicholson *et al.* 1996) Human alveolar macrophages in the bronchoalveolar lavage fluid of patients with active tuberculosis express increased numbers of iNOS positive macrophages.

Table 1.1b

Evidence of increased nitric oxide production by tissue macrophages to a wide variety of stimuli

Rat

In vitro activation

(Baydoun *et al.* 1999)	Rat aortic smooth muscle cells (RASMCs) in primary culture stimulated with LPS and IFNγ had increased iNOS protein expression and increased nitrite production. This effect relied on stimulation of the p38 mitogen activated protein kinase system.
(Durante *et al.* 1995)	RASMCs in primary culture with a mixture of proinflammatory cytokines demonstrate increased nitrite production.
(Gill *et al.* 1996)	RASMCs in primary culture with IL-1, TNF or a combination of the two increase nitrite production and iNOS mRNA.
(Hattori *et al.* 1999)	RASMCs in primary culture treated with LPS and IFNγ produced more nitrite and newly expressed iNOS mRNA.
(Kojima *et al.* 1998b)	RASMCs activated in an unspecified manner produce increased nitric oxide, as measured by DAF fluorescence.
(Kojima *et al.* 1998a)	RASMCs in primary culture activated with LPS and a mixture of proinflammatory cytokines produce more nitric oxide (as measured by DAF fluorescence) than unstimulated cells.
(Schwarz *et al.* 1999)	The medial layer of healthy rat aorta contained nNOS mRNA and protein. iNOS protein was induced in the aortic wall of these animals 'when exposed to lipopolysaccharide', but whether this was an in vitro or in vivo exposure, and where in the aortic wall the protein was found, is surprisingly not stated. eNOS protein was present in the endothelium but not the media or adventitia. Endothelium denuded aortic rings contracted more strongly in response to KCl when the nitric oxide synthase inhibitor L-NNA was added, demonstrating the media (which it would seem contained only nNOS) was producing functionally significant amounts of nitric oxide.
(Wileman *et al.* 1995)	RASMCs in primary culture activated using LPS with and without a mixture of proinflammatory cytokines. LPS alone increases nitrite production. Each of the cytokines by itself had no effect; however IFNγ in combination with TNFα, or TNFα + IL-1, had a substantially greater effect on nitrite production than LPS.

Endotoxin infusion in vivo

(Julou-Schaeffer *et al.* 1990)	Rats given intraperitoneal LPS 4 hours prior to removal of the thoracic aorta. Vessels were studied with and without endothelium. LPS treatment reduced the responsiveness of aortic rings to noradrenaline, redardless of whether endothelium was present. L-NMMA restored contractility to normal, and this effect was overcome with supplemental arginine, again irrespective of whether the endothelium was present or not. The only explanation for this result is that the arterial smooth muscle was making its own nitric oxide. Furthermore, in tissue with intact endothelium, relaxation induced by ACh was significantly less in LPS treated animals than in controls, suggesting that the endothelial NO production had been downregulated. ACh had no effect on endothelium denuded rings. Additionally, in aortic rings from control animals, L-NMMA increased the sensitivity to noradrenaline when the endothelium remained intact, but not when the endothelium had been removed, implying that in non-septic animals the arterial smooth muscle does not make nitric oxide.
(Biguad *et al.* 1990)	Rats given intraperitoneal LPS 4 hours prior to removal of the thoracic aorta. Very similar results to the above study (hyporesponsiveness of the media alone after LPS treatment) but to a variety of other contractile stimuli.
(Wakabayashi *et al.* 1987)	The thoracic aortae of rats given intraperitoneal LPS show decreased response to a variety of contractile agonists, and this effect persists if the endothelium is removed.

Clinical sepsis

(McKenna *et al.* 1986)	Thoracic aortae from rats made septic by caecal ligation and puncture were hyporesponsive to noradrenaline, and removal of the endothelium only partially restored the sensitivity to normal, suggesting the media was also making its own vasodilator.

Table 1.1c

Evidence of increased nitric oxide production by vascular smooth muscle (intact tissue or in culture) in response to a wide variety of stimuli

(Chen *et al.* 1994)	Mesenteric arterial rings from rats made septic by caecal ligation and puncture were studied with and without endothelium present: Septic rats had a greater relaxation to ACh than did control rats, in direct contrast to the findings of Julou-Schaeffer *et al.* (1990), whose study was of thoracic aorta. The effect observed in these mesenteric rings was due to increased endothelial NO production, as it was reversed by L-NNA. Removal of the endothelium significantly enhanced the contractile response to phenylephrine in both endothelium present and absent rings, again confirming that a vasodilator was being produced by the endothelium in both conditions. Moreover, the hyporesponsiveness of sepsis was present in vessels in which the endothelium had been removed, implying that the vascular smooth muscle as well as the endothelium was making nitric oxide.
(Vromen *et al.* 1996)	Thoracic aortae from rats made septic by caecal ligation and puncture have a markedly increased iNOS activity (measured as the calcium independent rate of conversion of arginine to citrulline), and a decrease in the contractile response to noradrenaline. However, surprisingly and in contrast to the results of Chen *et al.* (1994) and all the LPS models described above, treatment with a selective iNOS inhibitor, or a non-selective NOS inhibitor, failed to restore this contractility to normal. This suggests that although iNOS was present in these vessels, nitric oxide was not necessary for their hyporesponsiveness. There was no attempt to separate the response of vascular smooth muscle and endothelium in this study.
(Yaghi *et al.* 1995)	Pulmonary artery and aorta from rats made septic by caecal ligation and puncture were compared to those from healthy rats and rats which had undergone a sham operation: Pulmonary artery displayed decreased contractility after bowel puncture and sham operation, which was reversed by L-NAME. L-NAME had no effect on control contractility, suggesting the hyporesponsiveness observed was due to NO. Aorta displayed a trend to, but no significant decrease in, contractility after bowel puncture or laparotomy. L-NAME had little effect on this trend to reduced contractility. There was no attempt to separate the response of vascular smooth muscle and endothelium in this study. The effect of clinical sepsis is thus different in different vascular beds (see text).
Bovine	
In vitro activation	
(Brophy *et al.* 2000)	Stimulation in vitro with serotonin of bovine carotid ASM stripped of endothelium increases nNOS protein expression, NOS activity (assayed by rate of arginine to citrulline conversion, and confirmed to be due to NOS 1 using an isoform specific inhibitor) and cGMP activity. Immunohistochemistry of intact bovine carotid artery showed eNOS protein confined to the endothelium, no iNOS, and nNOS in the media.
Healthy	
(Zehetgruber, 1993 #358)	Bovine pulmonary arteries stripped of endothelium generated nitric oxide. The amount produced was not sufficient to relax detector vessels (stripped of endothelium), questioning whether this is a functionally significant amount.
Human	
In vitro activation	
(MacNaul & Hutchinson 1993)	Human aortic smooth muscle cells stimulated in primary culture with LPS and a combination of cytokines express iNOS mRNA. eNOS mRNA is never expressed.

Table 1.1c (continued)

Evidence of increased nitric oxide production by vascular smooth muscle (intact tissue or in culture) in response to a wide variety of stimuli

(Chester *et al.* 1998)	Internal mammary artery obtained at surgery and cultured for 48 hours with LPS and a mixture of cytokines newly expressed iNOS mRNA (though whether this was in the endothelium or smooth muscle was not determined). Immunocytochemistry showed induction of iNOS protein principally in the media, but also in some endothelial cells. There was an increase in the calcium independent rate of arginine to citrulline conversion, and in the amount of nitrite in the culture medium.
Endotoxin infusion in vivo	
(Bhagat *et al.* 1999)	In a unique experiment, IL-1, IL-1+TNF, or IL-1,+TNF+ IL-6 were infused into segments of vein in vivo, but isolated from the remainder of the circulation. On ex vivo analysis, the response to noradrenaline of these vessels was decreased. L-NMMA reversed this hyporesponsiveness while having no effect on healthy control vein, demonstrating the effect was due to the upregulation of nitric oxide. However, surprisingly, there was no iNOS or nNOS mRNA detectable in the vessel wall (which was analysed as endothelium + vascular smooth muscle together). eNOS was present but unchanged by activation. The hyporesponsivenes was explained on the basis of an increase in mRNA for BH_4, the cofactor for eNOS activity.
(Bhagat *et al.* 1996)	A very similar experiment to that described above was performed, using LPS rather than cytokines to activate the isolated vessel. The ex vivo contractility to noradrenaline was similarly reduced after activation, but in contrast this was not reversed by L-NMMA, suggesting LPS (as opposed to cytokines) is causing vascular hyporesponsiveness by a NO independent mechanism.
Clinical sepsis	
(Stoclet *et al.* 1999)	Omental arteries from patients with sepsis have 1. Increased iNOS protein in the intima, media and possibly adventita (though note the numbers in this study were very small). 2. Increased NO production (by the whole vessel wall; whether this was derived from the intima or media was not studied). 3. Reduced contractile response to higher concentrations of noradrenaline, which was reversed by L-NAME. 4. Endothelium dependent vasodilation (to bradykinin) was no different in the septic vessels, suggesting the endothelium was capable of producing the same amount of NO as in health (in contrast to some of the animal studies quoted in the text).
(Tsuneyoshi *et al.* 1996)	Mesenteric artery from 3 septic patients (compared to 4 healthy controls) develops reduced tension in response to noradrenaline, which was normalised by methylene blue or L-NAME. Methylene blue had only a minimal effect on control vessels.
Health	
(Buchwalow *et al.* 2002)	Human vascular smooth muscle cells from a variety of locations express all 3 isoforms of nitric oxide synthase.
(Bernhardt *et al.* 1991)	Human vascular smooth muscle cells in culture release detectable quantities of nitric oxide. The incubation medium from this cell culture causes relaxation of isolated rat mesenteric arteries.

Table 1.1c (continued)

Evidence of increased nitric oxide production by vascular smooth muscle (intact tissue or in culture) in response to a wide variety of stimuli

Rat

In vitro activation

Macrophages

(Chou *et al.* 1998)	Rat alveolar macrophages in primary culture with LPS and IFNγ had increased rates of membrane L-arginine transport, in parallel with increased nitrite production and calcium insensitive NOS activity.
(Hammermann *et al.* 2000)	Rat alveloar macrophages in primary culture with LPS or IFNγ (or both) show a time dependent increase in arginine transport (not kinetically identified) and an increased in the expression of CAT2B mRNA. A smaller quantity of CAT2B was present in healthy cells. CAT1 mRNA was present but unaffected by stimulation. iNOS mRNA and nitrite accumulation paralleled the increase in CAT2B and arginine transport; all of these effects were dependent on activation of the $NF\text{-}_KB$ pathway.
(Stevens *et al.* 1996)	Primary culture of rat brain astrocytes (the macrophage-like cell in the central nervous system) with LPS and IFNγ found increased arginine transport and increased CAT2B mRNA, which paralleled the increases in iNOS mRNA and nitrite production. An attempt was made to distinguish kinetically the type of arginine transporter present; however, the authors misinterpret their results due to misunderstanding the kinetic properties of y^+ and y^+L. The primary data presented suggest approximately half the arginine transport into unstimulated astrocytes is by y^+, and half by y^+L. The text states that this relationship is unchanged with activation; however the primary data are not presented and it is possible that a shift to y^+ transport could have been ignored. The upregulated nitrite production was completely dependent on the supply of arginine through membrane cationic amino acid transporters.

Vascular smooth muscle cells

(Baydoun *et al.* 1999)	Rat aortic smooth muscle cells (RASMCs) in primary culture, stimulated with LPS and IFNγ, have a greater rate of arginine transport (and nitrite production), and increased mRNA for proteins with y^+ activity: CAT1 increased 3x, CAT2A increased 6x, and CAT2B increased 7x. All of these proteins were, however, also expressed in unstimulated cells. No attempt was made to investigate the y^+L system.
(Durante *et al.* 1995)	RASMCs in primary culture activated with a variety of cytokines upregulated both nitrite production and arginine transport, though to a lesser degree than in studies of macrophages reported previously. Nitrite production was dependent on membrane arginine transport, as it was reduced to control levels by extracellular lysine. The arginine transport had the classically described properties of y^+ in that it was insensitive to neutral amino acids. It was also partially trans-stimulated: a property of CAT1 rather than CAT2A or B. Further kinetic analysis demonstrated the presence of a high and low affinity transporter: probably CAT1 and CAT2B. How the ratio of CAT1 to CAT2B changed with activation was not reported.
(Gill *et al.* 1996)	RASMCs in primary culture with IL-1, TNFα or a combination of the two display increase nitrite production and arginine transport 'via y^+', though in fact the study does not adequately distinguish between y^+ and y^+L. There was a simultaneous increase in CAT2 and iNOS mRNA. In contrast LPS and IFNγ increase nitrite production, but cause no change in arginine transport. This is one of the only demonstrations that arginine transport and nitric oxide production, while regulated in parallel, are not controlled by the same cellular mechanism. In an interesting aside, angiotensin II added to the cells increased their CAT1 mRNA but not CAT2B, indicating the upregulation of CAT2B was relatively specific to cytokine stimulation.

Table 1.1d

Evidence of increased rate of arginine transport, and increased arginine transporter mRNA and protein, in a variety of tissue types and species in response to a wide variety of stimuli

(Hattori *et al.* 1999) RASMCs in primary culture with and without LPS and IFNγ: mRNA for CAT-1 and CAT-2B are constitutively expressed in VSMC, and the expression of both mRNAs was rapidly stimulated by treatment with LPS-IFNγ. CAT1 mRNA expression peaked within 2 hours, and decayed to below basal levels within 8 hours after LPS-IFNγ exposure. CAT2B mRNA expression peaked at 4 hours, and declined over the next 24 hours. CAT-2A mRNA was not detectable in unstimulated or stimulated VSMC. Arginine transporter activity significantly increased 4-10 hours after LPS-IFNγ treatment, and intracellular arginine concentrations of arginine were significantly elevated at 12 and 24 hours. Nitrite production was reduced when the uptake of extracellular arginine was blocked.

(Low & Grigor 1995) RASMCs in primary culture, stimulated with angiotensin II, have increased y^+ activity and increased CAT1 and CAT2B mRNA. The CAT1 mRNA remained elevated throughout the time course of the study, whereas that of CAT2B soon fell, only to rise again after 16 hours of culture.

(Wileman *et al.* 1995) RASMCs in primary culture with LPS ± TNFα and IFNγ.have increased arginine transport, with the LPS and cytokines acting in a concentration dependent synergistic manner. Transport had the kinetic characteristics of y^+, and the balance of y^+ to y^+L was not altered by activation.

Other cell types, or cell type not specified

(Simmons *et al.* 1996) Rat ventricular myocytes in primary culture, when stimulated with IL-1 and IFNγ, approximately double their rate of arginine uptake and newly express a lower affinity transporter with a K_m closer to that of CAT2A than CAT2B, in addition to the higher affinity transport system which was present alone in unstimulated cells (which kinetically resembled either CAT1 or CAT2B).
The production of nitrite was dramatically increased, but reduced approximately 70% from this value when arginine transport was inhibited with lysine. This was a membrane transport effect as it did not persist when the cells were lysed.
Stimulation increased the mRNA for CAT1 10x. CAT2A and CAT2B were newly expressed after 24 hours stimulation.

In vivo activation / clinical disease

Other cell types, or cell type not specified

(Pacitti *et al.* 1992) TNFα was injected intraperitoneally into rats, whose hepatic plasma membrane vesicles were subsequently analysed ex vivo. TNFα increased total arginine transport, and this increase was principally caused by an increase in y^+ activity.

(Tabuchi *et al.* 2000) When rats were injected intraperitoneally with LPS, rat liver homogenate rapidly induced CAT2 mRNA, but this fell 6 hours after injection, and was below control levels at 12 hours, returning to normal at 24 hours. CAT2A was not distinguished from CAT2B. iNOS mRNA was induced over a similar time course.

Table 1.1d (continued)

Evidence of increased rate of arginine transport, and increased arginine transporter mRNA and protein, in a variety of tissue types and species in response to a wide variety of stimuli

Mouse

In vitro activation

Macrophages

(Caivano 1998)	RAW 264 macrophages activated with LPS and IFNγ newly express CAT2B mRNA, while the expression of CAT1 remains unchanged. The total uptake of arginine is 2.5 times greater in activated cells (though the kinetics of the transporters present were not determined). Interestingly, blocking activation of the MAPK signal transduction pathway returned arginine transport to normal, but has no effect on the increase in CAT2B mRNA, iNOS protein, or nitric oxide production.
(Bianchi *et al.* 1995)	RAW 264 macrophages activated with LPS and IFNγ have a greater rate of arginine uptake.
(Bogle *et al.* 1992)	J774 macrophages activated with LPS ± IFNγ had increased arginine transport, along with increased nitrite production. Nitrite production was dependent on membrane arginine transport. The arginine transport in both activated and control cells had the kinetic properties of y^+, though the contribution of y^+L cannot be stated from the results presented.
(Closs *et al.* 2000)	J774 macrophages stimulated with LPS had a transient increase in CAT1 mRNA expression (2x control) and a persistent increase in CAT2B mRNA expression (4x control) up to 18 hours incubation. CAT2AmRNA was not induced, which was surprising as a low affinity system (with a K_m of 5700 uM) was identified on kinetic analysis. CAT2A is the only transporter with such a low affinity; thus this report suggests there is another, unidentified, arginine transporter present in the membranes of these cells.
(Colton *et al.* 2001)	RAW 264 macrophages treated with IFNγ and polinosinic : polycytidylic acid had increased arginine transport, increased CAT2 mRNA expression, but decreased CAT1 mRNA expression. CAT3 mRNA was not expressed at any stage.
(Kakuda *et al.* 1998)	J774 macrophages activated with LPS had reduced CAT1 mRNA and increased CAT2 and iNOS mRNAs. No CAT2A was detected. In the only published study to claim successful use of an anti-CAT2 antibody, this study also demonstrated an increase in CAT2 protein expression in these cells.
(Kakuda *et al.* 1999)	J774 macrophages stimulated with IFNγ then LPS had a 5x increase in CAT2 mRNA, decreased CAT 1 mRNA decreased, iNOS mRNA increased 25x. CAT3 mRNA was not detected. Stimulation increased arginine transport, with nearly all of the transport into activated cells mediated by y^+. Presumably using the same antibody as in the authors' previous paper, the protein for CAT2 was found to be increased In an elegant series of experiments, oocytes injected with total mRNA from LPS + IFNγ stimulated cells had increased their y^+ activity, which was returned to control levels by CAT2 antisense oligonucleotides. The antisense oligos also decreased NO synthesis by oocytes back to normal. CAT2 expression was thus necessary for the increase in NO production stimulated by LPS.
(Kiemer & Vollmar 2001).	Mouse bone marrow macrophages in primary culture had a dose dependent increase in arginine transport when stimulated with LPS. A substantial proportion of the activated transport was surprisingly inhibited by leucine, suggesting y^+L rather than y^+ was the dominant transporter in these activated cells.
(Nicholson *et al.* 2001)	Macrophages from CAT2 knockout mice, when activated with cytokines in vitro, had 92% less NO production and 95% less arginine uptake than macrophages from wild-type mice. The reduction in NO production was not due to differences in iNOS protein expression, iNOS activity, or intracellular l-arginine content.
(Shibazaki *et al.* 1996)	Mouse peritoneal macrophages in primary culture were treated with LPS. LPS at very low concentration increased membrane arginine transport, whereas much higher concentration of LPS was required for the induction of nitrite production. Rate of arginine transport increased rapidly over the first 24 hours of culture with LPS, but then declined; in the case of the lower LPS concentration, returning to control values by 72 hours. LPS treatment also increased intracellular arginine concentration. Lysine reduced intracellular arginine concentration, and inhibited the nitrite production.

Table 1.1d (continued)

Evidence of increased rate of arginine transport, and increased arginine transporter mRNA and protein, in a variety of tissue types and species in response to a wide variety of stimuli

Other cell types / cell type not specified

(Kakuda *et al.* 1998) Mice underwent the stress of hepatectomy, splenectomy and fasting. mRNA levels for CAT1, CAT2A and CAT2B were then assessed in liver, skeletal muscle, and uterine smooth muscle. There was no change in CAT2A mRNA in the liver. CAT2A (but surprisingly not CAT2B) mRNA was increased in skeletal muscle, while CAT1 levels are low and unaltered by stimulation. The gene promoter which upregulated CAT2A expression was the same as that which activates CAT2B in other cell models, suggesting which protein is expressed is not controlled at a transcriptional level. CAT2A may be kinetically preferable if attempting to preserve muscle amino acid stores in the face of generalised catabolism. In uterine smooth muscle, there was no change in CAT1 or CAT2 mRNA.

Human

In vitro activation

(Irie *et al.* 1997) Human umbilical vein endothelial cells (HUVEC) in primary culture treated with TNFα increase their CAT2 mRNA, while CAT1 mRNA remains unchanged. CAT2 was essentially undetectable in normal HUVECs.

(Boyd & Crawford 1992) Human T cells activated in vitro with phytohaemaglutinin (PHA) show increased y^+ transport (5.1x), whereas B stimulated with the B cell mitogen Staphylococcus aureus cowan A have only a minimal increase (1.3x).

(Chen *et al.* 1996) Human PBMCs stimulated with PHA have increased y^+ activity. Confusingly, antisense oligonucleotides to 4F2hc (now termed CD98, the heavy chain of y^+L) inhibits this.

(Crawford *et al.* 1994) Human T cell y^+ transport is increased after activation with PHA. This is most marked in the $CD8^+$ $CD45RA^+$ sub-population.

In vivo activation / disease

(Brunini *et al.* 2002a) PBMCs from patients with either chronic renal or heart failure have increased arginine transport through y^+, while y^+L transport remains unchanged.

(Mendes Ribeiro *et al.* 1999) Platelets from healthy subjects transport virtually all arginine through y^+L. In end stage renal failure, total transport, and transport through y^+L, is markedly increased. There is no evidence for any significant contribution by y^+.

(Brunini *et al.* 2002b) NOS activity (measured by rate of conversion of arginine to citrulline) is increased in uraemic platelets, and this can be significantly inhibited by competitively blocking y^+L transport with leucine.

Table 1.1d (continued)

Evidence of increased rate of arginine transport, and increased arginine transporter mRNA and protein, in a variety of tissue types and species in response to a wide variety of stimuli

Kinetic classification	Properties
y^+	*Cationic amino acid transporter* Compared to y^+L, has a lower affinity but higher capacity for cationic amino acids Na^+ independent when transporting cationic amino acids. Low affinity transport of small neutral amino acids, with Na^+ dependence. No transport of large neutral amino acids. Electrogenic: when a positively charged amino acid is transported, there is no other movement of a charged particle to balance this current. Selectively inhibited by N-Ethylmaleimide Several proteins are known to have y^+ activity: **CAT1** Ubiquitous expression except in hepatocytes Higher affinity for L-arginine, but lower capacity than other CATs Highly dependent on substrate concentration on the trans side of the membrane (trans-stimulated) pH independent K_m (arginine) 70-250 uM **CAT2A** Constitutively expressed in hepatocytes; also expressed in other cells Lowest affinity for L-arginine, but highest capacity, of all the CATs. Optimised for uptake of CAA at the high plasma concentrations in gut derived blood. Largely independent of the concentration on the trans side of the membrane pH dependent in the range 5.5-7.5, distinguishing it from CAT-1 K_m (arginine) 2150-5200 uM **CAT2B** Induced in T cells, macrophages, and other cells, perhaps to provide a substrate for induced iNOS. Lower affinity for L-arginine than CAT1, but higher than CAT2A. Higher capacity than CAT1 but lower than CAT2A. Less dependent than CAT1 on the concentration on the trans side of the membrane pH dependent in the range 5.5-7.5, distinguishing it from CAT-1. Differs by 42 amino acids to CAT2A. K_m (arginine) 190-250 uM **CAT3** Found in rats, but not in any human cell studied at the time these experiments were performed (see discussion). Rat CAT3 is apparently localised to brain, and has different K_ms for arginine and lysine – in contrast to all of the above. Kinetic characteristics are not well defined; Human K_m (arginine) 40-120 uM **CAT4** Nothing was known of the kinetic characteristics of this transporter at the time these experiments were performed; is was identified using sequence homology alone (though it is only 40% homologous with the other CAT proteins). It is now questioned whether CAT4 is in fact a functional membrane transport protein (see discussion). Found exclusively in human cells.
y^+L	*Cation-modulated broad scope transporter* High affinity, low capacity compared to y^+ Transports neutral and cationic amino acids with comparable affinities when Na^+ is present Affinity and capacity for neutral amino acids is markedly reduced in the absence of Na^+. Electroneutral: when a positively charged amino acid is transported, a Na^+ ion is transported in the reverse direction. Two proteins are known to have y^+L activity: **CD98 + y^+LAT1** (heavy + light chain) K_m (arginine) = 340uM (though reported elsewhere as 3uM; this may in fact have been CD98 + y^+LAT2) **CD98 + y^+LAT2** (heavy + light chain) K_m (arginine) = 6-10uM
$B^{0,+}$	*Na^+ dependent broad scope amino acid transporter* Transports both cationic and neutral amino acids, both of which are dependent on the concentration of Na^+. Cl^- stimulated (in contrast to all the others) Electroneutral Present in blastocysts and small intestine **ATB$^{0,+}$** is a protein with $B^{0,+}$ activity
$b^{0,+}$	*Na^+ independent broad scope amino acid transporter* Transports both cationic and neutral amino acids, independent of the concentration of Na^+ Electrogenic: exchanges cationic for neutral amino acids Present in blastocysts, renal brush border membrane, and small intestine One protein is known to have $b^{0,+}$ activity: **rBAT + $b^{0,+}$** (heavy + light chain)

Table 1.1e

Properties of the known cationic amino acid transporters (based on Deves & Boyd 1998, Closs et al. 1997, and Mann et al. 2002).

1.2
Aims

Noting the data showing inter-species differences, and differences in responses to various means of mimicking the septic condition, the experiments presented in this thesis have been exclusively performed on human cells, from patients with septic shock and from healthy controls. Peripheral blood mononuclear cells (PBMCs) have been studied as there is preliminary evidence that their cationic amino acid transport, via y^+, is increased in clinical human sepsis (Clark 1998). They are also relatively easily obtained, and may be physiologically relevant in the development of hypotension in sepsis if they marginate in sufficient numbers next to the vessel wall, or infiltrate the adventitia. Vascular smooth muscle cells from small arteries in the bowel mesentery have also been studied. Abnormal production of vasoactive substances by these cells is likely to have the greatest physiological significance of any cell type: the bulk of the resistance in the vascular system is in small arteries, and those of the mesentery contain a large quantity blood which is least susceptible to manipulation by simple physical means (in contrast to the legs, for example, which can be raised to return blood to the central circulation).

Studies have been performed to first confirm the hypothesis that these cells do indeed produce more nitric oxide in sepsis. To assess to what extent this production is influenced by the availability of enzyme cofactors, assays of nitric oxide synthase

activity in the presence of more than sufficient quantities of necessary cofactors have been performed. The expression of nitric oxide synthase mRNA and protein has been assessed.

Immunohistochemistry has allowed the location of eNOS, iNOS and HO-1 in the vessel wall to be determined. This was done to validate the decision to study in detail only the media of these vessels in the other experiments described. The hypothesised decrease in endothelial eNOS expression was also investigated.

In PBMCs, the rate and kinetic nature of arginine transport have been determined, and the expression of mRNA for known arginine transporters assessed. Arginine transporter mRNA has also been quantified in arterial smooth muscle. Attempts have been made to develop antibodies which recognise the cationic amino acid transporter proteins, to facilitate their localisation and quantitation. The ability of a putative inhibitor of the upregulated component of arginine transport in activated cells, resveratrol, has also been investigated.

The importance of the heme oxygenase system in human sepsis has been investigated by determining the HO-1 mRNA and protein expression in both PBMCs and arterial smooth muscle. An attempt has been made to assess the heme oxygenase activity of these cells.

Chapter 2:

Preparation of patient samples

2.1
Diagnostic criteria for severe sepsis and septic shock

Patients were included in these studies if they met the Society of Critical Care Medicine / American College of Chest Physicians (SCCM/ACCP) consensus definition of septic shock (1992). These are the currently accepted guidelines for the definition of sepsis. However these guidelines have been criticised for lacking specificity: postoperative patients, and those with trauma, burns or pancreatitis, for example, may fulfil the criteria if they are concurrently infected, though the infection is not the primary cause of their inflammatory symptoms. The recent observation that serum procalcitonin concentration specifically reflects severity of illness in sepsis (rather than infection or inflammation in isolation) suggests this marker should be included in future studies (Marik 2002). Unfortunately the evidence supporting the use of procalcitonin was not available at the time the current experiments were designed.

In these studies of PBMCs, samples were taken from the patients within 24-72 hours of first meeting the SCCM/ACCP criteria. For the ASM studies, samples taken at surgery were included in the study if the patient met these definitions in the 24 hours before or after the procedure.

The definitions require that the patient have both evidence of infection (as defined) and the signs of a systemic inflammatory response.

Evidence of infection

- Clinical suspicion of infection OR
- microbiologically proven infection OR
- the introduction or change of systemic antimicrobial therapy within the previous 72 hours for suspected infection.

Severe sepsis

A systemic inflammatory response as evidenced by 2 or more of the following (each documented within a 24 hour period):

- Core temperature less than 36°C (96.8°F), or greater than 38°C (100.4°F).
- Tachycardia, as defined by a heart rate greater than 90 beats/min.
- Tachypnoea, as defined by a respiratory rate greater than 20 breaths/min OR
  a $PaCO_2$ less than 4.3 kPa (32 mmHg) during spontaneous ventilation OR
  the requirement for mechanical ventilation.
- A white blood cell count greater than $12x10^9$/L OR
  less than $4x10^9$/L OR
  greater than 10% immature (band) forms.

AND:

Acute onset of end-organ dysfunction, unrelated to the primary septic focus and not explained by any underlying chronic disease, as indicated by one or more of the following:

- Acute deterioration in mental state, not due to sedation or primary underlying disease of the central nervous system.
- Hypoxaemia as defined by PaO_2/FiO_2 ratio less than 40 kPa (300 mmHg) in the absence of primary underlying pulmonary disease.
- Oliguria as defined by a urine output of less than 0.5 mL/kg/h for at least 2 consecutive hours OR a rise in serum creatinine concentration of greater than or equal to 177 µmol/L (2.0 mg/dL) within the previous 48 hours, in the absence of primary underlying renal disease.
- Thrombocytopaenia as defined by a platelet count less than $75x10^9$/L, OR

  an acute decrease of greater than or equal to 50% within the previous 24 hours, in the absence of primary underlying bone marrow disease.
- Acute hepatic dysfunction as defined by at least 2 of the following, in the absence of primary underlying hepatic disease:
  - A serum bilirubin concentration greater than 43 mmol/L (2.5 mg/dL).
  - Serum alanine aminotransferase (ALT) concentration greater than twice the upper limit of the normal range.
  - International normalised ratio (INR) greater than 1.5 OR

    prothrombin time (PT) greater than 1.5 times the control value OR

    PT greater than 1.5 times the mid-point of the reference range, in the absence of systemic anti-coagulation.
- Disseminated intravascular coagulopathy as defined by at least 2 of the following:
  - Platelet count less than $75x10^9$/L, OR

    an acute decrease of greater than or equal to 50% within the previous 24 hours in the absence of primary underlying bone marrow disease.
  - International normalised ratio (INR) greater than 1.5 OR

PT greater than 1.5 times the control value OR

PT greater than 1.5 times the mid-point of the reference range, in the absence of systemic therapeutic anti-coagulation.

- D-dimer titre greater than 0.5 mg/mL OR

  fibrin split product titre greater than 10 µg/mL.

- A plasma lactate greater than the upper limit of the normal range (as defined by a particular laboratory) OR

  a base deficit greater than 5mmol/L not explained by a metabolic derangement other than severe sepsis (e.g. acute renal failure).

Septic shock

- Mean arterial pressure (MAP) <70 mmHg for >30 minutes despite fluid resuscitation OR
- Adrenaline > 0.05 µg/kg/min AND/OR
- Noradrenaline > 0.05µg/kg/min AND/OR
- Dopamine > 5µg/kg/min, to keep MAP > 90 mmHg.

2.2
Preparation of the peripheral blood mononuclear cell fraction from patient blood samples

10-20ml of blood was collected from the indwelling arterial cannula of patients within the first 24-72 hours of their fitting the Society of Critical Care Medicine / American College of Chest Physicians definition of septic shock (1992)(Chapter 2.1), and by venipuncture of healthy control subjects. Exclusion criteria included a pre-morbid history of a systemic inflammatory condition (such as rheumatoid arthritis or ankylosing spondylitis), treatment with systemic corticosteriods at any time in the preceding 6 months, or known infection with a transmissible blood borne virus such as HIV or Hepatitis C.

Heparin sodium (1000IU/ml, 10ul per ml whole blood)(CP Pharmaceuticals) was used as an anticoagulant for all samples other than those to be analysed by reverse transcription polymerase chain reaction, in which EDTA (7.5mg per 4ml whole blood)(Vacutainer) was substituted, as heparin is known to inhibit the action of Taq polymerase.

The blood was diluted 1:1 with an aerated Ringer's physiological salt solution (Appendix II) and layered over the manufacturer's recommended volume of Ficoll/sodium diatrizoate (LSM, Cappel). The tubes were centrifuged at 240g for 40 minutes. The PBMC layer was aspirated, and the cells washed a further two times

with Ringer's solution. All of these procedures were conducted at room temperature. We have previously shown by FACS analysis that PBMCs isolated in this manner are typically 15% monocytes, 15% B lymphocytes and 70% T lymphocytes (Chen 1996).

In the DAF, ^{3}H arginine to citrulline conversion (NOS activity), membrane arginine transport and Western blotting experiments, the PBMC concentration was estimated by counting an aliquot of cells suspended in trypan blue using a haemocytometer. The remaining cells used in these experiments were prepared as follows:

- In the DAF and membrane arginine transport experiments: PBMCs were pelleted by centrifugation then diluted to a concentration of 10 million viable cells/ml using aerated Ringer's solution, and the experiment performed with the least possible delay to ensure maximum cell viability
- In the Western blotting and ^{3}H arginine to citrulline conversion experiments: PBMCs were pelleted by centrifugation then diluted to a concentration of 100 million viable cells/ml using phosphate buffered saline (Gibco) containing 1mM AEBSF protease inhibitor (Sigma). The cell suspension was placed in sterile, protease free tubes at –80°C and stored until sufficient samples had been accumulated to allow processing as a single batch. The protein concentration of one of these the aliquots was measured using the bicinchoninic acid technique described in Appendix III. For Western blotting, these values were used to adjust the results of these two assays for starting protein concentration; for the ^{3}H arginine to citrulline conversion experiments samples were first diluted to a standard protein concentration.

In the RT-PCR experiments, RNA concentration rather than cell count was used to standardise sample quantity between subjects. The PBMCs from 8ml whole blood were pelleted by centrifugation, resuspended in 3ml cell lysis solution (Gentra Purescript RNA extraction kit), immediately frozen at –80°C and stored until sufficient samples had been accumulated.

The ages of the patients and volunteers (mean, standard deviation and range) included in the various studies are summarised in table 2.2a.

Experimental series	**Control**	**Septic**
DAF	38.5 ± 12.1 years (23-56) (n=6)	58.5 ± 23.67 years (17-77) (n=6)
Arginine to citrulline conversion	33.2 ± 7.4 years (23-43) (n=5)	63.6 ± 8.14 years (55-76) (n=5)
Arginine uptake	42.8 ± 13.0 years (30-56) (n=5)	65.2 ± 6.8 years (59-76) (n=5)
RT-PCR: nitric oxide synthases, heme oxygenase and arginine transporters	47.5 ± 10.6 years (30-58) (n=8)	64.4 ± 11.47 years (43-76) (n=7)
Western blot: nitric oxide synthases and heme oxygenase	33.2 ± 7.4 (23-43) (n=5)	70.4 ± 12.9 (55-87) (n=5)

Table 2.2a
Ages of patients and volunteers in the various studies of PBMCs (mean ± SD (range)(number of subjects))

2.3
Preparation of isolated arterial smooth muscle from patient bowel samples

Arterial smooth muscle was dissected from the mesentery of bowel sections taken from two groups of patients:

1. those undergoing laparotomy for peritonitis secondary to bowel perforation who also met the Society of Critical Care Medicine / American College of Chest Physicians definition of severe sepsis or septic shock (1992):
   a. within the 24 hours before surgery, or
   b. who it was suspected would meet these criteria in the 24 hours post surgery. Only those patients who subsequently did meet these criteria were included in the study.
2. those who were otherwise well undergoing large bowel resection for known carcinoma.

Exclusion criteria included a pre-morbid history of a systemic inflammatory condition (such as rheumatoid arthritis, ankylosing spondylitis, or inflammatory bowel disease), treatment with systemic corticosteriods at any time in the preceding 6 months, or known infection with a transmissible blood borne virus such as HIV or Hepatitis C.

Each length of vessel was divided into a number of parts, depending on the study to which it had been allocated. For the immunocytochemistry protocol, a length of vessel was placed intact into 4% paraformaldehyde for 24 hours, followed by 24

hours in 20% sucrose, and then snap frozen in tissue mounting medium (TissueTek, Sakura). Further processing of these samples for immunohistochemistry is described in Chapter 8. For all the other protocols, vessel rings were opened along their longitudinal axis and scraped on both sides to remove both the endothelial cells and the remaining adventitia. The effectiveness of this technique is demonstrated in figures 2.3a and 2.3b. Some of these smooth muscle strips were placed in the preservative *RNA Later* (Ambion) and after allowing time for permeation, were frozen at -80°C for up to 6 months. Strips to be used for the DAF-2DA experiments were kept alive in aerated Ringer's solution at 4°C for up to 30 minutes, until the DAF-2DA protocol was commenced. Other strips were chopped into fine pieces then mechanically homogenised in a 1 mM solution of the protease inhibitor AEBSF in PBS, before similarly being frozen in aliquots at –80°C. The protein concentration of one of these AEBSF aliquots was measured using the bicinchoninic acid technique described in Appendix III. For Western blotting, these values were used to adjust the results of these two assays for starting protein concentration; for the ^{3}H arginine to citrulline conversion experiments samples were first diluted to a standard protein concentration.

The ages of the septic and control patients (mean, standard deviation and range) are summarised in table 2.3a.

Experimental series	**Control**	**Septic**
DAF	50 ± 24 years (33-67) (n = 3) (only 2 ages recorded)	68.3 ± 8.02 years (60-76) (n = 3)
RT-PCR (all but HO-1)	64.2 ± 24.6 years (36-88) (n = 8) (only 5 ages recorded)	69.0 ± 11.6 years (54-89) (n = 8)
RT-PCR (HO-1)	65 ± 20.2 years (36-88) (n = 11) (only 7 ages recorded)	69.8 ± 11.09 years (54-89) (n = 9)
Arginine to citrulline	69 ± 2.0 years (69-71) (n = 5) (only 3 ages recorded)	77.6 ± 6.58 years (54-89) (n = 5)
Western blot: nitric oxide synthases	66.8 ± 14.9 (33-88) (n = 13)(only 9 ages recorded)	71.5 ± 9.2 (54-89) (n = 11)
Western blot: heme oxygenase	66.7 ± 16.9 (33-88) (n = 10)(only 7 ages recorded)	71.5 ± 9.2 (54-89) (n = 11)

Table 2.3a

Ages of patients in the various studies of arterial smooth muscle (mean ± SD (range)(number of subjects))

There were no significant differences in the ages of the control and septic groups in any of the studies, although unfortunately the age of some of the patients was not recorded at the time of collection. The requirement to collect samples without patient identification data makes retrospectively obtaining this information from the clinical medical record impossible.

A number of the studies incorporate different numbers of subjects in the two groups. In the case of RT-PCR for HO-1, this was because the difference between the two groups when 8 + 8 subjects were analysed just failed to reach statistical significance. A further 3 control subjects and 1 septic subject were studied; the uneven numbers reflect the difficulty in obtaining septic tissue. Western blotting was performed for 13

control and 11 septic subjects (as 24 subjects could be analysed simultaneously on the same gel); the difference again reflects the lesser availability of septic tissue. In the HO-1 blot, three of the control lanes were excluded from the analysis of the results, as protein from the positive control lane had spilled over to give artefact bands. This was apparent from the decrease in intensity from left to right on the membrane, and the lack of any inter-lane gap – as is clear in the upper panel of figure 8.2b.



Figure 2.3a
H&E stained human arterial smooth muscle, prior to longitudinal opening and stripping of adventitia and endothelium

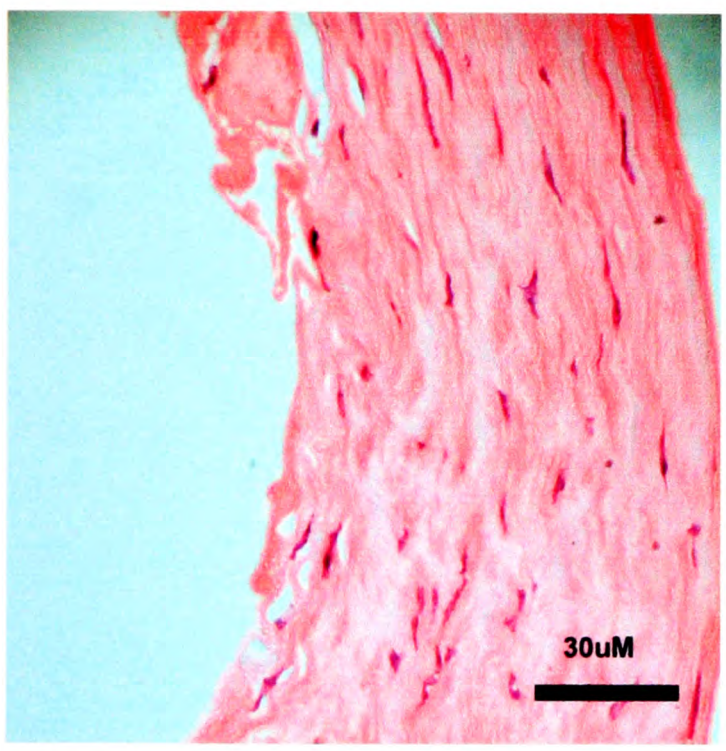


Figure 2.3b
H&E stained human arterial smooth muscle, after removal of endothelial and adventitial layers

Chapter 3:

Nitric oxide production: Diaminofluorescein

3.1
Quantitation of nitric oxide production using diaminofluorescein: Method

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4,5 diaminofluorescein diacetate (DAF-2 DA)(Calbiochem) is a recently described molecule which diffuses freely into cells, where it is esterified to DAF-2 (Kojima *et al.* 1998 a and b). As DAF-2 it cannot cross the cell membrane, and so the dye is concentrated inside the cell. In the presence of molecular oxygen, NO inside the cell reacts with DAF-2 to form DAF-2T, a product with similar fluorescent excitation and emission maxima to fluorescein (figure 3.1a). The amount of DAF-2T is proportional to the amount of NO within the cell.

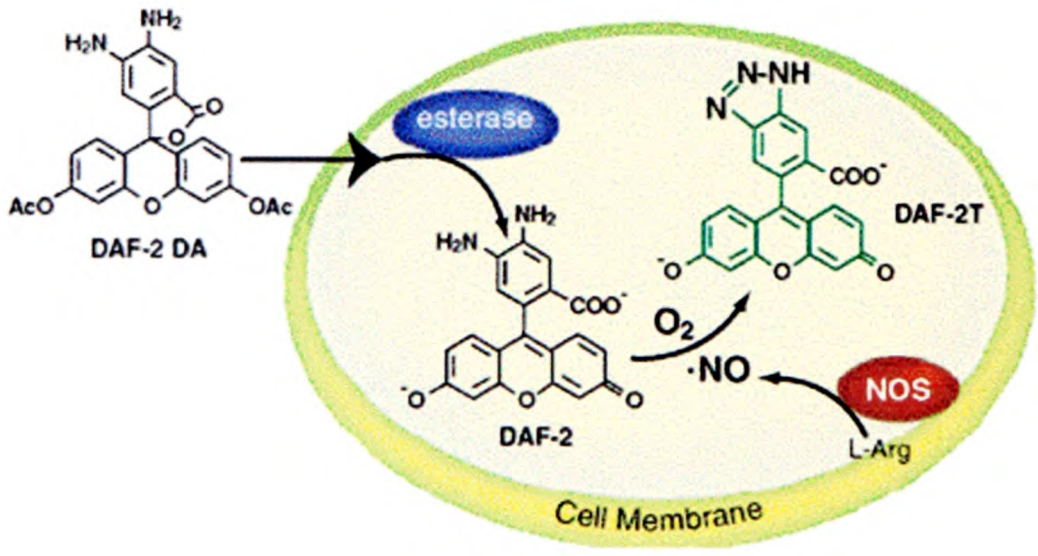


Figure 3.1a
Mechanism of action of the nitric oxide sensitive dye DAF-2DA

DAF-2DA in peripheral blood mononuclear cells

45ul aliquots of a 10^6 cells/ml suspension of PBMCs were added to wells of a clear 96 well plate, which contained DAF-2DA at a final concentration of 10uM, superoxide dismutase (Sigma) at a final concentration of 1000 units/ml, and L-arginine (Sigma) at a final concentration of 30uM (the lower end of the normal plasma arginine concentration range). Where there were sufficient cells, each condition was performed in triplicate. Cells from four patients and four controls were also incubated with 10mM of the nitric oxide synthase inhibitor N-nitro-L-arginine methyl ester (L-NAME) in addition to the 30uM supplemental arginine. The 96 well plate was incubated for 3.5 hours at 37°C in the dark, agitating for 5 seconds every minute. The fluorescence was read at the start and end of this incubation period on a plate reader using an excitation wavelength of 485nm and detection wavelength of 535nm. The mean fluorescence for each condition at the start of the incubation was subtracted from that at the end.

3.5 hours was chosen for the duration of the incubation as this has been shown in preliminary experiments to be just prior to the time at which the increase in fluorescence started to plateau (figure 3.1b)

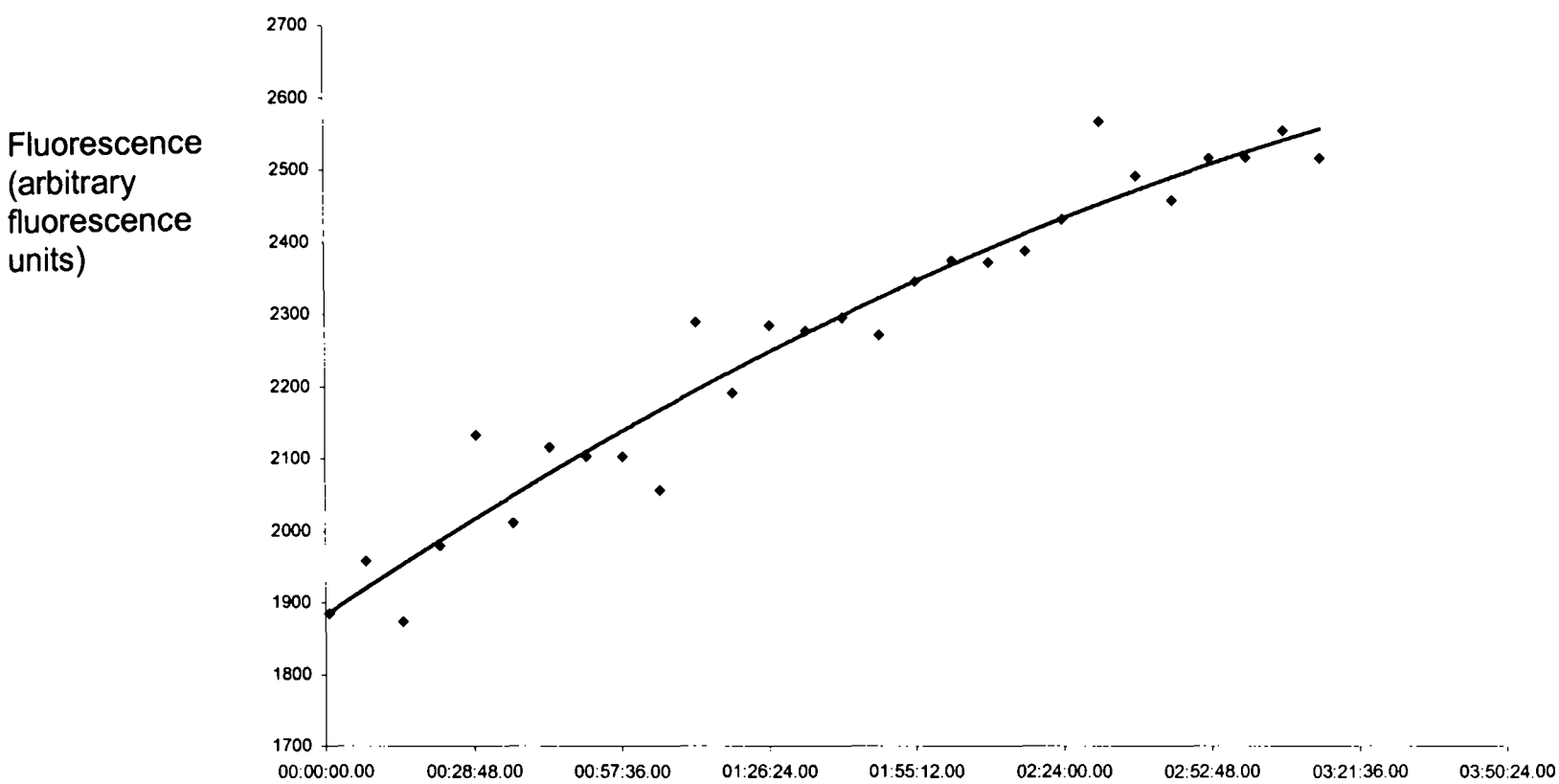
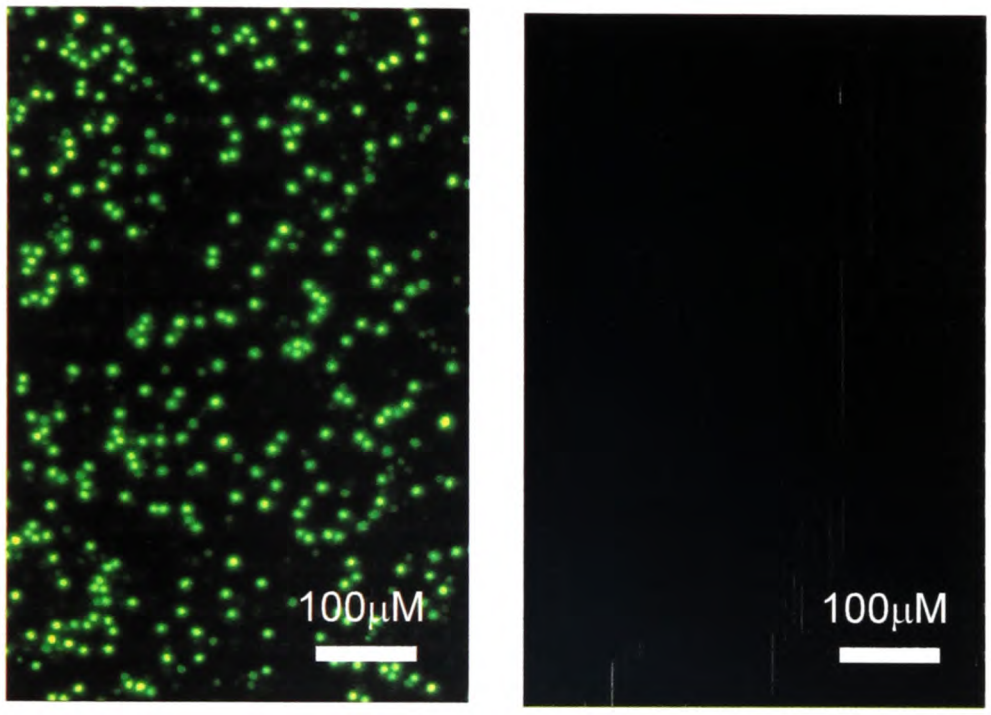


Figure 3.1b

Preliminary experiment showing the increase in fluorescence over time in PBMCs from a control subject in the presence of DAF-2DA. At approximately 3.5 hours the rate of increase in fluorescence is beginning to decline; hence this time point was selected for the estimate of nitric oxide production by the experimental cells.

Control wells containing DAF-2DA and superoxide dismutase but no cells showed essentially no increase in fluorescence over the time course of the assay, demonstrating the cell-dependence of the change in fluorescence. In a preliminary experiment, control wells containing cells but no DAF-2DA produced a low baseline signal on the plate reader, which was also essentially unchanged over the course of the assay. The detailed results of these experimental controls are presented in Chapter 3.2.

At the conclusion of the assay described above, cells from one of the septic patients which had been incubated with DAF-2DA were removed from the 96 well plate, smeared on a glass slide, and visualised using a standard fluorescence microscope with filters for fluorescein. The fluorescence of this image was not quantified, but does demonstrate the cellular origin of the fluorescent signal measured by the plate reader (figures 3.1c and d).

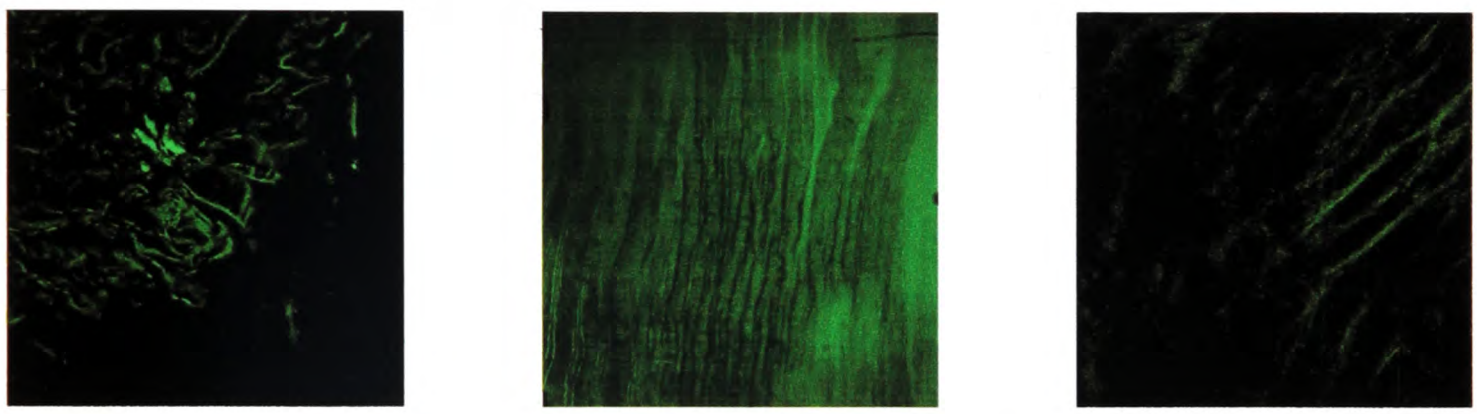


Figures 3.1c and d
Peripheral blood mononuclear cells from a patient with septic shock, loaded with DAF-2DA (L), and to which no DAF-2DA has been added(R), following 3.5 hours incubation as described in the text. Green fluorescence indicates the presence of DAF2T – the product of the reaction of DAF-2 with nitric oxide. The fluorescence of this image was not quantified, but it does demonstrate the cellular origin of the signal measured by the plate reader

DAF-2DA in arterial smooth muscle

A length of artery from each subject was opened along its longitudinal axis, scraped free of endothelium and adventitia, and cut into two segments. The weight of these segments was 4-20mg. One of the segments was placed in aerated Ringer's solution

and incubated for 8 hours 30 minutes at 37°C in the dark. The second was loaded with 10uM DAF-2DA in aerated Ringer's for 30 minutes at 37°C, then placed in fresh aerated Ringer's for a further 8 hours. 8 hours was selected on the basis of preliminary experiments, where the fluorescence of tissue following varying incubation times was examined using a confocal laser microscope. Unfortunately, as noted below, it was not possible to accurately quantify the fluorescence of tissue studied in this manner; however 8 hours appeared to provide the maximal signal, whereas by 24 hours the fluorescence had begun to decline (figures 3.1 e, f and g).



Figures 3.1 e, f and g (left to right)
Arterial smooth muscle from control patients after 1, 8 and 24 hours incubation with DAF-2DA. On the basis of this preliminary experiment, it was decided 8 hours was the optimal time over which to incubate tissue with DAF-2DA.

When the incubations were complete, both tissues were placed in 4% buffered paraformaldehyde and kept at 4°C in the dark until analysed.

It was initially hoped that the fluorescence of these tissues would be readily quantified by analysis of the images acquired using a laser confocal microscope. In practice it proved impossible to find a standard plane in which to record a fluorescent signal representative of the whole tissue. To overcome this difficulty, the total fluorescence

of each tissue was measured on the same plate reader as had been used for the PBMC experiment. Unfortunately the analysis was only undertaken after all of the tissues had been collected, at which time the DAF-2DA incubations were complete. It was thus not possible to subtract the fluorescence of the tissue prior to the DAF-2DA incubation from that at the end, as was done in the PBMC experiments. Rather, the fluorescence of wells with tissue to which no DAF-2 had been added was subtracted from the DAF-2 signal. The fluorescence was normalised to each tissue's weight. To tissue from one patient, a third condition was added: 100uM L-NAME was included in the DAF and 8 hour incubation buffers, in order to demonstrate the NO dependence of the signal. The results of these experiments are presented in Chapter 3.3.

All of the tissue segments were imaged by scanning confocal laser microscopy, with the same excitation and emission spectra as was used by the plate reader. While attempts to directly quantify the fluorescence in these optical sections were unsuccessful, these images do confirm that the fluorescent signal recorded by the plate reader did in fact originate from smooth muscle cells, and that the signal was due to the presence of DAF (figures 3.1 h and i).

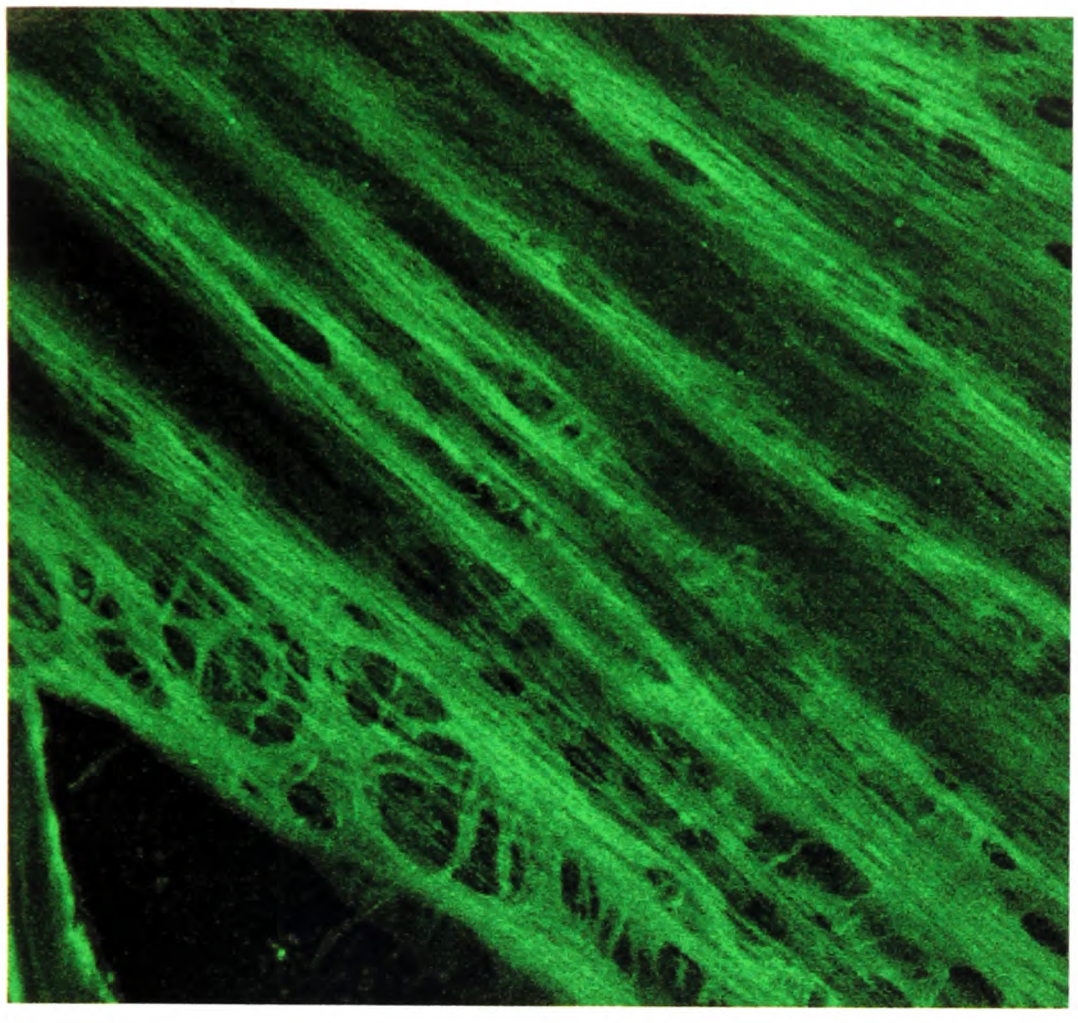


Figure 3.1h
Mesenteric arterial smooth muscle from a control patient, which has been loaded with DAF-2DA for 30 minutes and incubated in the dark for 8 hours prior to fixation in paraformaldehyde. The fluorescence observed clearly originates from the vascular smooth muscle cells in the tissue.

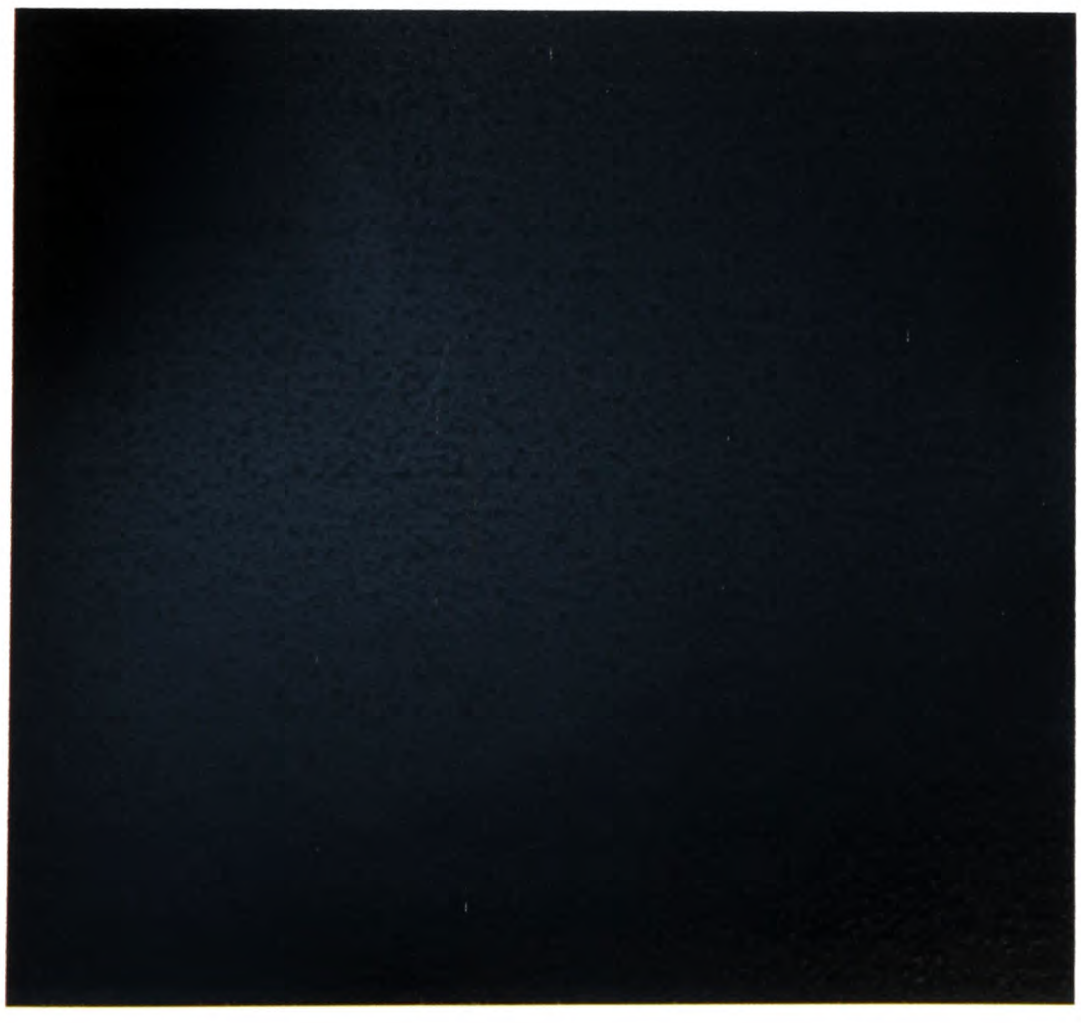


Figure 3.1i
A contiguous segment of mesenteric arterial smooth muscle to that shown in Figure 3.1h, which has not been loaded with DAF-2DA but which in every other respect has been treated in the same manner. The fluorescence observed in figure 3.1h is dependent on the presence of DAF-2DA.

3.2
Nitric oxide production by peripheral blood mononuclear cells

The fluorescence measurements of DAF-2DA loaded PBMCs from the 6 septic and 6 control subjects studied are shown in table 3.2a.

PBMCs from patients with sepsis produced more fluorescence than those from control subjects (n = 6 in each group, p=0.004, Student's *t* test)(figure 3.2a). Whenever 10mM L-NAME was added as an extra experimental condition, the change in fluorescence was decreased. The mean effect of L-NAME in reducing fluorescence was significant in the four septic patients ($p<0.03$) and four controls ($p<0.05$) studied, suggesting that NO was at least in part responsible for the signal observed (summary results not shown).

	Start of incubation	**After 3.5 hours incubation**	**Difference**
Septic 25	1737	2936	1199
Septic 26	1798	2914.5	1116.5
Septic 28	1906	2816	910
Septic 28 + L-NAME	*1930*	*2715*	*785*
Septic 29	1973	3272	1299
Septic 29 + L-NAME	*1776*	*2745*	*969*
Septic 31	1894	3063	1169
Septic 31 + L-NAME	*1850*	*2737*	*887*
Septic 32	1900	3163	1263
Septic 32 + L-NAME	*1908*	*2925*	*1017*
Control 1	1826	2629	803
Control 2	1796	2667.5	871.5
Control 3	1856	2607	781
Control 3 + L-NAME	*1779*	*2362*	*583*
Control 4	1842	2699	857
Control 4 + L-NAME	*1850*	*2521*	*671*
Control 5	1847	2682	835
Control 5 + L-NAME	*1871*	*2387*	*516*
Control 6	1992	2608	616
Control 6 + L-NAME	*1966*	*2325*	*359*
Experimental controls			
No cells + DAF-2DA	1782	1895	113
Cells but no DAF-2DA	1736	1844	108
Sodium nitroprusside, DAF-2DA, no cells	2050	4268	2218
Sodium nitroprusside, DAF-2DA, no cells, + L-NAME	*3309*	*14511*	*11202*

Table 3.2a

Fluorescence (in arbitrary fluorescence units) of wells containing PBMCs + DAF-2DA ± L-NAME, and experimental control wells, prior to and at the completion of 3.5 hours incubation, as described in the text. The increase in fluorescence over the period is taken to indicate nitric oxide production, even though this was incompletely blocked by this dose of L-NAME. There appears to be a non-cell dependent interaction of NO, L-NAME and DAF-2DA (demonstrated when the nitric oxide donor sodium nitroprusside is added to the wells) which may account for the high residual signal in cells treated with L-NAME.

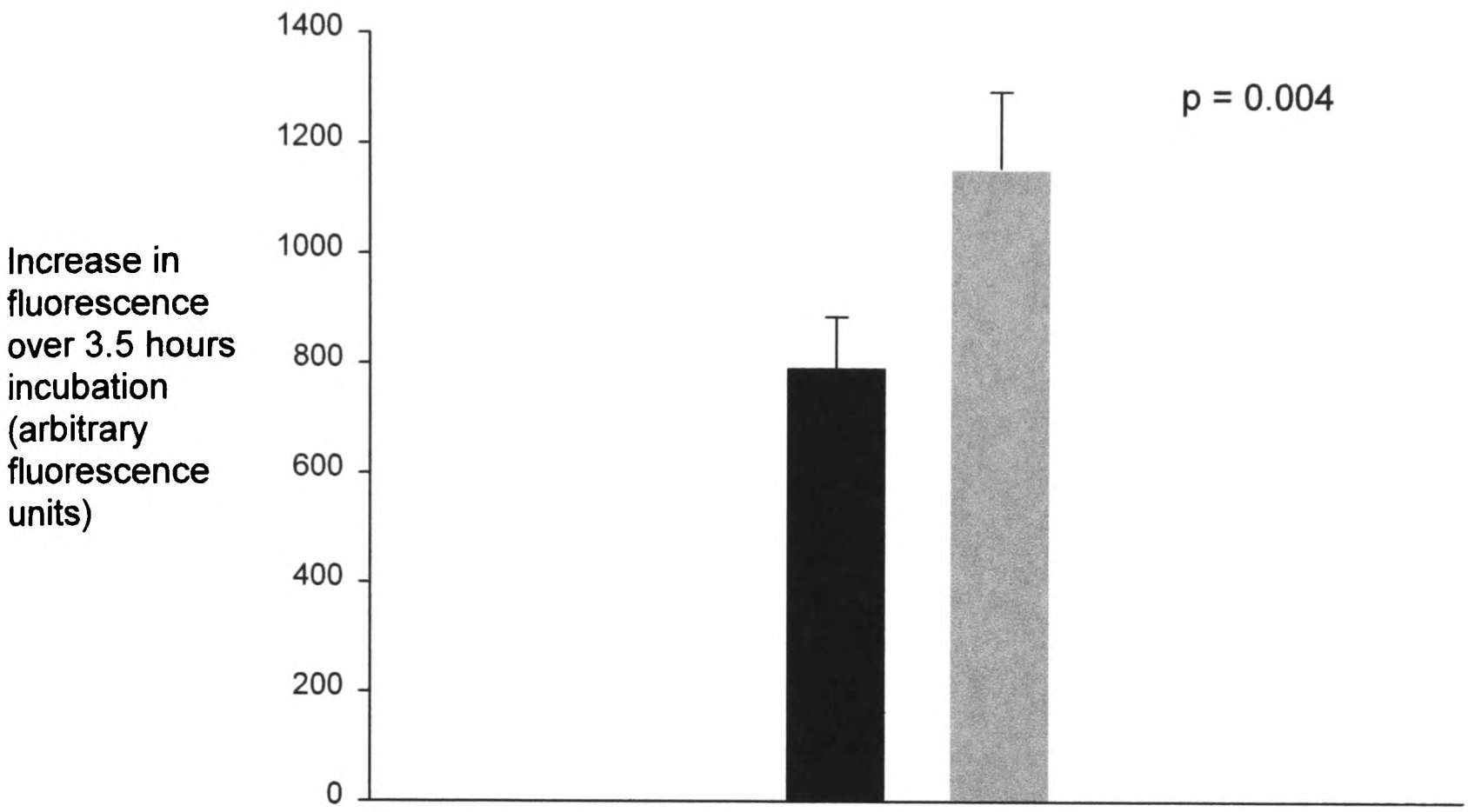


Figure 3.2a
NO production (as measured by an increase in DAF fluorescence over 3.5 hours) in PBMCs from six septic patients and six healthy controls. Error bars represent standard deviation.

Alternate analysis

The high fluorescence in the presence of L-NAME is surprising. If the NO-dependent fluorescence of the cells is taken to be (increase in fluorescence in the absence of L-NAME) – (increase in fluorescence in the presence of L-NAME), the difference between the cells from the 4 septic and 4 control subjects disappears (septic 246 ± 88,

control 240 ± 61, mean ± standard deviation, n = 4, 4, p = 0.92). This may reflect the smaller number of samples or the additive effect of experimental error in two measurements. However, as noted in table 3.2a, L-NAME appears to interact with NO and DAF-2DA to produce an artifactual fluorescent signal at this excitation and emission wavelength. It may be that this was responsible for the high fluorescence in the L-NAME wells in this experiment, and obscured the effect of sepsis on these cells.

3.3
Nitric oxide production by arterial smooth muscle

The fluorescence of arterial smooth muscle from septic and control patients is shown in tables 3.3a and b.

In contrast to the findings in PBMCs, the fluorescence of DAF-2DA loaded arterial smooth muscle was decreased in tissues from patients in septic shock (table 3.3b; figure 3.3a). This result is significant ($p<0.03$) when the signal is not corrected for the background fluorescence of tissue not loaded with DAF-2DA. However when this correction is made the difference just fails to reach significance ($p<0.12$), which probably reflects the addition of experimental errors required to make this correction. Unfortunately it was not possible to collect more tissue for this experiment in the time available.

L-NAME decreased the fluorescence of the one tissue to which it was added, confirming the NO dependence of the signal. Fluorescence in the presence of L-NAME did not reduce to zero, but in a separate experiment it was demonstrated that L-NAME interacted with DAF and a NO donor to produce a fluorescent signal independent of any cellular activity (see chapter 3.2). Hence much of the signal observed in the presence of L-NAME may not represent cellular NO production.

	Recorded fluorescence (+DAF-2DA)	**Tissue weight (mg) (+DAF-2DA)**	***Weight adjusted fluorescence (+DAF-2DA)***	**Recorded fluorescence (no DAF-2DA control)**	**Tissue weight (mg) (no DAF-2DA control)**	**Weight adjusted fluorescence (no DAF-2DA control)**	***Weight adjusted fluorescence (+DAF-2DA) - weight adjusted fluorescence (no DAF-2DA control)***
Control 12	21858	11.8	*1692797*	17190	20.7	739469	*953328*
Control 13	21781	7.2	*2763611*	4569	4.6	583913	*2179698*
Control 14	20310	7.0	*2632429*	15109	7.7	1717662	*914766*
Septic 13	7439	10.3	*539417*	4908	10.0	302500	*236917*
Septic 14	6821	4.3	*1148372*	4281	4.8	499583	*648789*
Septic 15	9811	6.3	*1258413*	5500	5.3	682453	*575960*
Experimental controls							
No L-NAME	108001	5.6	*18949643*	8700	5.4	1611111	*17687235*
100uM L-NAME	94726	6.2	*14974677*	-	-	-	-

Table 3.3a

Fluorescence (in arbitrary fluorescence units) of wells containing arterial smooth muscle + DAF-2DA, background wells containing tissue but no DAF-2DA, and experimental control wells, at the completion of 8 hours incubation, as described in the text. The tissue weight-adjusted fluorescence, or the weight adjusted fluorescence of DAF-2DA loaded tissue less than of non-DAF-2DA loaded tissue, is taken to indicate nitric oxide production, even though (in the one control tissue studied) this was only incompletely attenuated by L-NAME. This may be due to a non-cell dependent interaction between L-NAME, NO and DAF-2DA, as demonstrated in chapter 3.2 and discussed above.

	Mean	**SD**	**n**	**p**
Weight adjusted fluorescence (+DAF-2DA: control)	2362946	584060	3	
Weight adjusted fluorescence (+DAF-2DA): septic	982067	387274	3	0.03
Weight adjusted fluorescence (+DAF-2DA) - weight adjusted fluorescence (no DAF-2DA control): control	1349264	719435	3	
Weight adjusted fluorescence (+DAF-2DA) - weight adjusted fluorescence (no DAF-2DA control): septic	487222	219807	3	0.12

Table 3.3b

Summary statistics for Table 3.3a, with analysis of the results with and without correction for the background fluorescence of the tissue without any DAF loaded.

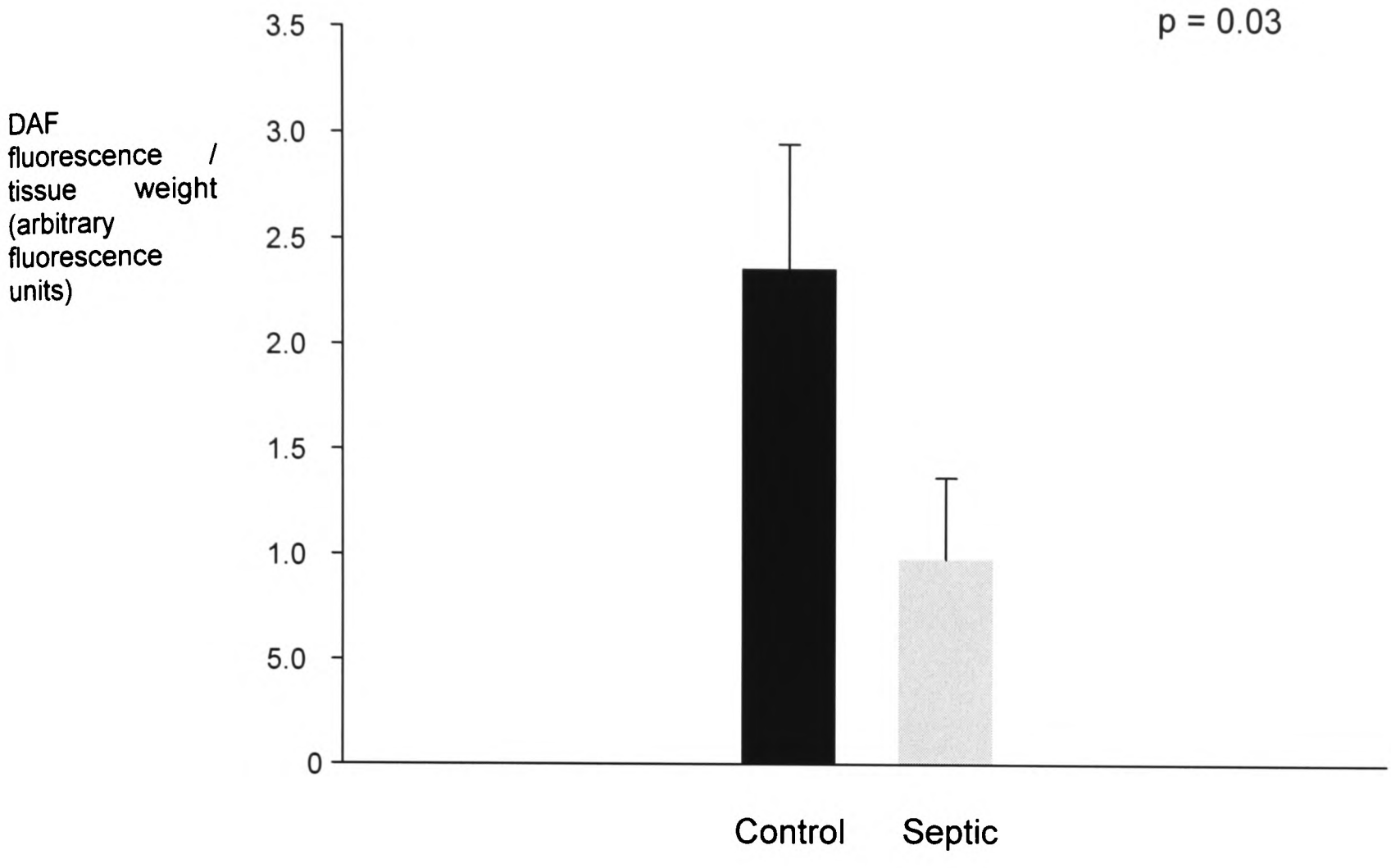


Figure 3.2a

Fluorescence of arterial smooth muscle from 3 septic patients and 3 controls with bowel carcinoma but no inflammatory disease loaded with DAF-2DA, without correction for the background fluorescence of the tissue without any DAF loaded. Error bars represent standard deviation.

Chapter 4:

Nitric oxide synthase activity: ^{3}H arginine to ^{3}H citrulline conversion

4.1 Quantitation of nitric oxide synthase activity by rate of ^{3}H arginine to ^{3}H citrulline conversion: Method

The assay relies on the differential binding of cationic ^{3}H arginine and neutral ^{3}H citrulline to Dowex cation exchange resin (Sigma Dowex 50X8-400)(Combet *et al.* 2000; Knowles & Salter 1998). Separation of substrate from product allows the rate of the reaction, and hence enzyme activity, to be quantified.

In brief, a cell homogenate was mixed with ^{3}H labelled arginine and high concentrations of all the cofactors for nitric oxide synthase. A high concentration of valine was also added to the reaction mix to inhibit any arginase present. As the valine concentration (which was taken from a published protocol) was so high, its effect was tested in a preliminary experiment. The change in rate of citrulline formation caused by valine was minimal (2217 counts per minute formed in 2 hours in the absence of valine; 2104 counts per minute formed in the presence of valine; single experiment). After 2 hours, a period of time demonstrated to be within the linear phase of product accumulation (figure 4.1a), the Dowex resin was added to the mixture. Dowex binds the remaining ^{3}H labelled arginine, leaving only the ^{3}H labelled reaction product, citrulline, free in solution. The radioactivity of the solution containing only the labelled citrulline was measured by liquid scintillation counting to provide an indication of NOS activity.

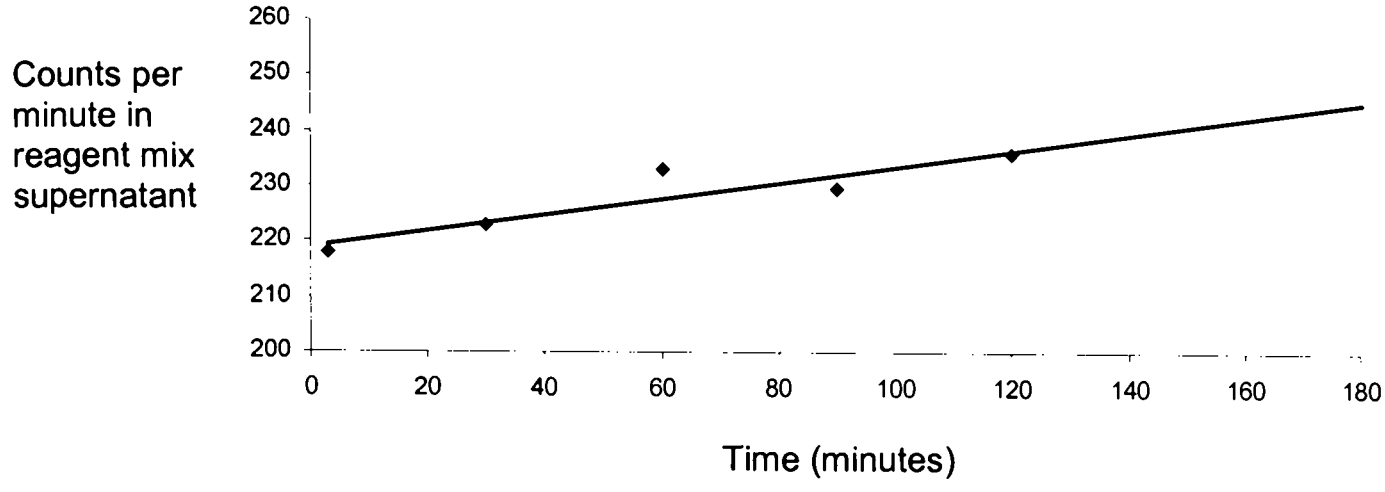


Figure 4.1a

Accumulation of ^{3}H labelled citrulline (represented by radioactive counts per minute in the reaction mix supernatant) as a function of reaction time, using placental homogenate as a source of enzyme. The reaction is linear until at least 120 minutes, which was thus used as the time period of all the reactions using this technique.

Rate of arginine to citrulline conversion in PBMCs

PBMCs stored frozen in AEBSF at 100 x 10^6 cells/ml were thawed, and an aliquot used for estimation of protein concentration (using the method outlined in Appendix III). Using this data, another aliquot of PBMCs was diluted to a protein concentration of 0.5mg/ml with assay buffer (25mM potassium phosphate, pH 7.2). The detergent CHAPS (Sigma) 20 mmol/L and a protease inhibitor cocktail (containing AEBSF, aprotinin, leupeptin, bestatin, pepstatin A and E-64)(Sigma) 0.8ul/100ul were added to this PBMC solution, which was mechanically homogenised by repeated passage through a 23 SWG needle and hypodermic syringe. 100ul of the PBMC homogenate was added to 18ul of the 'reaction mix' containing $MgCl_2$, $CaCl_2$, FAD, FMN, NADPH, BH_4, calmodulin, valine and ^{3}H arginine (NEN Life Sciences) in 50 mM

Tris buffered saline pH 7.4 (see Appendix V for details)(protocol modified from Combet *et al.* (2000)). The reaction tubes were incubated for 2 hours at 37°C.

The reaction was terminated by the addition of 1.5ml 50% water / 50% Dowex 50W 200-400 mesh, 8% cross-linked (Sigma), which had been previously converted from the H^+ form to the Na^+ form by repeated washing in 1M NaOH then water. 2.5ml extra water was added to each test tube to provide sufficient volume, and the Dowex allowed to settle in the tube over 30 minutes. 2 x 1ml volumes of the liquid above the Dowex were placed in 3ml ACS II aqueous scintillant, and the scintillation tubes counted with a Beckman LS1701 scintillation counter to an accuracy of 2% or over 20 minutes, whichever was the lesser period of time. The radioactivity in 5ul aliquots of the 'reaction mix' was counted in a similar manner, which, using the manufacturer's quoted specific activity of the arginine solution, enabled the results of the experiment to be expressed as fmol citrulline formed/mg cell homogenate/minute. The specific activity of NOS (as distinct from non-specific arginine breakdown) was calculated by subtracting the residual rate of citrulline formation in the presence of the potent non-isoform specific NOS inhibitor SEITU (2M final concentration).

Rate of arginine to citrulline conversion in arterial smooth muscle

Protein homogenate from arterial smooth muscle was prepared as described in Chapter 3.1, from 5 septic patients and 5 controls. The conduct of the experiment was essentially as described for PBMCs in Chapter 2.3, with the exception that in these ASM experiments, because of a greater availability of tissue, a third condition was added for each patient sample: 2.4ul of a 100mM EGTA solution was added to the tube, in order to chelate the available Ca^{2+} and hence selectively inhibit eNOS, but not Ca^{2+} independent iNOS, activity. This was in addition to the SEITU (non-specific NOS inhibitor) and no-inhibitor conditions described previously. The total activity of NOS enzymes (as distinct from non-specific arginine breakdown) was calculated by subtracting the residual rate of citrulline formation in the presence of SEITU (2M) from the rate of formation with no inhibitor present. The iNOS activity was calculated by subtracting the residual rate of citrulline formation in the presence of SEITU from the rate of citrulline formation in the presence of EGTA. The eNOS activity was determined by subtracting the rate of citrulline formation in the presence of EGTA from the total uninhibited rate of citrulline formation.

To summarise:

Total NOS activity = no inhibitor – SEITU

iNOS activity = EGTA – SEITU

eNOS activity = no inhibitor – EGTA.

(figure 4.1b)

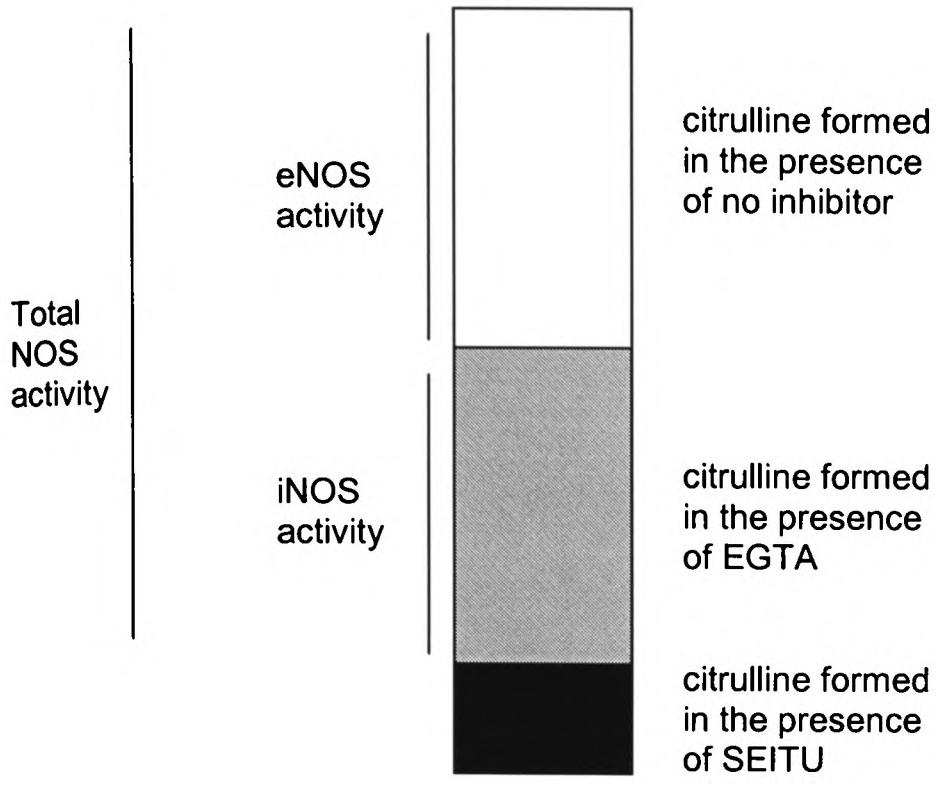


Figure 4.1b
Diagrammatic representation of the process to distinguish iNOS and eNOS from total NOS activity by means of the inhibitors EGTA and SEITU

4.2
Nitric oxide synthase activity in peripheral blood mononuclear cells

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The raw data for this experiment are presented in table 4.2a (in ^{3}H counts per minute). It is immediately apparent that the 'background' counts (in the presence of the NOS inhibitor SEITU) approach (but in no case reach) the counts in the enzyme + no inhibitor reactions. The activity of NOS in these protein homogenates is thus barely detectable using this technique.

	No inhibitor present (CPM)	**+ 2M SEITU (CPM)**	**Difference (α NOS activity)(CPM)**	**NOS activity (fmol citrulline formed/min/mg protein)**
Control 11	438	433	5	0.032
Control 12	471	446	25	0.149
Control 13	458	411	47	0.288
Control 14	553	497	56	0.341
Control 15	445	432	13	0.079
Septic 27	563	531	32	0.190
Septic 32	464	453	11	0.065
Septic 33	431	419	12	0.077
Septic 34	479	377	102	0.622
Septic 39	494	431	63	0.386

Table 4.2a

Raw data (^{3}H counts per minute) quantifying the amount of citrulline in the reagent mix supernatant. The rate of citrulline formation, less the rate of citrulline formation in the presence of the NOS inhibitor SEITU, is taken to indicate NOS activity. This difference is expressed in raw CPM, and also as fmol citrulline formed/min/mg protein present. The amount of specific NOS activity is a small fraction of the background counts, indicating the NOS activity approaches the lower limit of sensitivity of this assay. Each data point represents the mean of two reaction tubes for each condition.

There was a trend towards increased NOS activity in PBMCs from septic patients, but this difference did not reach statistical significance (0.18 ± 0.13 control, 0.27 ± 0.24 septic, fmol citrulline formed/min/mg protein; mean ± standard deviation)(figure 4.2a).

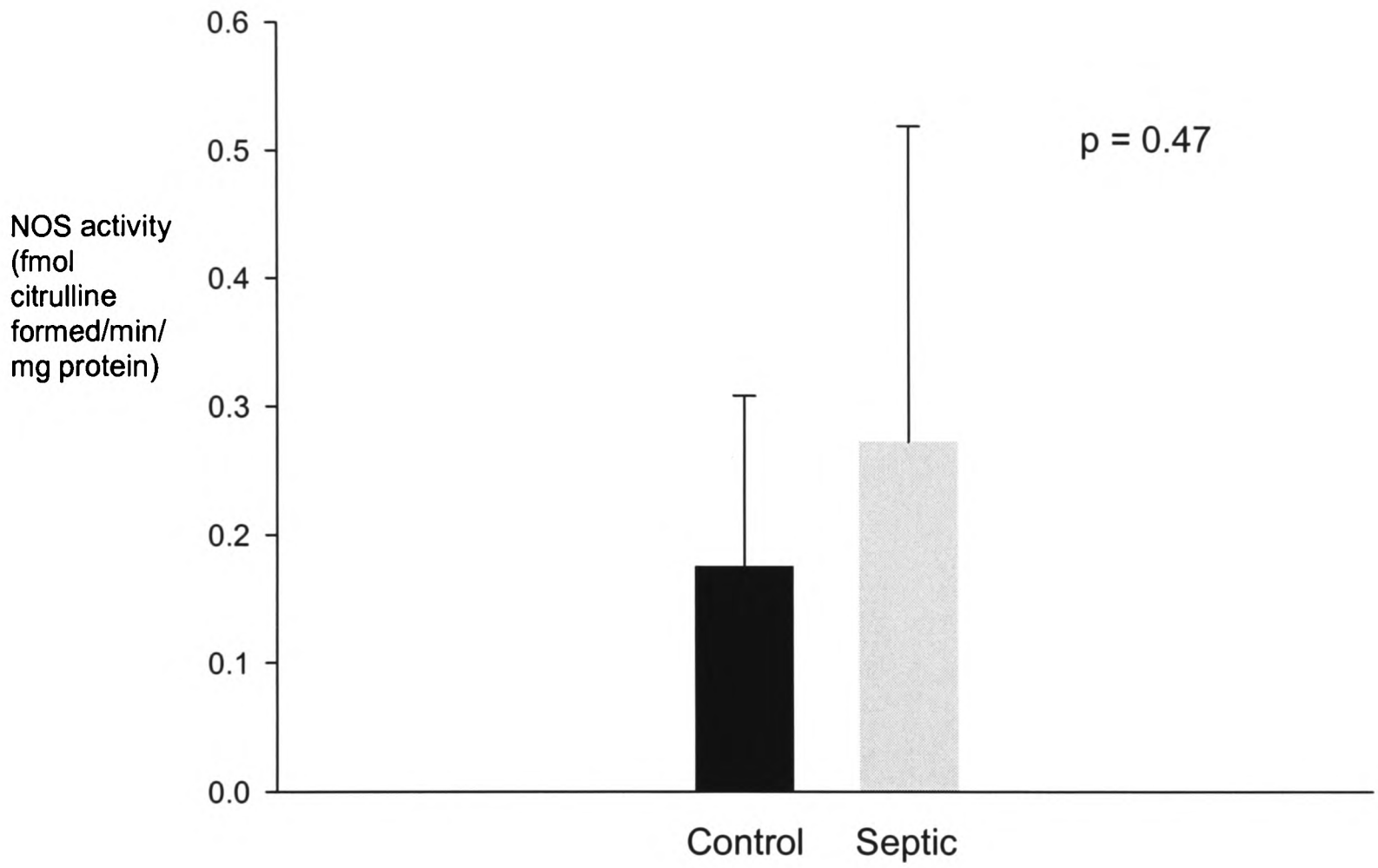


Figure 4.2a
NOS activity (measured as rate of arginine to citrulline conversion) in PBMCs from 5 septic patients and 5 controls. Error bars represent standard deviation.

The result of this experiment is thus equivocal. It is possible that the variability of NOS activity within each sample group is such that to conclude there is no difference between the groups is to make a Type II error. If the difference between the means of

the two samples remains 0.1 pmol/mg tissue/min, and the standard deviation of the measurements remains 0.19 pmol/mg tissue/min, the predicted number of samples required in each group to give an 80% certainty of achieving $p = 0.05$ is 59 (Using the statistical program of Chang (2000)). As this is an impractical number of samples in the context of these studies, no further NOS activity experiments have been performed.

4.3
Nitric oxide synthase activity in arterial smooth muscle

The raw data (in counts per minute formed over the 2 hour incubation period) for NOS activity in the presence of EGTA, SEITU, and no inhibitor are shown in table 4.3a.

	No inhibitor present	**+ 2M SEITU**	**+100m M EGTA**	**No inhibitor - SEITU (=total NOS activity)**	**EGTA - SEITU (=iNOS activity)**	**No inhibitor - EGTA (=eNOS activity)**
Control 10	426.73	435.66	432.6	0	0	0
Control 11	494.56	505.4	480.31	0	0	14.5
Control 14	366.14	457.52	451.02	0	0	0
Control 16	424.2	428.75	421.47	0	0	2.73
Control 23	425.55	456.03	418.9	0	0	6.65
Septic 2	633.39	379.18	402.26	254.21	23.08	231.13
Septic 6	542.61	489.86	554.03	52.75	64.17	0
Septic 12	522.79	475.81	502.97	46.98	27.16	19.82
Septic 14	511.22	473.34	613.44	37.89	140.1	0
Septic 16	787.49	713.43	764.16	74.06	50.73	23.33

Table 4.3a

Raw data (^3H counts per minute) quantifying the amount of citrulline in the reagent mix supernatant. As was the case for PBMCs, the amount of specific NOS activity is a small fraction of the background counts, indicating the NOS activity approaches the lower limit of sensitivity of this assay. Indeed, in tissue from healthy controls, the citrulline formed in the presence of the NOS inhibitor was greater than when no inhibitor was present (in which case the NOS activity is recorded as 0, a negative activity being logically impossible). Each data point represents the mean of two reaction tubes for each condition.

No NOS activity was detected in arterial smooth muscle from control patients, but was seen in all 5 samples from patients with septic shock (0.865 ± 0.845 fmol citrulline formed/min/mg protein, mean ± SD) (figure 4.3a). This was probably due to an increase in the iNOS fraction, which was also only detected in the septic cells (0.567 ± 0.439 fmol citrulline formed/min/mg protein, mean ± SD)(figure 4.3b). While there was a trend to increase in eNOS activity in sepsis, this difference did not reach significance (control 0.044 ± 0.055, sepsis 0.471 ± 0.942 fmol citrulline formed/min/mg protein, mean ± SD)(figure 4.3c).

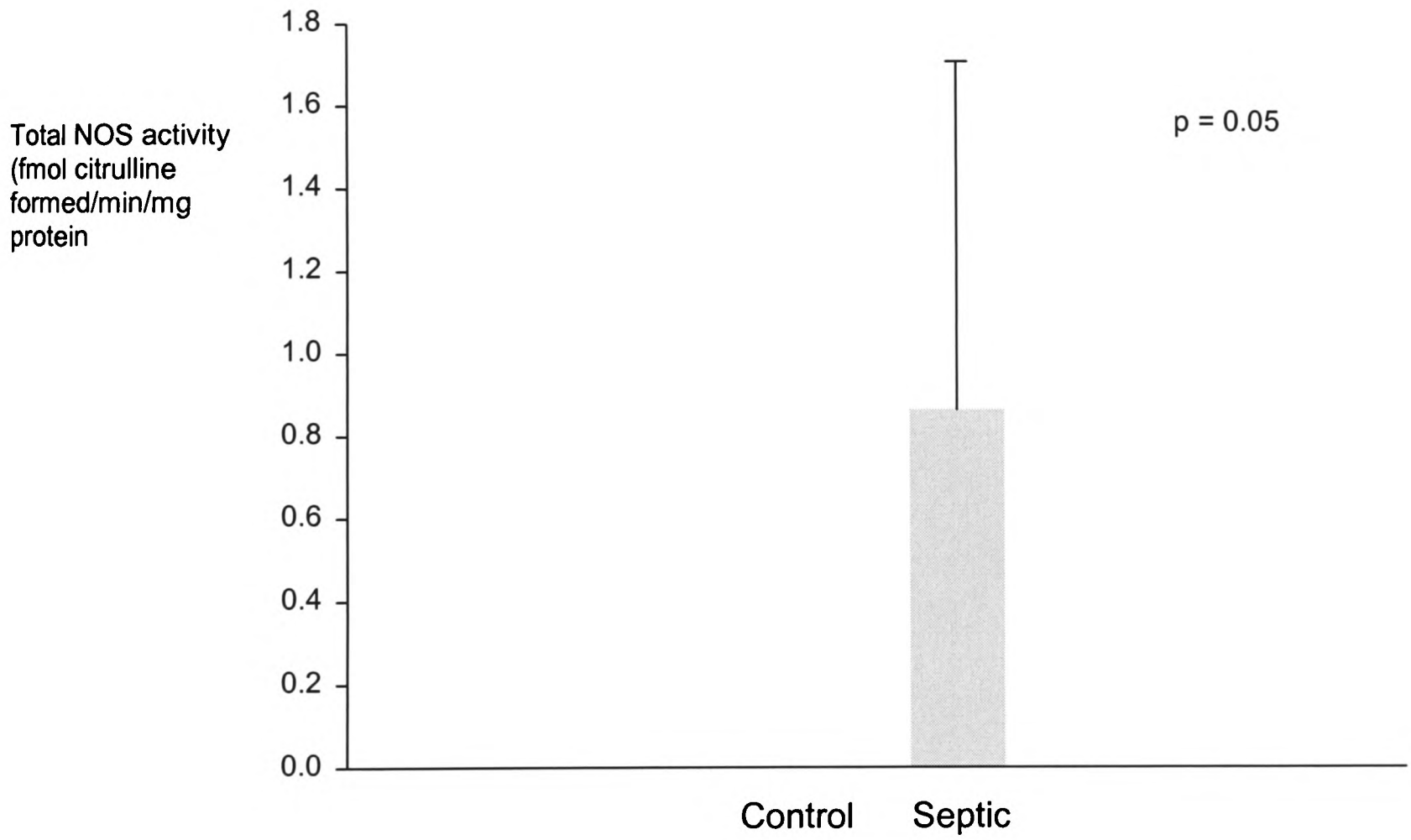


Figure 4.3a
Total NOS activity in arterial smooth muscle from 5 septic patients and 5 controls. Error bars represent standard deviation.

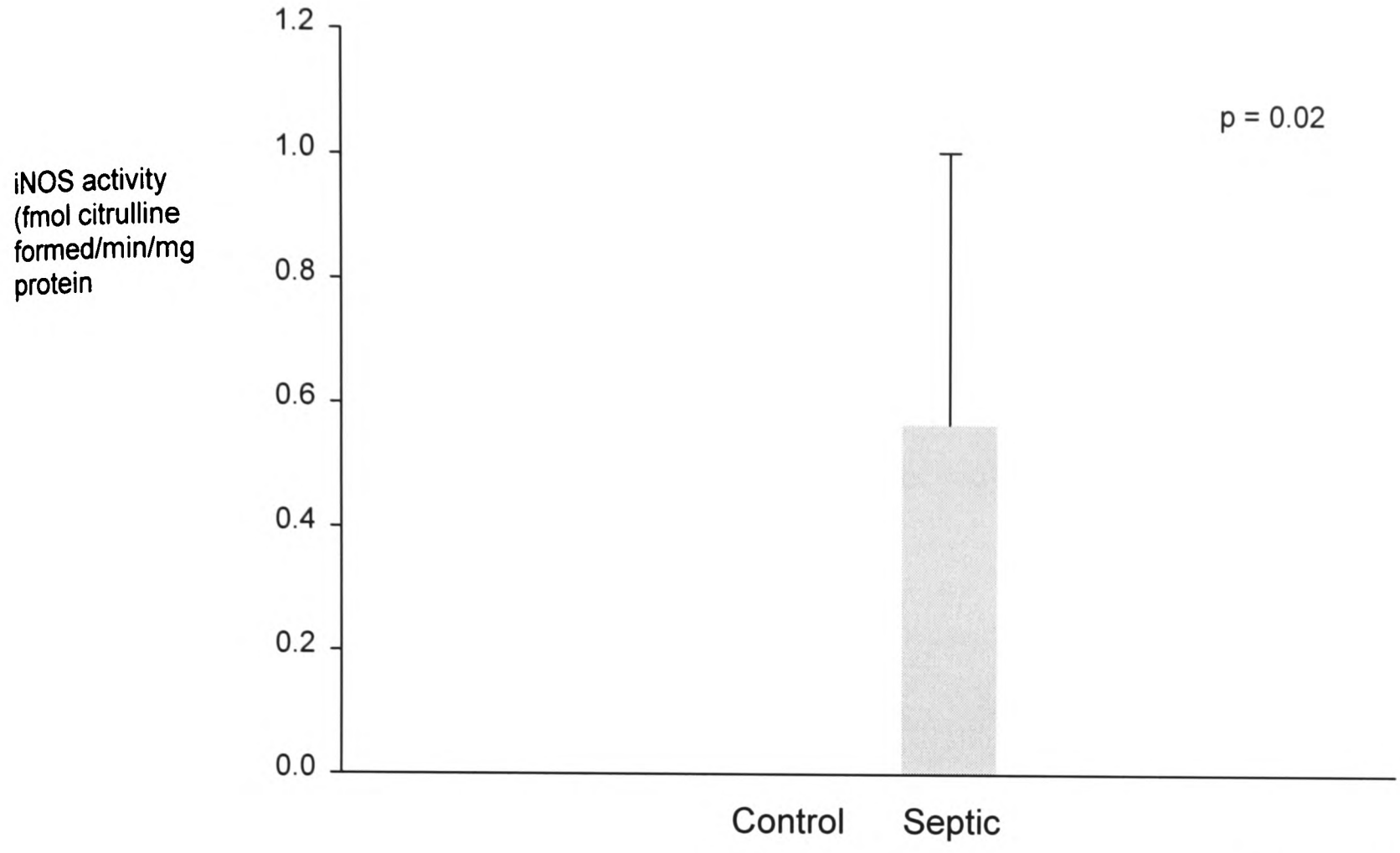


Figure 4.3b
iNOS activity in arterial smooth muscle from 5 septic patients and 5 controls. Error bars represent standard deviation.

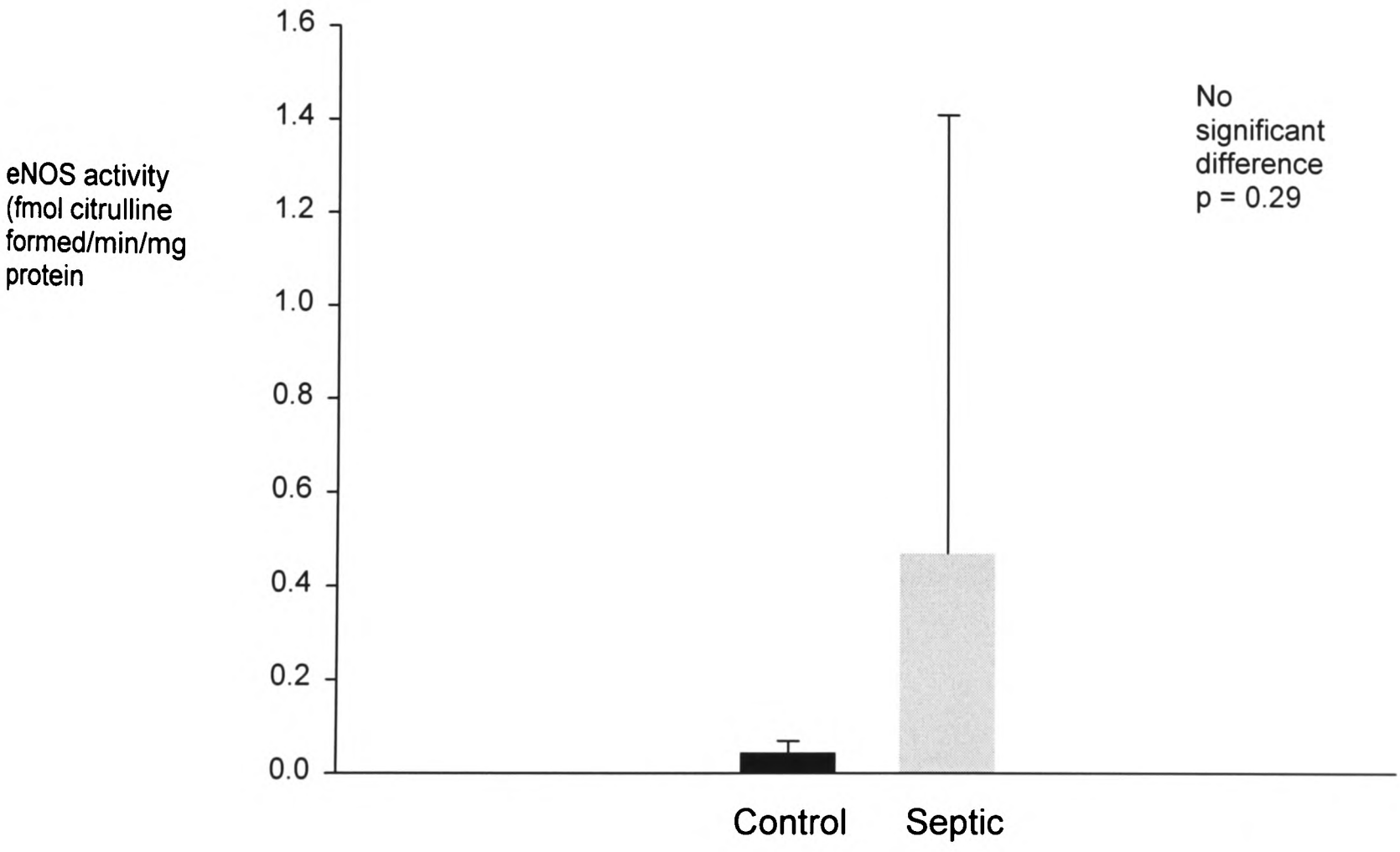


Figure 4.3c
eNOS activity in arterial smooth muscle from 5 septic patients and 5 controls. Error bars represent standard deviation.

The total (uninhibited) activity of NOS in arterial smooth muscle from septic patients is a little higher than that found in PBMCs from either septic patients or controls (Chapter 2.3). This is perhaps surprising, in that PBMCs are a known source of nitric oxide (which they are assumed to release for its cytotoxic effect). In contrast vascular smooth muscle is thought to be the target, rather than the source, of nitric oxide.

No NOS activity, and no iNOS activity, were seen in arterial smooth muscle from control patients. A very small value was recorded for the eNOS activity in these cells, which seems at odds with the finding of no uninhibited NOS activity. This apparent anomaly is almost certainly due to the very low rates, and hence low signal to noise ratio, of citrulline formation by control tissue in the presence of both EGTA and SEITU and the addition of these two errors in the calculation of the components of NOS activity.

How is it that sepsis causes an increase of NOS activity in these cells, which in Chapter 3.3 were seen to produce less nitric oxide than controls? The two results are not necessarily irreconcilable. It is quite possible that the nitric oxide production was reduced in sepsis due to insufficient cofactor availability for the enzyme, or due to a reduction in substrate supply, while in fact the NOS activity remained increased. The endogenous supply of cofactors would not affect the NOS activity assay.

Chapter 5:

Arginine transporter function: labelled amino acid uptake

5.1 Characterisation of arginine transport through y^+ and y^+L in peripheral blood mononuclear cells: Method

PBMCs from septic and control patients were studied to determine the half maximal inhibition constant, K_i, of glutamine for the uptake of ^3H arginine; and also to determine the relative contributions of y^+ and y^+L to the total rate of arginine transport in these cells. The K_i of glutamine was of interest for 2 reasons: firstly, any change in arginine transporter expression might be reflected in the K_i; and secondly, knowledge of the K_i facilitated later experiments where a single high concentration of glutamine was used to inhibit all y^+L activity with minimal effect on y^+ (see Appendix IV).

20ul of cell suspension (1 x 10^6 cells/ml; 200 000 cells) was aliquoted into test tubes, and maintained on ice until immediately before use so as to maximise their viability. Cells were suspended in Ringer's solution, or in Ringer's solution in which Na^+ had been replaced with K^+. Ringer's solution (± Na^+) containing ^3H arginine (NEN Life Sciences) (such that the final concentration of ^3H arginine was 0.2uM) was added to the tube, and briefly vortexed. In some cases this labelled arginine solution also contained varying concentrations of the neutral amino acid glutamine, a competitive inhibitor of arginine transport through y^+L but not y^+. The tube was incubated at 37°C for 3 minutes, a time period which preliminary experiments had shown to be within the linear part of the arginine uptake vs. time curve, and hence which approximated initial rate conditions (see below). At the end of these 3 minutes, 4 ml

of ice cold phosphate buffered saline (Sigma) was added to the tube to stop the arginine uptake, and the whole suspension was passed through a 0.65uM mixed cellulose ester filter under vacuum. The tube was rinsed twice with 4ml of ice cold PBS to ensure the maximum number of cells were transferred to the filter.

Having separated the cells from the remaining extracellular ^{3}H arginine, the filters were dissolved in 4ml ACSII aqueous scintillation fluid (Amersham) and their radioactivity counted over 5 minutes using a Beckman LS1701 scintillation counter. The radioactivity of filters though which the ^{3}H arginine solution but no cells had passed was also measured, and these blank counts were subtracted from each of the test counts. The amount of ^{3}H arginine used in different experiments was standardised by measuring the radioactivity of 5ul aliquots of the addition solutions. Each condition was performed in triplicate. Using the manufacturer's quoted specific activity of the arginine solution, the uptake in each condition was expressed as the mean of each of these triplicates, as picomoles of arginine per 10^{7} cells over 3 minutes.

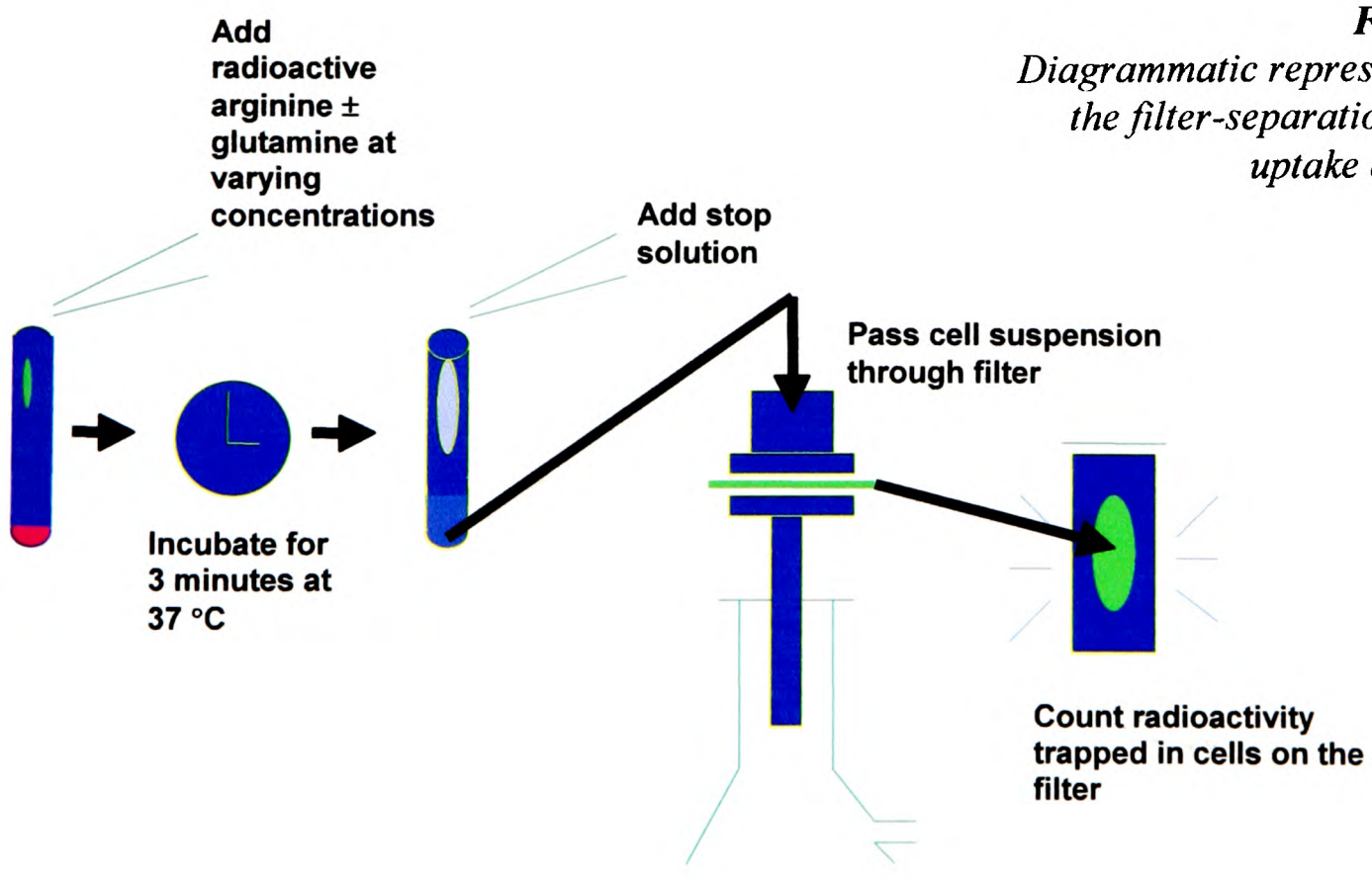


Figure 5.1a
Diagrammatic representation of the filter-separation arginine uptake experiment

A publication in Appendix VII (Reade *et al.* 2002) also reports results of studies of arginine transport in PBMCs from septic patients, using a different technique for separation of PBMCs from extracellular radioactivity. These experiments were performed by another member of our laboratory. As my only contribution to the arginine transport experiments reported in this paper was in the analysis of the results, and as the conclusions are exactly the same as for my own experiments described here, these experiments are not otherwise reported in this thesis.

Results of preliminary experiments

It was necessary to perform a number of preliminary experiments to validate aspects of the technique described above. These included:

a. Demonstration that 3 minutes is within the 'initial rate' phase of arginine uptake
b. Determination of the optimal means of transfer of PBMCs onto the nitrocellulose filter
c. Demonstration of the kinetic equivalence of labelled arginine and lysine, and glutamine and leucine (noting the paper (Reade et al. 2002) in Appendix VII reports experiments using lysine and leucine rather than the arginine and glutamine of chapter 5.2).

The results of these experiments are presented in this section.

a. 'Initial rate' kinetics of arginine uptake

The experiments described in chapter 5.2 involve assays of the uptake of radiolabelled arginine into PBMCs over a period of 3 minutes. The rate of arginine uptake must eventually decline with time, until the intracellular and extracellular concentrations of the labelled amino acid have reached equilibrium, at which point there will be no net uptake. The only valid indication of arginine transporter function is the rate of labelled arginine uptake prior to this equilibrium beginning to establish. In theory this is an infinitely short time after the cells are first exposed to the labelled amino acid, but in practice there is a short period of time during which the accumulating intracellular label has negligible effect on the rate of uptake. The rate of uptake at this time is the 'initial rate'; characterised by an essentially linear plot of uptake vs. time. However, a longer time period for uptake increases the signal to noise ratio of the measurement, and this must be balanced against the desire to keep the time period as short as possible to ensure 'initial rate'. This experiment was performed to determine the optimal balance of these two factors.

PBMCs were prepared and aliquoted as in the standard experiments described in chapter 5.2. In addition to the standard incubation with radiolabelled arginine for 3 minutes, incubations were also performed for periods ranging from 15 seconds to 60 minutes. Measurements at each time point were performed in triplicate. The radioactivity captured on the filters (in DPM) was adjusted for the specific activity of the arginine and the number of cells in each aliquot, as in chapter 5.2, and plotted against the duration of incubation in figures 5.1a and b.

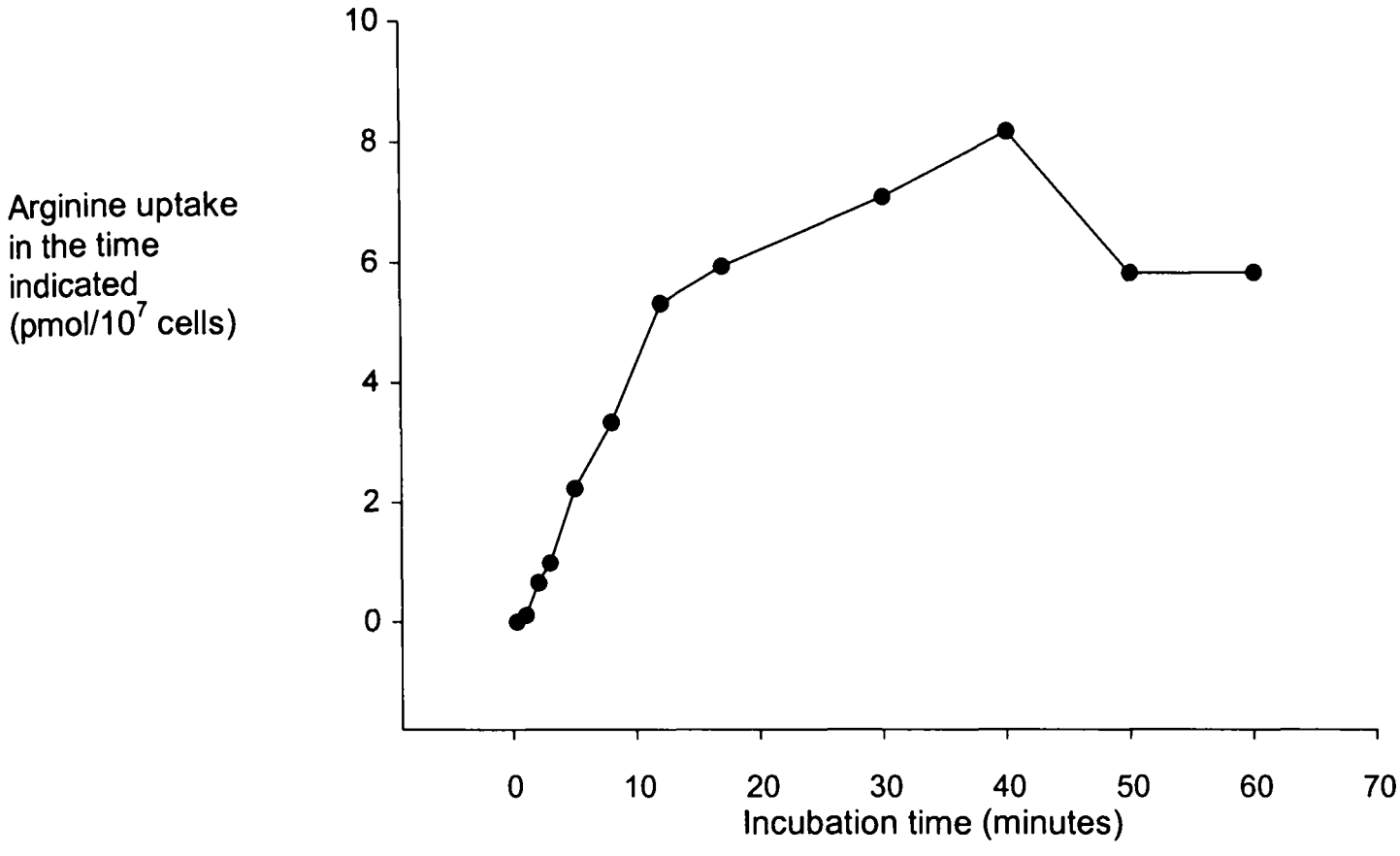


Figure 5.1a
Arginine uptake by PBMCs after varying incubation time periods.

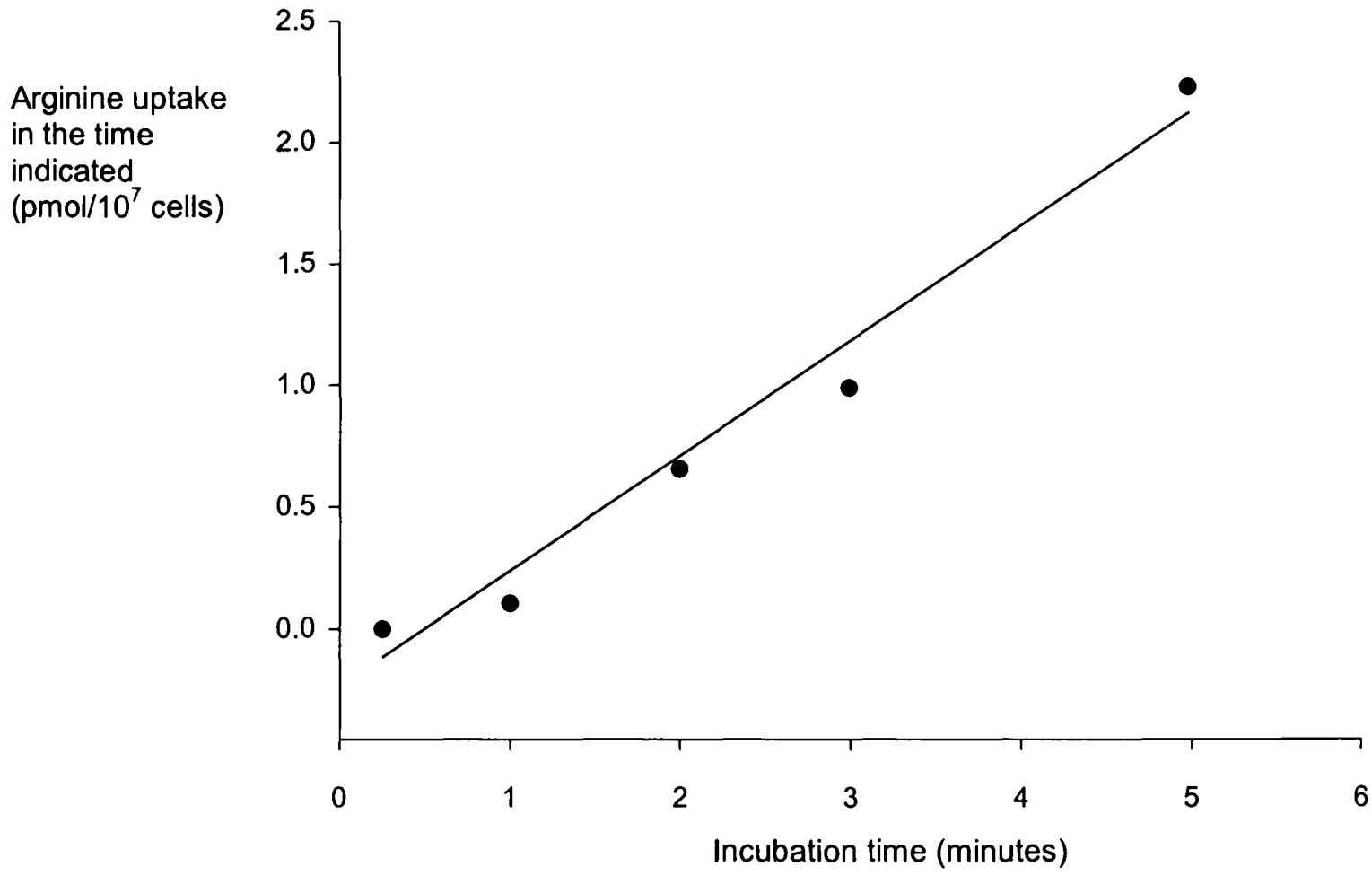


Figure 5.1b
Arginine uptake by PBMCs after incubation time periods from 15 seconds to 5 minutes (in detail).

It is apparent from these two graphs that the uptake of arginine is linear with respect to time for at least the first 5 minutes. Thus experiments performed after 3 minutes incubation are sure to be an accurate estimate the initial rate of uptake.

b. Optimal method of transfer of cells from suspension to nitrocellulose membrane

This is a simple experiment to determine the best volume of wash solution with which to rinse the test tubes, to facilitate their transfer onto the nitrocellulose membrane. The competing influences are the tendency for the PBMCs to clump and adhere to the test tube wall (necessitating greater wash volume), and the effect of reduced radioactivity retained on the filter if too great a wash volume is used, possibly due to cell lysis.

Human PBMCs from a healthy subject were prepared and aliquoted into test tubes, as described above. After incubation with the standard ^{3}H arginine solution for 3 minutes, 4ml of iced PBS 'stop solution' was added to the tube, then one of the following was performed:

a. The contents of the tube were poured onto the filter under vacuum. The tube was rinsed with a further 4ml iced PBS, which was again poured onto the filter.
b. as for a, followed by 10ml iced PBS directly onto the filter
c. as for a, followed by 20ml iced PBS directly onto the filter
d. 2ml x 2 of the PBS was aspirated from the tube and passed through the filter, followed by 5ml iced PBS directly onto the filter.

e. as for d, but using 10ml wash onto the filter

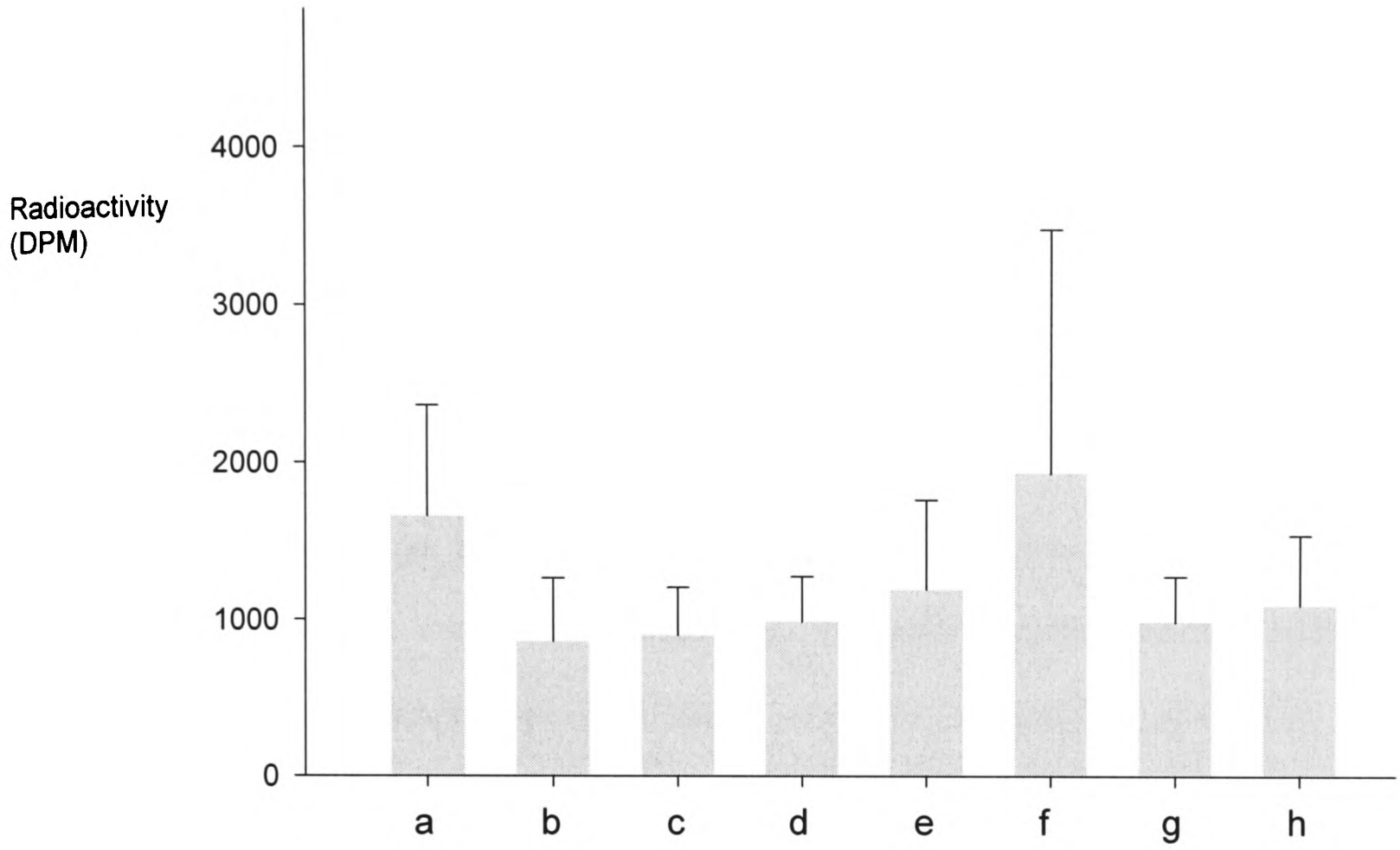
f. as for e, but using 20ml wash onto the filter

g. as for a, but the 4ml wash was repeated once more.

h. as for a, but the 4ml was repeated twice more

Radioactivity caught on the filters was analysed as described above. Each condition was repeated three times, and the radioactivity of filters which had been treated in the same manner but through which no cells had passed was subtracted from the results.

There was an almost 2 fold difference in the radioactivity retained on the filters using the different wash methods (figure 5.1c). The best wash method was that which gave the highest mean number of counts and the smallest standard deviation for the three replicates; a small standard deviation being the more important parameter. These being equal between two methods, the simpler technique was selected. This was chosen to be method g in the table above.



a. The contents of the tube were poured onto the filter under vacuum. The tube was rinsed with a further 4ml iced PBS, which was again poured onto the filter.
b. as for a, followed by 10ml iced PBS directly onto the filter
c. as for a, followed by 20ml iced PBS directly onto the filter
d. 2ml x 2 of the PBS was aspirated from the tube and passed through the filter, followed by 5ml iced PBS directly onto the filter.
e. as for d, but using 10ml wash onto the filter
f. as for e, but using 20ml wash onto the filter
g. as for a, but the 4ml wash was repeated once more.
h. as for a, but the 4ml was repeated twice more

Figure 5.1c

Radioactivity of filters with (through which cells have passed – blanks) processed using the various wash methods identified by letter in the text above (mean of three replicates; error bars represent standard deviation). The best method is the simplest, with the greatest DPM and least SD. This was judged to be method g (see text)

c. Demonstration of the kinetic equivalence of different cationic and neutral amino acids.

These experiments were performed in response to a number of theoretical concerns:

- Arginine is a component of many biochemical pathways (eg. the urea cycle, and via transaminase to the tricarboxylic acid cycle) which cause transfer of the ^{3}H label to $FADH_2$ and eventually water. The water can diffuse out of the cell, causing the measured uptake to be artificially reduced.
- Glutamine is an important energy substrate for leukocytes, in particular during inflammatory conditions. Moreover, extracellular glutamine concentration modulates lymphocyte proliferation, cytokine production, phagocytic and secretory activities. Thus high concentrations of extracellular glutamine, even if only for the brief period of these assays, may alter cell function in ways other than competitively blocking $y^{+}L$ transport (Abcouwer 2000). Glutamine is also an inhibitor of NO synthesis. This inhibition appears dependent on the activity of glutamine - fructose-6-phosphate transaminase (GFAT), which metabolises glutamine to glucosamine (Wu *et al.*, 2001). Glucosamine reduces the availability of NADPH (a NOS cofactor) by inhibiting pentose cycle activity. Reduced NO production might have a number of influences on measured arginine transport. NO has a negative feedback effect on arginine transport. Additionally reduced NO synthesis might increase the intracellular arginine concentration, leading to increased activity of those cationic amino acid transporters which are trans-stimulated.
- Transport proteins are likely to have slightly different affinities for arginine and lysine (in particular $y^{+}L$; C.A.R. Boyd, unpublished results, pers. comm.).

Arginine, not lysine, is the physiologically relevant amino acid in terms of nitric oxide production.

The method used to determine whether labelled arginine is the kinetic equivalent of lysine, and whether glutamine can inhibit transport through y^+L in a manner equivalent to leucine, was entirely different to that used in the arginine uptake studies described thus far. Instead of using a filter under vacuum to separate cells from their radioactive milieu, the cells were layered over a oil, centrifuged and frozen; the tip of the microcentrifuge tube containing the cell pellet was cut off, and its radioactivity measured by liquid scintillation counting as had been previously done for cells trapped on filters. This was the technique used previously in our laboratory, and reported in the paper in Appendix VII (Reade *et al.* 2002).

In detail, 200ul of cell suspension was added to 700ul of Ringer's solution containing both ^3H arginine and ^{14}C lysine, and various concentrations of glutamine, leucine, or no neutral amino acid. The amount of ^3H arginine used was such that its radioactivity was 5x that of the ^{14}C lysine, to prevent the ^3H signal being obscured by the more powerful ^{14}C signal. The results were mathematically adjusted for this, and also the experimentally determined difference in scintillation quench values for ^{14}C and ^3H. 200ul aliquots of the cell / amino acid mixture were removed at 15 seconds, 1, 2 and 3 minutes, layered over 50ul oil (3:2 dibutyl phthalate (Sigma):dinonyl phthalate (BDH)) in a 0.4ml tube, and centrifuged for 10 seconds at 10 000g. The tubes were then frozen and the bottom of each tube containing the cell pellet was cut off; the radioactivity in this pellet was measured by liquid scintillation counting. The background activity was taken to be the uptake in the presence of 10mM non-

radioactive arginine, which would competitively inhibit all relevant transporters, meaning any radioactivity in the cell pellet under these conditions would be non-transport specific. Each data point was performed in duplicate. Results are expressed as rate of uptake of radioactivity (corrected CPM/second) corrected for differences in total radioactivity added and different quench values. The experiment was performed using cells from a septic patient (septic 30) and a healthy control subject (control 1, repeated), to allow for the possibility that transporter affinity might be different in sepsis.

The results are shown in tables 5.1a and b.

		No inhibitor (CPM/s)
Control	^{3}H arginine	88.6
	^{14}C lysine	56.4
Sepsis	^{3}H arginine	57.4
	^{14}C lysine	38.2

Table 5.1a

Rate of uptake of ^{3}H arginine or ^{14}C lysine into PBMCs from a control patient and a patient with septic shock, demonstrating the apparent higher uptake of ^{3}H arginine compared to ^{14}C lysine, a relationship which is not altered in sepsis. Data represent mean of 2 observations on PBMCs from the same patient.

		Glutamine (CPM/s)	**Leucine (CPM/s)**
Control	^{3}H arginine	17.8	17.1
	^{14}C lysine	20.9	18.6
Sepsis	^{3}H arginine	27.5	30.7
	^{14}C lysine	15.2	17.0

Table 5.1b

Rate of uptake of ^{3}H arginine or ^{14}C lysine into PBMCs from a control patient and a patient with septic shock, demonstrating the equivalence of glutamine and leucine as inhibitors of $y^{+}L$ transport. When maximally inhibited by either glutamine or leucine, there appears little difference between the uptake of ^{3}H arginine and ^{14}C lysine in control cells, but, as is the case when no inhibitor is present, in sepsis the ^{3}H arginine uptake is the greater. Data represent mean of 2 observations on PBMCs from the same patient.

Tables 5.1 a and b show a number of interesting results, though it is important to highlight the data are derived from only one septic and one control subject. That the total (uninhibited) uptake into control cells is greater than into septic cells does not necessarily contradict the conclusion of chapter 5.2. However, the proportion of total uptake which remains in the presence of either glutamine or leucine is substantially greater in sepsis than control cells, confirming the result of chapter 5.2.

Glutamine and leucine inhibit the uptake labelled amino acid in a very similar manner, irrespective of whether ^{3}H arginine or ^{14}C leucine is used as the marker.

When no inhibitor is present, and with either inhibitor in control cells, the rate of arginine uptake appears substantially greater than that of lysine. This may possibly relate to differences in the ability of the scintillation counter to detect ^{14}C and ^{3}H, although (in a preliminary experiment) this difference was quantified, and a correction factor applied to the results above. If arginine was being metabolised at a greater rate than lysine, the ^{3}H water so produced would leak from the cell, causing the arginine counts to be decreased rather than increased. The apparent higher rate of arginine uptake thus remains unexplained. Most importantly, though, is that the proportion of total uptake which remains in the presence of inhibitor is substantially greater in sepsis than control cells regardless of whether arginine or lysine are used. The conclusions of chapter 5.2 would probably be unchanged if lysine rather than arginine had been selected.

5.2
Arginine transport through y^+ and y^+L in peripheral blood mononuclear cells

Figures 5.2a and 5.2b are representative of the study of 5 septic patients and 5 healthy controls.

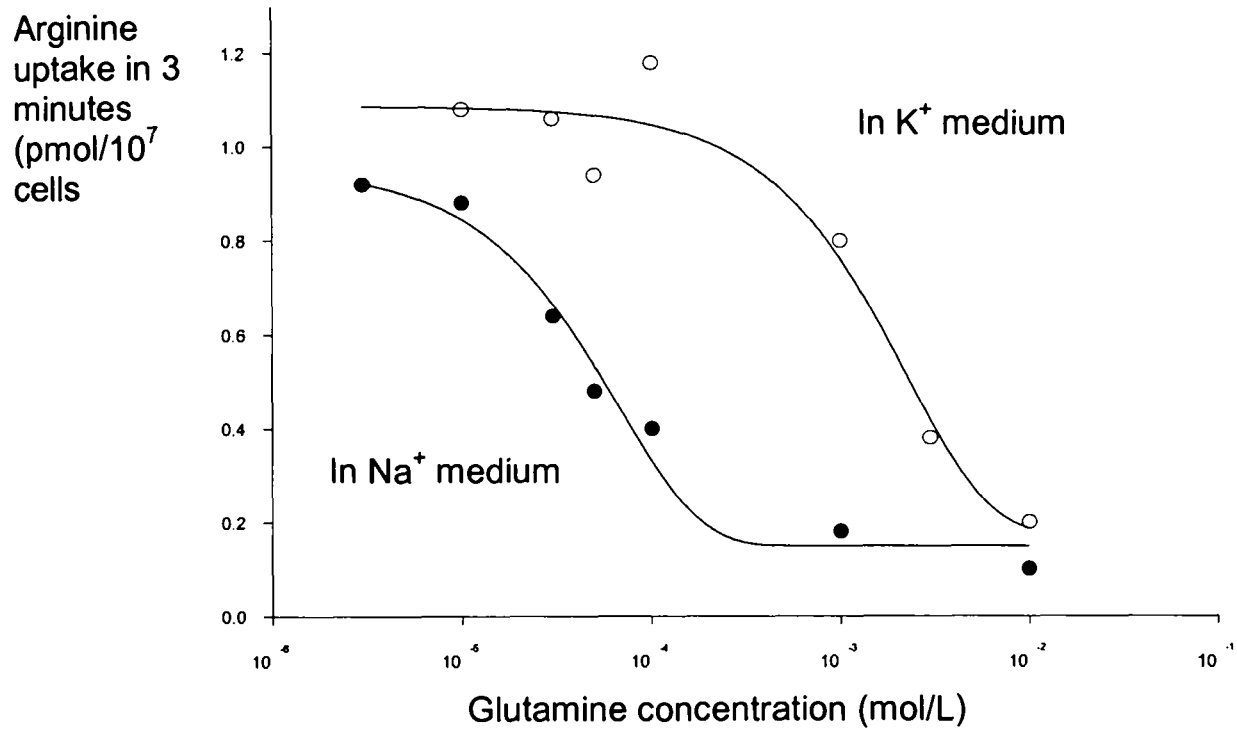


Figure 5.2a
Healthy control peripheral blood mononuclear cells

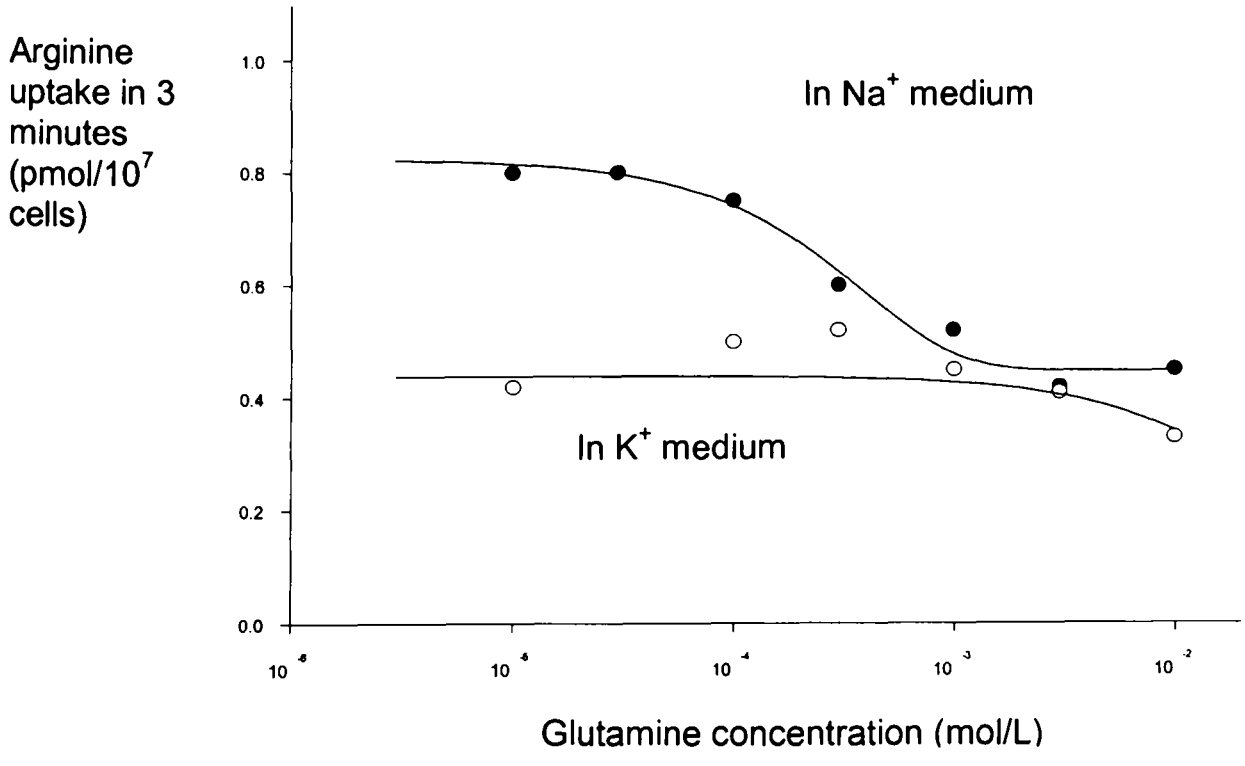


Figure 5.2b
Peripheral blood mononuclear cells from a patient with septic shock

In PBMCs from a healthy individual, the bulk of arginine is transported by the y^+L system, as shown in figure 5.2a. Thus neutral glutamine is able to block nearly all (cationic) arginine uptake in Na^+ containing medium. In the absence of Na^+, the affinity of glutamine for y^+L, and hence its potency in inhibiting arginine influx, is substantially reduced. Figure 5.2a is thus an elegant demonstration of the known Na^+ dependence of neutral amino acid transport through y^+L.

Figure 5.2b shows the same experiment conducted with PBMCs from a patient with sepsis. A large component of arginine transport in the presence of sodium is now unaffected by glutamine. Furthermore, transport is now less when K^+ replaces Na^+ in the medium. This suggests the presence in sepsis of an additional transporter with different characteristics to y^+L. This transporter has the properties of y^+, which is known to be selective for cationic amino acids and hence unaffected by glutamine. In contrast to y^+L, y^+ is a sodium independent, electrogenic transporter. Thus transport of arginine is reduced when the cell is depolarised by K^+, even in the absence of glutamine. Of additional note is the apparent change in the K_i for glutamine in the presence of sodium: however, this result should be interpreted with caution, as in later experiments it became apparent that uptakes recorded with the lowest concentrations of glutamine (which were performed at the end of the experiment) were artifactually depressed, possibly due to cell death or loss of membrane integrity. The effect of this time-dependent reduction of uptake may have been greater in the septic cells than the controls.

The total (uninhibited) transport of arginine is significantly greater in PBMCs from septic patients (1.0980 ± 0.3563 pmol/10^7 cells in 3 minutes) than those from controls

(0.6780 ± 0.2050 pmol/10^7 cells in 3 minutes) (mean ± standard deviation)(n = 5,5; p = 0.05). When y^+L is inhibited with the highest concentration of glutamine used, the residual transport (attributable to y^+), expressed as a proportion of the maximal uninhibited transport, was markedly increased: from 0.07 ± 0.06 to 0.36 ± 0.13 (mean ± standard deviation)(n=5, 5; p = 0.002). Thus y^+ activity has increased from 7% of total transport in healthy cells, to become responsible for over one third of the arginine transport into PBMCs from patients with septic shock (figure 5.2c).

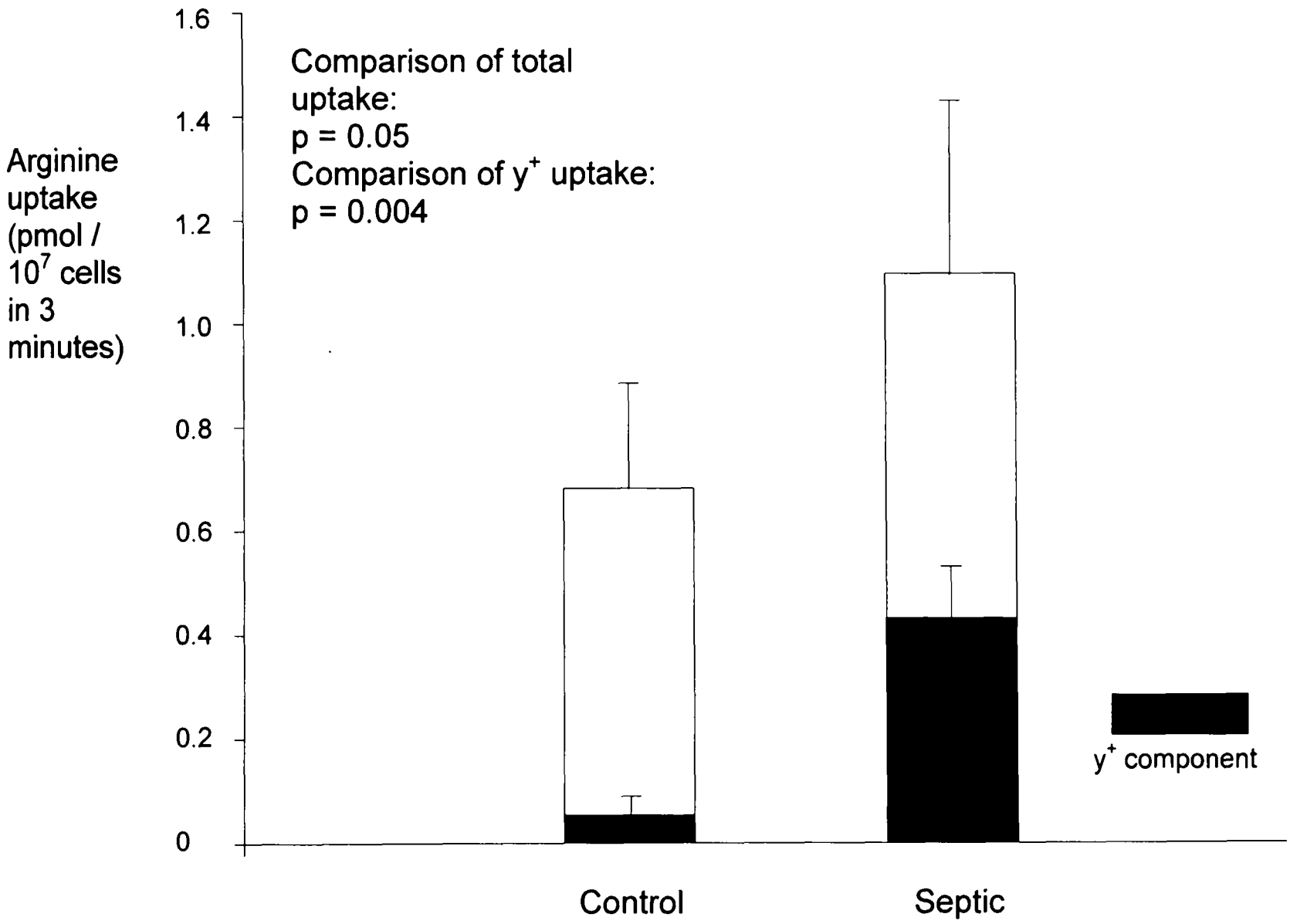


Figure 5.2c

Maximum (uninhibited) arginine uptake, and arginine uptake in the presence of maximum inhibition by glutamine (ie. y^+ transport), in PBMCs from 5 septic patients and 5 controls. Error bars represent standard deviation of total transport, and transport through y^+.

This new expression of y^+ activity in PBMCs from septic patients is quantitatively strikingly similar to the results of previous experiments in our laboratory using different cells and a different experimental technique (Reade *et al.* 2002). The proportion of total transport due to the y^+ transporter in that experimental series increased from 0.14 ± 0.04 in control PBMCs to 0.50 ± 0.18 in septic PBMCs (n= 7 septic patients and 6 controls, p = 0.0005) – very similar figures to those demonstrated here.

Chapter 6:

Arginine transporter and nitric oxide synthase mRNA quantity: real-time reverse transcription polymerase chain reaction

6.1 Real-time reverse transcription polymerase chain reaction: Method

PBMCs isolated from 10ml blood were resuspended in 1.5ml of a proprietary cell lysis solution containing citric acid, EDTA, and SDS (Gentra) and stored at –80°C. When sufficient samples had been accumulated, they were thawed on ice, and the remainder of the manufacturer's total RNA extraction protocol (involving protein precipitation with citric acid and NaCl followed by isopropanol precipitation of RNA) completed.

Arterial smooth muscle was stored at –80°C in *RNA Later* (Ambion), as described in detail in Chapter 2, until sufficient samples had been accumulated to allow processing as a single batch. When thawed, the tissues were weighed; the weights ranged from 5 – 77 mg. The tissues were then semi-frozen on dry ice, finely chopped with a sterile scalpel blade, and homogenised using a ceramic mortar and pestle. A volume of cell lysis solution (Gentra) appropriate to the tissue weight was added, and the remainder of the RNA extraction, DNAse treatment, reverse transcription and real time PCR performed in the same manner as described above for PBMCs.

Once the RNA had been extracted, both PBMC and arterial smooth muscle samples were treated with DNase (*DNA-free*, Ambion) to remove contaminating genomic DNA, and the concentration of RNA was calculated from its absorbance at 260nm.

After dilution to a standard concentration of 25ug/ml, the RNA was reverse transcribed using random hexamer primers and MultiScribe reverse transcriptase (TaqMan Gold RT reagent kit, Applied Biosystems), using the manufacturer's recommended protocol of 10 minutes at 25°C, 30 minutes at 48°C, and 5 minutes at 95°C to inactivate the enzyme.

Segments of the cDNA produced were amplified using primers designed specifically for use in the TaqMan real-time PCR reaction, the concentration of which had been optimised in preliminary experiments (described in detail below). Each primer had been checked against all known mRNA sequences to ensure it would bind only to the intended sequence. The sequences for the primers and probes used are shown in table 6.1a.

For most of the primer/probe combinations, there was an exon/exon boundary present between the two primers (ideally in the probe sequence itself), making it highly unlikely that anything other than cDNA would be amplified. Notably, because of the nature of the sequences, it was not possible to design the primers/probes in this way for y^+LAT1, y^+LAT2, eNOS or CD98, though one of the primers did cross such a boundary in all of these cases.

Target sequence	GenBank identification		Sequence	Position	Amplicon length
CAT1	4507046	Forward	TGTGAACATTTCAACCAGCCTTA	1632-1654	78 bp
		Reverse	AGAGCCTCCCTTCCAAGCA	1709-1691	
		Probe	AGCTGTTCTCATCATCACCTTCTGCATTGTG	1656-1686	
CAT2	4507048	Forward	GATGTCCATTGGCACACTCATG	1430-1451	84 bp
		Reverse	TCGTAAGATAAGCCAGGCTGGTA	1513-1491	
		Probe	CCTACTCTCTGGTGGCAGCCTGTGTTCT	1453-1480	
CAT2B	484049	Forward	GTAAACTATGCTATGGCGGAGGAT	1086-1110	143 bp
		Reverse	GCGCCTTCAGGTCAAACAGA	1228-1208	
		Probe	TTGCTTTTCAAATGTCTAGCTCAAATCAATTCCA	1114-1147	
CAT4	4759137	Forward	CCTGAGCATCGTCCTCAACA	1731-1750	174 bp
		Reverse	AATACCACGTAGTGTGTGGAGTTC	1904-1881	
		Probe	CTGCCTCATGCTGAAACTTAGCTATCTGACCTG	1752-1784	
CD98hc	182864	Forward	TGGTGCAGCTGGAGTTTGTCT	1094-1114	77 bp
		Reverse	AGCTGGTAGAGTCGGAGAAGTTGA	1170-1147	
		Probe	AGGCAAGGCTCCTGACTTCCTTCTTGC	1116-1142	
y^+LAT1	3982909	Forward	AAGGAGGCAATGCCAGGAA	240-258	72 bp
		Reverse	CTTCATACTCAGTGCTGTCAACCA	311-288	
		Probe	CTGGTGAAGGGTTTCCTCTCCTCCACC	260-286	
y^+LAT2	1665758	Forward	ACAGGCAGTGGCGTGATCT	135-153	105 bp
		Reverse	GTGTTTGCTGTGGCCTGTAATC	239-218	
		Probe	CTCACTGCAACCTCCGACTCCCTGG	157-181	
iNOS	951320	Forward	GCAGGTCGAGGACTATTTCTTTCA	3429-3453	80bp
		Reverse	CGTAAGGAAATACAGCACCAAAGAT	3508-3483	
		Probe	CTCAAGAGCCAGAAGCGCTATCACGAAG	3454-3482	
eNOS	10835160	Forward	GGAGAATGGAGAGAGCTTTGCA	1760-1782	96bp
		Reverse	CGGATCTTATAACTCTTGTGCTGTTC	1855-1829	
		Probe	CTGCCCTGATGGAGATGTCCGGCC	1783-1807	
18S	--		Purchased from Applied Biosystems (P/N 4308329). No data on sequence available from company for commercial reasons.		

Table 6.1a.
Sequences of the primers and probes used in this study.

In the experiments described in this thesis, there was no amplification in the no-RT controls using the cDNA specific primers described above – with the exception of primers for 18S, y^+LAT1 and y^+LAT2, as described in Chapters 6.2 and 6.3. In some of the samples amplified using the 18S primers, which could not be cDNA specific, there was a small quantity (between 1/100 and 1/1 000 000) of product in the no-RT controls, indicating that there was some genomic DNA contamination of these samples even after DNAse treatment. The no-RT controls for y^+LAT1 and y^+LAT2 in arterial smooth muscle experiments produced significant amplification, which was

true to a lesser extent in PBMCs as well. Unfortunately it was not possible to mathematically subtract the no-RT amount from that in the 'test plates', as the no-RT controls were performed some months after the initial experiments, and the integrity of the cDNA standard curve had degraded. The reported results for y^+LAT1 and 2, especially in arterial smooth muscle, are thus not corrected for genomic DNA amplification, and must be interpreted with caution.

In the 18S, CAT4, y^+LAT1 and y^+LAT2 reactions of a separate experimental series (Reade, Sibley & Speake, Universities of Oxford and Manchester, unpublished results), there was even greater amplification in the no RT controls, suggesting that despite DNAse treatment this tissue remained heavily contaminated with genomic DNA. That these four primers produced amplification in the no RT controls of these samples suggests they lack true cDNA specificity, but more importantly confirm the other sequences are indeed highly cDNA specific. The lack of cDNA specificity undoubtedly reflects the fact that one of the primer sequences itself, rather than the probe or the segment of DNA between the primers, bound to the exon/exon boundary. Notably in the case of CAT4 this should not have been the case if the sequence reported in the literature is correct – but in that the sequence for CAT4 has only recently been described and is not yet independently verified this is perhaps not surprising.

The PCR mixture comprised a master mix of AmpliTaq Gold DNA polymerase, dNTPs, a passive reference fluorochrome (ROX), and the necessary buffer (Applied Biosystems). The potential problem of contamination with product from previous PCR reactions was minimised by the use of heat inactivated uracil-N-glycosylase in

the PCR master mix, which contained dUTP rather than dTTP. The reaction was cycled according to the manufacturer's standard protocol: 2 minutes at 50°C, 10 minutes at 95°C, and then 40 or 50 cycles of 15 seconds at 95°C and 1 minute at 60°C.

The quantity of PCR product was detected during and after each PCR cycle using an Applied Biosystems 7700 Sequence Detector. In addition to acting as a thermal cycler, this device monitors the increase in fluorescence in each reaction well. A sequence specific probe is bound between the two primers, which during the extension phase is cleaved by the 5' exonuclease activity of the Taq polymerase. In the process of cleavage, the quencher (TAMRA) is removed from its position next to the fluorescent marker (which in these experiments was carboxyfluorescein (FAM) for the mRNAs of interest, and the proprietary VIC dye for the 18S endogenous control). Hence with each new strand of DNA produced, another quanta of fluorescence is detected (figure 6.1a).

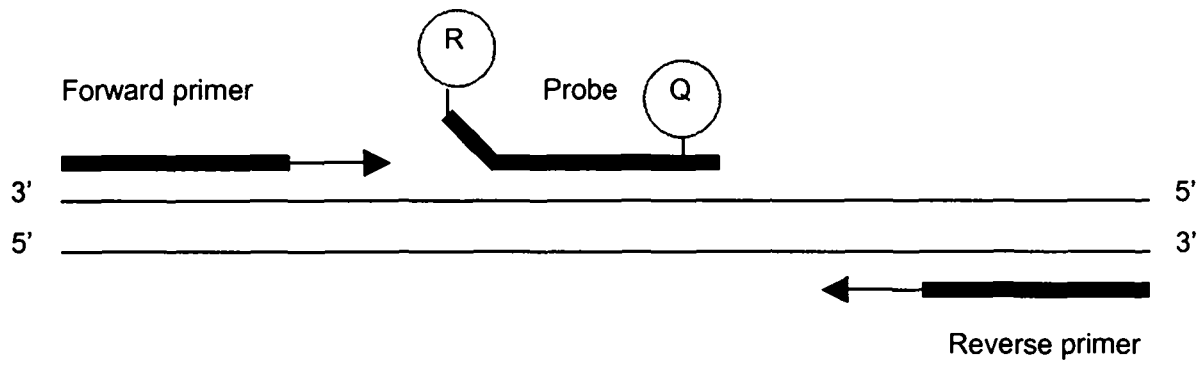


Figure 6.1a
Mechanism of the 5' nuclease assay for PCR product detection. As the forward primer is extended, the 5' exonuclease activity of the Taq polymerase cleaves the sequence specific probe, which removes the reporter dye, R, from its close proximity to the quencher, Q.

Small variations in the starting quantity of cDNA following reverse transcription of each sample were standardised by reference to the amplification of cDNA by primers and probe for the 18S component of ribosomal RNA. PCR reactions for cells and muscle from each subject were performed in quadruplicate. Whilst it is almost certain that the fluorescence detected was due to amplification of the expected cDNA sequence, this was confirmed by visualisation of the products after electrophoresis on a 3% agarose gel stained with ethidium bromide.

The starting quantity of cDNA in each sample is indicated by the number of cycles of PCR required to reach a threshold fluorescence intensity. For each sample, this was referred to the amplification of a serial dilution of human placenta cDNA (Clontech), a tissue known to express all of these mRNA species at high levels. The amount of each mRNA transcript present is thus expressed as a proportion of the expression of that mRNA in placenta.

Rationale for using this real-time PCR method

There are a number of different options when performing real-time RT-PCR analysis. This section describes these options and the reasons for the method selected.

a. Method of detection of reaction products

Real time PCR detects products as they accumulate during each PCR cycle. This eliminates the need for preliminary experiments with each primer set to determine the number of cycles of PCR in the exponential phase of amplification for all experimental samples, as is the case for semi-quantitative PCR. There is also no need to quantitate PCR produce by band intensity on an electrophoresis gel.

There are two methods for detecting PCR products as they are formed. The simpler uses SYBR Green I dye, a marker which binds double stranded DNA in a highly specific manner. In that it detects amplification of non-specific reaction products as well as the intended sequence (akin to including any spurious bands or streaking on an agarose electrophoresis gel in the quantification of the intensity of bands at the expected molecular size), experiments using SYBR Green I must be performed using rigorously optimised conditions, and arguably the product of each reaction should be run on an electrophoresis gel to confirm amplification specificity. The alternative is the system used in the experiments described in this thesis: the TaqMan, or fluorogenic 5' exonuclease assay, described in Chapter 1.5. The use of a probe with a sequence complementary to that of the template between the two primers ensures the fluorescence generated by the reaction is highly specific. While it is reassuring that

the product of the PCR reaction has the expected molecular weight, as seen on the electrophoresis gels presented in chapters 6.2 and 8.1, it is highly unlikely that even if there had been amplification of other products these would have been detected by the oligonucleotide probe. The manufacturers similarly consider it unnecessary to confirm the product of the TaqMan PCR reaction by sequencing. The only disadvantage compared to SYBR Green I is the greater cost.

b. Use of Uracil-N-glycosylase

The PCR reaction mixture used in these experiments contained the enzyme uracil-N-glycosyalse (UNG), which destroys cDNA sequences containing uracil during the 50°C incubation prior to thermal cycling. The dNTP mixture used for PCR in all these experiments contained dUTP rather than dTTP, so this preliminary step destroyed any products of previous TaqMan PCR experiments which may have contaminated the reaction. The UNG was inactivated in the 95°C step which followed, immediately prior to thermal cycling, thus allowing PCR product of the subsequent reaction to remain intact. It was of course necessary to set up these experiments in an environment away from other (non-TaqMan) PCR reactions to allow this method to work. The only disadvantage of using the UNG system was its cost.

c. Selection of reporter dye, and use of multiplex PCR

A number of dyes (FAM, TET, HEX, JOE, VIC, etc) are available to attach to the 5' end of the oligonucleotide probe. FAM is the most commonly used, as its emission maximum (approx. 540nm) is furthest from that of the passive reference dye ROX (600nm) and the quencher dye TAMRA (575nm), and it produces a strong fluorescent signal. FAM is sufficiently distinguishable from VIC (555nm) to allow both to be used ('multiplexed') in the same reaction tube: the VIC attached to a probe for the endogenous control gene product, and the FAM attached to the probe for the gene of interest, with both sets of primers also present in the tube. For this reason, the endogenous control gene product primer/probe sets are supplied by the manufacturer labelled with VIC, not FAM.

The main advantage of multiplex PCR is removal of any error caused by inaccurate pipetting of the same sample into 2 separate wells: the gene of interest and the endogenous control gene. Amplification of the cDNA at the higher starting concentration (invariably the endogenous control) should have reached the plateau phase before there is any significant signal from amplification of the cDNA of interest. It is necessary to perform an extra preliminary experiment to determine the primer concentration to best achieve this. In some cases it is not possible to separate the amplification of the two sequences in this manner, in which case the multiplex technique cannot be used.

The main disadvantage of multiplex PCR in the context of these experiments is its cost. It would be necessary to run the endogenous control primer / probe in every

reaction plate, rather than just a single plate the results of which are used in the interpretation of all others. This would increase the total cost of the experiments by approximately 35% - which was not considered acceptable given the marginal extra benefit of this technique.

d. **One step vs. two step RT-PCR**

Applied Biosystems manufacture an enzyme for use in the TaqMan assay which has both reverse transcriptase and DNA polymerase (with 5'-3' exonuclease) activity, allowing the RT and PCR for each sample to be completed in one reaction well. This has the main disadvantage of being less efficient at both reverse transcription and PCR than the enzymes developed with those specific single functions. It is also possible to perform one-step RT-PCR using a mixture of the standard RT and PCR enzymes; however the composition of the reaction buffer is a compromise between the optimal conditions of each, again producing reduced efficiency. The RT-only enzyme must be allowed to work at 48°C, which is close to the optimal temperature of UNG; UNG cannot therefore be included in this mixture as it will destroy the cDNA copy as soon as it is made. On top of these difficulties, if one step RT-PCR is to be used, the samples must be stored as RNA rather than the much more stable cDNA. It was therefore decided that in these experiments the best course was to make a 'bank' of cDNA for all samples, and perform PCR in a separate reaction.

e. Method of relative quantitation of products: standard curve or $^{\Delta\Delta}C_t$.

There are two methods used for quantitation of gene expression: reference to a standard curve, or use of what the manufacturer terms the $^{\Delta\Delta}C_t$ method.

The standard curve method is the more powerful technique, and the simpler to understand. There are two variants: the absolute and relative standard. It is possible to give an absolute quantitation of starting RNA quantity in the samples tested if a standard curve is used, the quantity of RNA in which is known by some independent means. A single pure sequence of RNA is difficult to obtain, and even if this is possible, the efficiency of the reverse transcription is likely to be different to that in the biological samples of interest. Fortunately it is of no value in these experiments to know the absolute quantity of RNA; all that is necessary is to determine if RNA expression is greater in one group of samples than another. Reference to a relative standard curve achieves this. This involves serial dilution of cDNA from a positive control tissue – that is, a tissue known to express the mRNA of interest at a reasonably high level. Human placenta was shown in preliminary semi-quantitative PCR experiments (not reported in this thesis) to express all the mRNAs of interest in these studies. The expression of RNA in a patient tissue sample, as determined by its C_t, is compared to the C_ts of the various dilutions of placenta. The result is thus reported as a proportion of the expression of that mRNA in placenta.

The $^{\Delta\Delta}C_t$ method removes the need for a standard curve. The C_t vaules for each patient sample are simply compared to one another, and usually expressed as a multiple of one of the samples (or a positive control). However, to incorporate

correction for differences in starting cDNA quantity using the endogenous control, a preliminary experiment must be performed to prove the efficiency of the test and endogenous control amplifications are equivalent; this must be done for each mRNA of interest. The mathematics which allow incorporation of this endogenous control correction factor are complex, though described in detail in the manufacturer's documentation.

The $^{\Delta\Delta}C_t$ method has the small advantage of not requiring a standard curve on each experimental plate, which in the case of the experimental design used here would free 24 wells. However, there is then no control for plate to plate variability – which may be particularly important if the experiments are performed over a prolonged period of time, when the cDNA used at the beginning of the experiments may have deteriorated by the time it is used again some months later. For this reason, these experiments use the relative standard curve method throughout.

Preliminary Experiments

This section outlines:

- the results of experiments to determine the optimal relative concentrations of primers in each reaction, and
- the results of the preliminary experiments to determine the best endogenous gene to standardise starting cDNA quantity between samples.

a. Optimisation of relative primer concentrations

The TaqMan software supplied by the manufacturer designs primers and probes using a highly stringent algorithm, which combined with the very short amplicon size used in real-time PCR means there is no need to optimise melting or annealing temperature, or magnesium concentration, as is traditionally required in semi-quantitative PCR. However, the ability of the computer programme to predict the melting temperature of the primers (the temperature at which half of the oligonucleotides dissociate from the template) is not perfect. For maximum efficiency the forward and reverse primers should bind to the sequence of interest with the same affinity. That this is unlikely to be true in practice, despite the best efforts of the computer programme, necessitates a primer optimisation experiment for each primer combination. The principle of this experiment is that the *effective* primer melting temperature can be modified by the primer concentration. Reducing the concentration of one primer (relative to the other) has the same effect as reducing its melting temperature. The experiment uses three different concentrations of forward and reverse primer, with positive control placental cDNA as the template, to determine the combination which produces the lowest C_t and the highest plateau (which the software designates ΔR_n).

An example of the result of such an experiment, in this case for the optimisation of heme oxygenase 1, is shown in figures 6.1b and c.

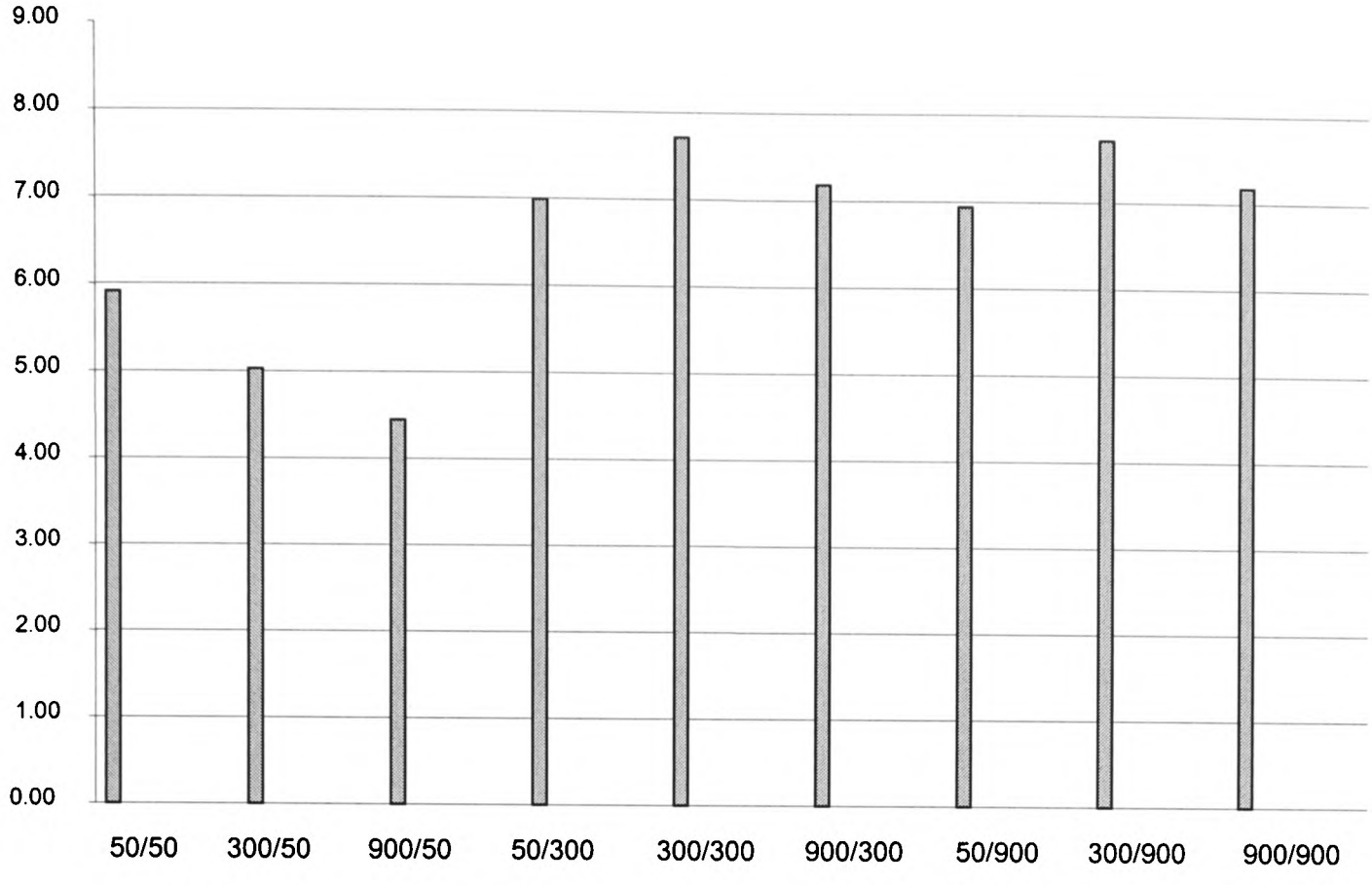


Figure 6.1b

ΔR_n values for each combination of heme oxygenase 1 forward and reverse primer concentration (fwd/rvs, nM).

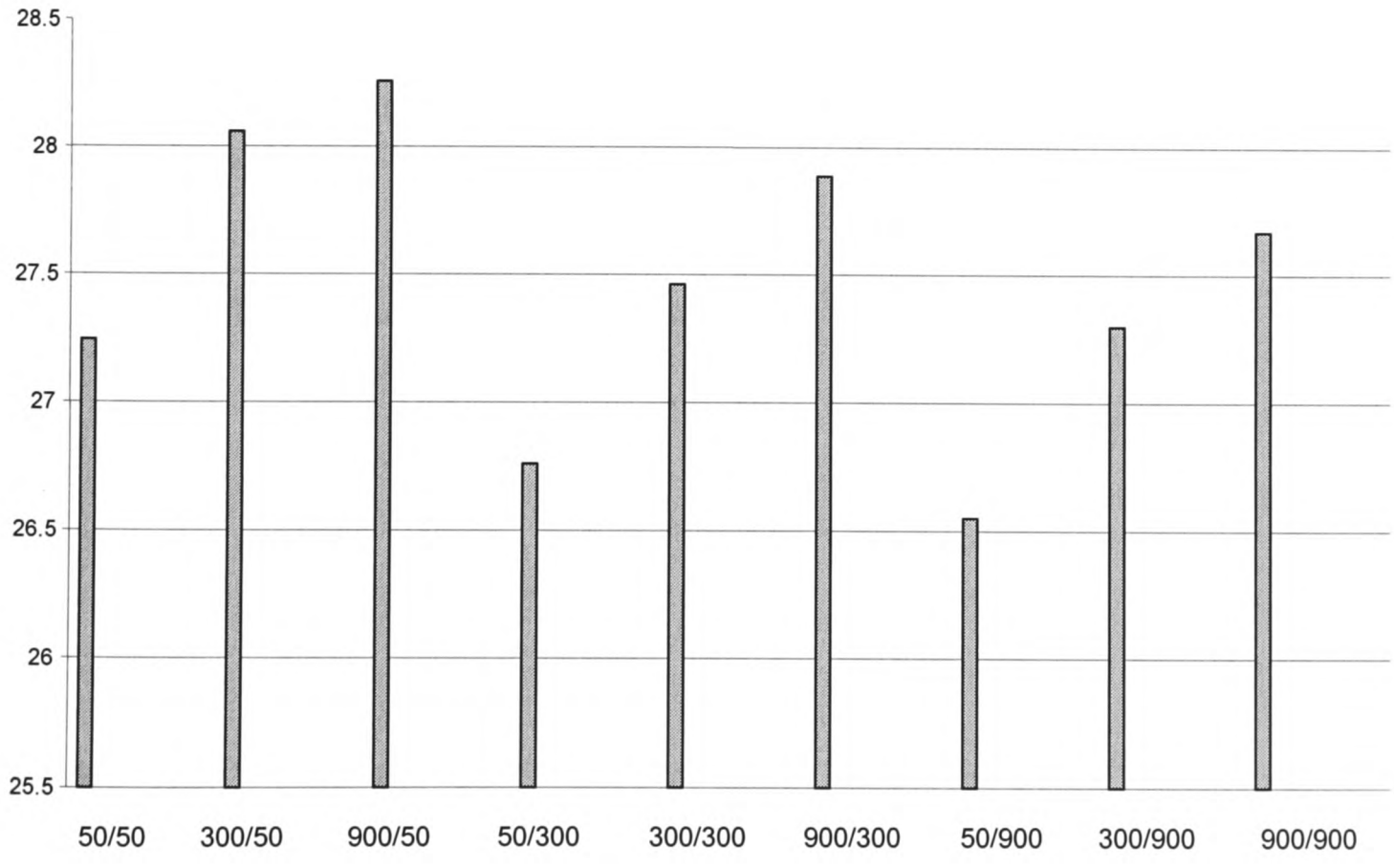


Figure 6.1c

C_t values for each combination of heme oxygenase 1 forward and reverse primer concentration (fwd/rvs, nM).

It is clear from figures A-IVa nd b that the combination of 50/900nm produces the lowest C_t and an acceptable ΔR_n. This was the combination chosen for subsequent experiments. The results of all the experiments using the other primer pairs are listed in table 6.1b.

	Forward primer concentration (nM)	***Reverse primer concentration (nM)***
CAT1	*900*	*900*
CAT2 (non-specific)	*300*	*300*
CAT2B	*300*	*900*
CAT4	*300*	*900*
CD98	*300*	*900*
y^+LAT1	*300*	*900*
y^+LAT2	*300*	*900*
iNOS	*300*	*900*
eNOS	*900*	*900*

Table 6.1b
Optimised forward and reverse primer concentrations

b. Determination of the best endogenous control gene

The purpose of amplifying an endogenous control gene is to allow standardisation between samples for small variations in starting cDNA quantity. These differences should not be large, as the amount of RNA (though incorporating residual DNA contamination) was standardised to 25ug/ml following determination of the A_{260} of each sample. However, the spectrophotometer technique is relatively inaccurate, does not correct for differences in the RT efficiency, and does not take into account the possibly variable quality of the RNA in the different samples. Ideally what is

required is some measure of the total, intact, reverse transcribed mRNA or total RNA quantity in each sample. This is accomplished by amplifying a gene which is expressed at a high and constant level (relative to the entire mRNA pool): when all samples are normalised to this quantity, a specific mRNA can be appreciated to increase or decrease in proportion to this total pool.

There are many such 'housekeeper' genes which have been proposed as candidates, of broadly two categories. Firstly, messenger RNAs for structural or metabolically essential proteins present in every cell: for example actin or GAPDH, and secondly the smaller subunit of ribosomal RNA, 18S. Whereas mRNA constitutes only about 1% of the total RNA content of a cell, rRNA is over 90%, and is much more likely to remain constant. 18S allows standardisation to cell number more than the total mRNA pool, but it is not unreasonable to assume that the relative proportions of mRNA to rRNA remain relatively constant.

It is particularly important that, even if there are small variations in the expression of the endogenous control between cells, there be no systematic difference in its expression in the two conditions studied. Some authors, particularly if using the highly accurate real-time technique, state that because the C_t values for their endogenous controls show no significant difference between the two groups studied, their selection is valid. This is an illogical argument. There could be a difference in the expression of the endogenous control gene in the two groups, even if they amplify to a similar degree, if the quantification of amplification is not sufficiently accurate to detect a difference in the number of samples tested, or if one of the groups is

amplified in a systematically different way to the other: for example, if one condition produces more fragile RNA.

A better, though still imperfect, justification for the use of an endogenous control is to observe its expression in the two sample groups in the context of a large number of other control cDNAs considered potentially suitable. The best selections will be those with the least differences in expression between groups. This experiment has been performed using one septic and one control PBMC sample and the array of 11 possible endogenous controls supplied loaded onto a 96 well plate, purchased from Applied Biosystems. The results of this experiment are shown in Figure 6.1d.

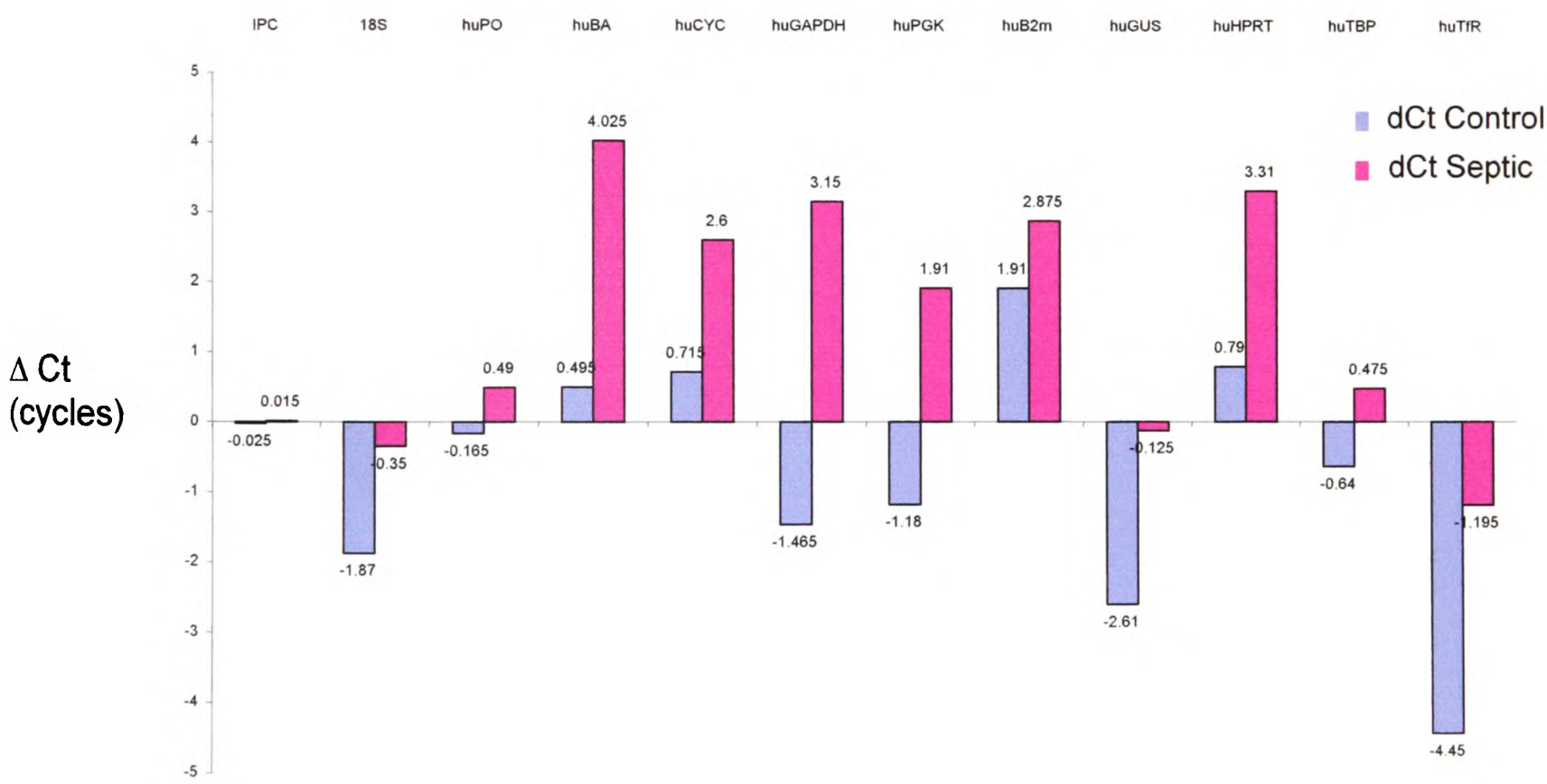


Figure 6.1d

Results of the plate comparing the expression of putative endogenous control RNA sequences in PBMCs from a septic patient and a healthy control subject. The best endogenous controls are those in which the expression in sepsis and control is similar, and not dissimilar to the expression in the positive control tissue (placenta), indicated by the arbitrary value of 0 in the above graph. huPO (acidic ribosomal protein) and huTBP (transcription factor IID, TATA binding protein), along with 18S, are the most acceptable candidates by these criteria. Notably, huGAPDH is shown to be a particularly poor choice.

Figure 6.1d demonstrates that 18S, acidic ribosomal protein (huPO) and transcription factor IID (huTBP) are the best candidates for use as endogenous controls. huPO and huTBP are very infrequently used in published studies of mRNA expression, and it was therefore decided to use 18S as the endogenous control for the experiments described in this thesis.

The use of 18S as the endogenous control requires one alteration to the standard RT-PCR procedure. The primer for the RT reaction in RT-PCR is usually oligo-dT, allowing selective amplification of mRNA, which is distinguished from other RNA types by its poly-A tail. 18S RNA is rRNA, not mRNA, and so lacks the poly-A tail. The RT reaction for all the samples and the positive control tissue used in the standard curve must therefore be primed with random hexamers, rather than oligo-dT.

6.2
Nitric oxide synthase and arginine transporter mRNA in peripheral blood mononuclear cells

Arginine transporters

All six arginine transporter primers strongly amplified cDNA from the placental standard, over a 1-1/1000 range of dilutions and with acceptable correlation coefficients. An example of the amplification plot (which displays fluorescent intensity increasing with cycle number) for the CAT2 primer/probe is shown in figure 6.2a. The cycle number at which the fluorescence intensity exceeds a defined threshold, C_t, is taken as the indicator of starting mRNA quantity. A lower C_t therefore implies a greater amount of starting material. The standard curve constructed using serial dilutions of placental cDNA, in this case amplified by the non-specific CAT2 primer, is shown in figure 6.2b. The quantity of CAT2 cDNA from the 'unknown' PBMC samples is read from this curve.

The identity of the amplified sequence was confirmed by visualisation of fragments of the predicted size on an agarose electrophoresis gel (figures 6.2c and d).

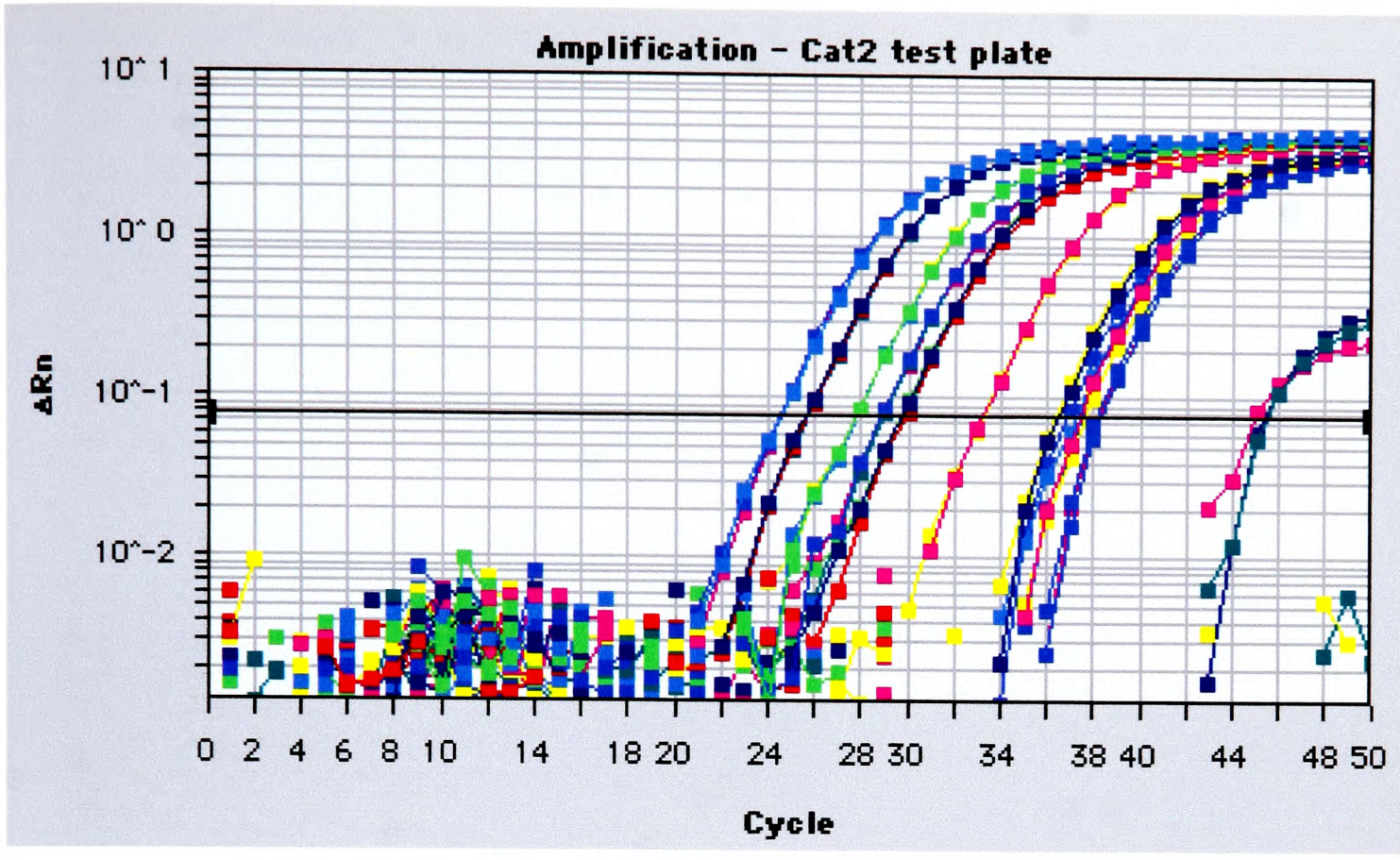


Figure 6.2a

Raw data from the real-time quantitative RT-PCR assay of CAT2 gene expression in PBMCs, demonstrating the easy identification of the linear phase of PCR product accumulation.

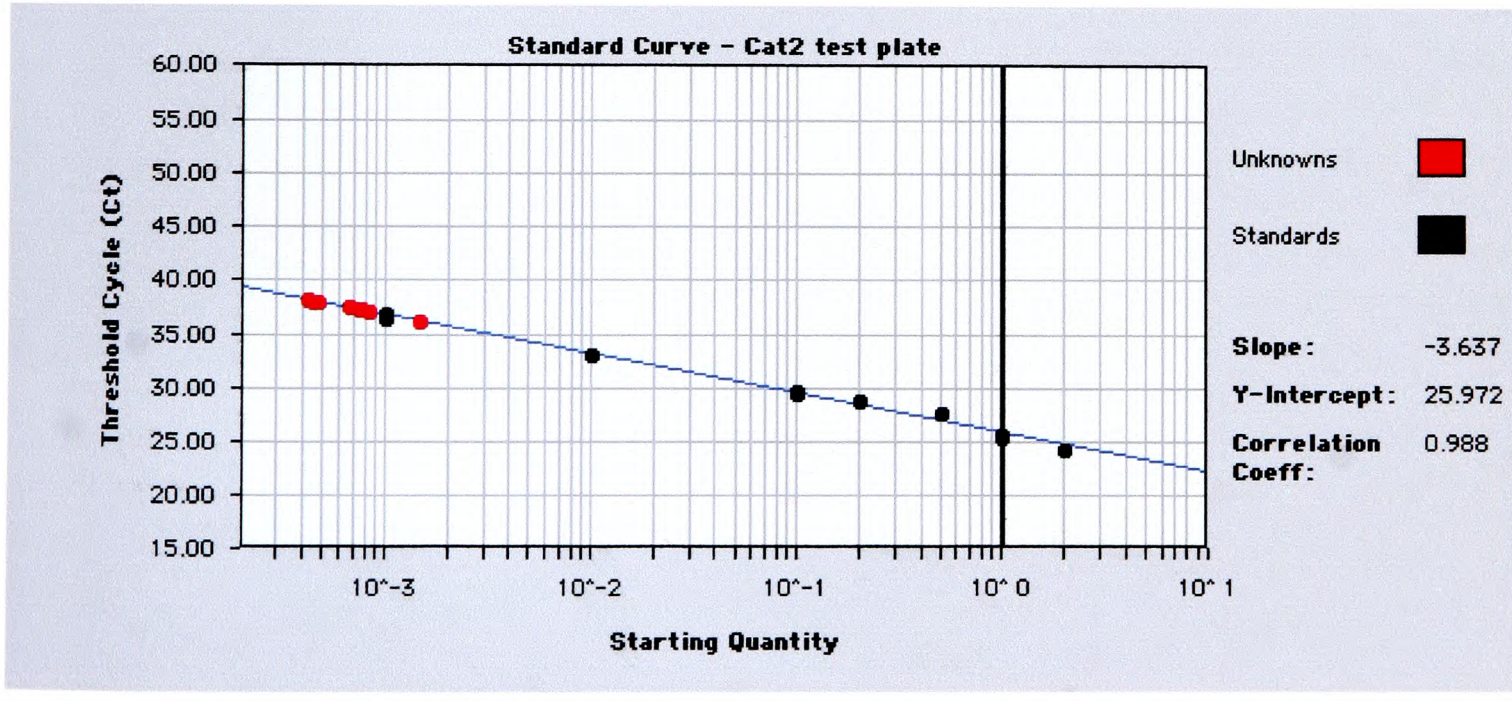
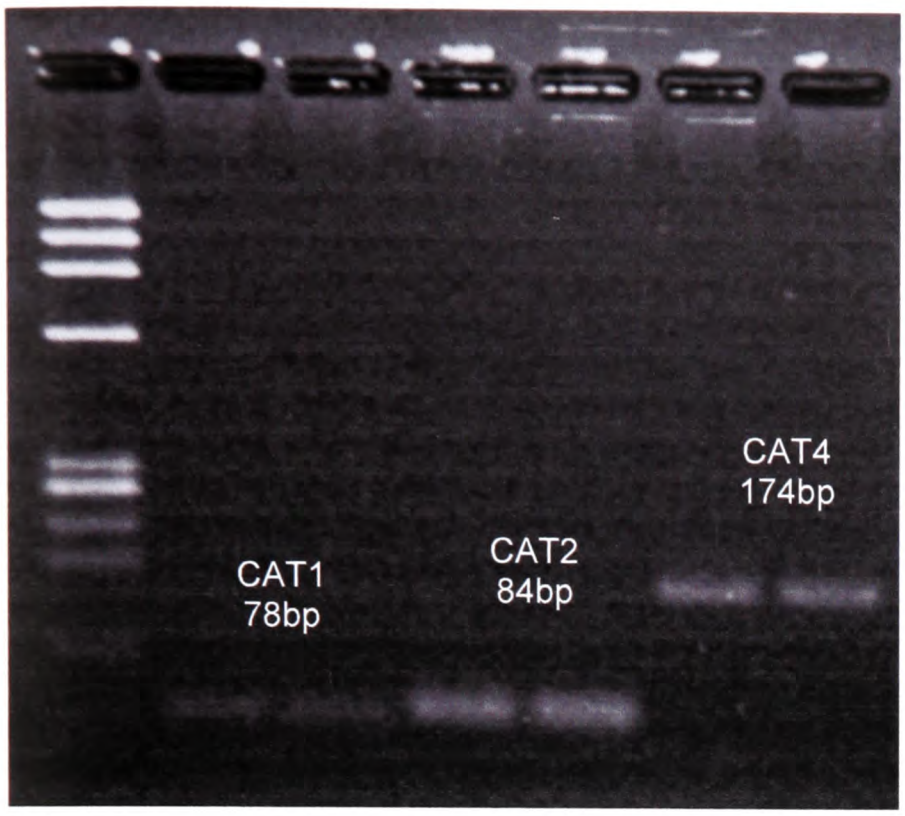
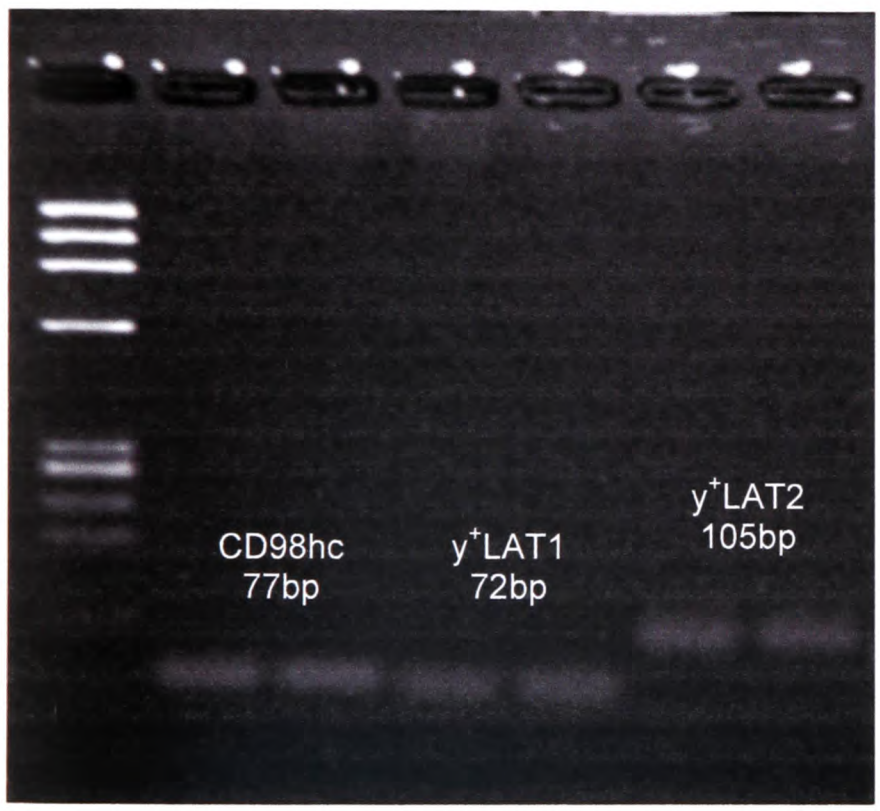


Figure 6.2b

Standard curve showing serial dilutions of placental control tissue, with overlaid data showing amplification of CAT2 mRNA. Only PBMCs from septic patients produced any CAT2 amplification (see below). Note the correlation coefficient of 0.988, indicating highly efficient amplification by this primer set.





Figures 6.2c and d
Agarose gels stained with ethidium bromide, demonstrating PCR amplicons of the expected sizes for all of the arginine transporter mRNAs studied.

The only significant difference between PBMCs from septic and control patients was an increase in CAT2 mRNA in sepsis. No CAT2 mRNA was detected in any of the control PBMCs, while it was present in 5 of the 7 septic samples (p=0.02, Fisher's exact test).

There were no significant differences in the amounts of the other transporter mRNAs studied (Table 6.2a)

Transporter system	mRNA encoding protein with this activity		Control (n=8)	Septic (n=7)
y^+	CAT1		79±40	84±33
	CAT2		none detected in any sample	0.02 ± 0.03; present in 5/7 samples*
	CAT4		6.5±10	0.0050±0.01
y^+L	heavy chain	CD98hc	90±40	141±56
	light	y^+LAT1	12±7	13±5
	chain	y^+LAT2	5±2.5	3±1.5

Table 6.2a

mRNA expression in PBMCs from 7 septic patients and 8 controls, expressed as mean % ± standard deviation of that found in placental reference tissue.
** p=0.02, Fisher's exact test*
The possible decrease in CAT4 and increase in CD98hc expression in sepsis did not reach statistical significance.

While the quantity of CAT2 mRNA appears small, it is not possible to conclude from this experiment whether this is a functionally significant amount. The value reported may be low due to a relative abundance of CAT2 mRNA in the placental calibrator tissue; the absolute amount of CAT2 in placenta is not known. Even if the CAT2 mRNA level in these PBMCs is lower than that of the other transporter mRNAs, this

does not necessarily reflect protein amount or correlate with its functional significance. No relationship of amino acid transporter mRNA to protein quantity or function has been described to date. However, the amounts of many other proteins are known to be poorly correlated with their mRNA level; thus, even if the absolute amount of CAT2 mRNA in these cells is small, the amount of functional protein may be large. What is certain is that these cells display increased y^+ transporter activity, and the increase in CAT2 was the only change observed at the mRNA level that could contribute to this.

The CAT2 gene encodes two different proteins with y^+ activity: CAT2A (found in liver, constitutively expressed, with a lower affinity for arginine and a greater maximal transport velocity) (Closs *et al.* 1997), and CAT2B. The kinetic characteristics of the CAT2B gene product more closely resemble CAT1 than CAT2A. While CAT2B is constitutively expressed in some cells, it is characterised by increased expression (in animal cells at least) in response to stimuli which also increase nitric oxide production and NOS expression (Gill *et al.* 1996) (Hattori *et al.* 1999). The primers used in this experiment will have amplified both CAT2A and CAT2B. Though it is therefore not possible to conclude which of these two is upregulated, evidence from studies of animal cells strongly suggests it is the CAT2B spice variant.

Nitric oxide synthases

The identities of the PCR products were confirmed by visualisation of fragments of the expected size by agarose gel electrophoresis (figure 6.2e).

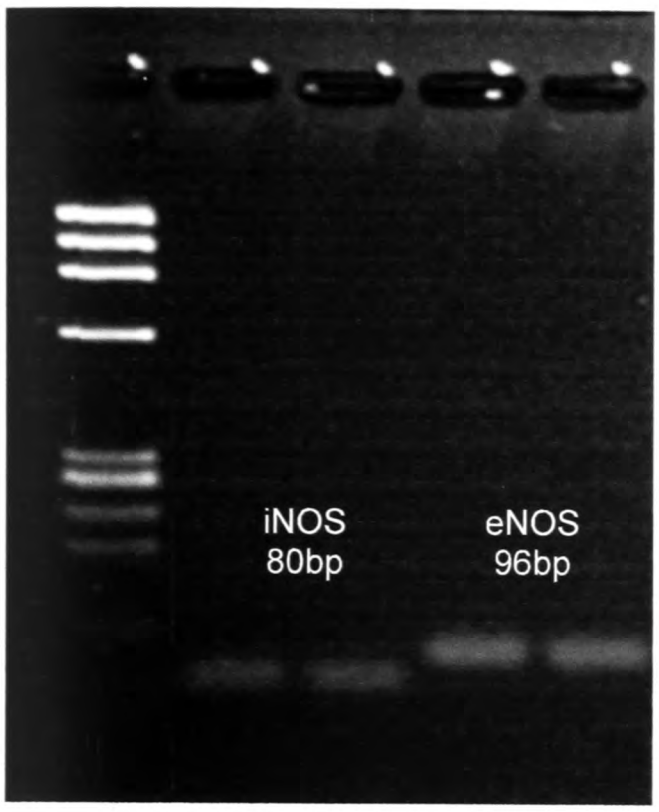


Figure 6.2e
Agarose gels stained with ethidium bromide, demonstrating PCR amplicons of the expected sizes for the mRNA of iNOS and eNOS.

The mRNA for both iNOS and eNOS were significantly reduced in sepsis (table 6.2b).

	Control (n=8)	**Septic (n=7)**	
iNOS	55 ± 31	10 ± 12	p = 0.03
eNOS	14 ± 4	7 ± 3	p = 0.002

Table 6.2b
mRNA expression in PBMCs from 7 septic patients and 8 controls, expressed as mean % ± standard deviation of that found in placental reference tissue. iNOS and eNOS were significantly decreased in sepsis.

6.3
Nitric oxide synthase and arginine transporter mRNA in arterial smooth muscle

Arginine transporters

The only difference between septic and control arterial smooth muscle was a decrease in the expression of CAT1 in sepsis (Table 6.3a).

Transporter system	mRNA encoding protein with this activity		Control (n=8)	Septic (n=8)
y^+	**CAT1**		194 ± 113	101 ± 43*
	CAT2		115 ± 65	113 ± 78
	CAT2B		163 ± 146	208 ± 160
	CAT4		27 ± 55	31 ± 58
y^+L	**heavy chain**	**CD98hc**	108 ± 50	83 ± 41
	light chain	**y^+LAT1**	1531 ± 835	1758 ± 1314
		y^+LAT2	3264 ± 2153	5197 ± 5928

Table 6.3a

mRNA expression in arterial smooth muscle from 8 septic patients and 8 controls, expressed as a % ± standard deviation of that found in placental reference tissue.
** The only difference between septic and control tissue was a decrease in CAT1 mRNA: p=0.05, Student's unpaired t test.*

Interestingly, the expression of all of the y^+ transporter mRNAs, as well as the y^+LAT1 and y^+LAT2 mRNAs, was substantially greater in arterial smooth muscle than in PBMCs, while there was little difference in the mRNA for CD98. In both septic and control arterial smooth muscle, CAT2 mRNA expression is equivalent to

the other y^+ mRNAs, whereas it is extremely low in PBMCs (indeed undetectable in healthy PBMCs).

Nitric oxide synthases

The pattern of mRNA expression for iNOS and eNOS exactly paralleled that found in peripheral blood mononuclear cells (Table 6.3b). Arterial smooth muscle has significantly decreased amounts of iNOS and eNOS mRNA. Interestingly, and in concert with the results of the NOS activity assay, there was substantially more iNOS and eNOS in arterial smooth muscle than in peripheral blood mononuclear cells. PBMCs are well recognised as producers of nitric oxide, whereas before this study this had not been established for arterial smooth muscle cells.

	Control (n=8)	Septic (n=8)	
iNOS	202 ± 92	85 ± 98	p = 0.03
eNOS	48 ± 44	12 ± 9	p = 0.04

Table 6.3b

mRNA expression in PBMCs from 8 septic patients and 8 controls (NOS), expressed as % ± standard deviation of that found in placental reference tissue.

CAT1 protein has been shown to co-localise with eNOS in plasma membrane associated caveolae (McDonald *et al.* 1997). eNOS mRNA is decreased in arterial smooth muscle from septic patients, so it is perhaps not surprising that CAT1 mRNA will similarly be decreased. It would be most interesting to determine the CAT1 protein levels in these cells, if indeed eNOS protein is increased. This experiment

awaits the development of a suitable antibody against CAT1. Attempts to do this have unfortunately to date met with little success (Appendix V).

CAT2B is conventionally thought to be the y^+ isoform upregulated in cells under oxidative or other stress (Hammermann *et al.* 2000) (Hattori *et al.* 1999), although rat vascular smooth muscle cells stimulated with lipopolysaccharide and IFNγ (which increased their CAT2B mRNA levels 7-fold) also upregulated their CAT1 (3 fold) and CAT2A (6 fold) mRNA (Baydoun *et al.* 1999). It that CAT1 was decreased in these cells lends further weight to the argument that studies of animal cells and cells activated by exposure to exclusively pro-inflammatory cytokines in vitro do not represent the pathology of clinical human sepsis.

Total RNA content of septic tissue

It is noteworthy that the amount of RNA extracted from arterial smooth muscle from patients with sepsis (normalised to tissue weight) was significantly greater than that from controls (control 200 ± 146, septic 815 ± 806 ng/ml/mg tissue)(n= 11 controls, 9 septics, p = 0.02, Student's unpaired *t* test)(figure 6.3a). This was also the case in a study of placenta from patients with pre-ecclampsia, compared to healthy pregnant controls (Reade, Speake & Sibley, Universities of Oxford and Manchester, unpublished results). Cells from patients with pre-eclampsia are thought to share many of the inflammatory changes present in sepsis.

The equivalent analysis of total RNA content was not possible for the PBMCs studied, as the cell count was not recorded in the PBMCs to be processed for RT-PCR.

A likely explanation is that the arterial smooth muscle has become metabolically more active in sepsis. Presumably other cellular pathways are also upregulated in sepsis. Though this phenomenon has not previously been reported in the literature, its significance would be better understood if the identity of the upregulated genes were known. This would be best analysed by RNA microarray (see Chapter 11 – Future studies).

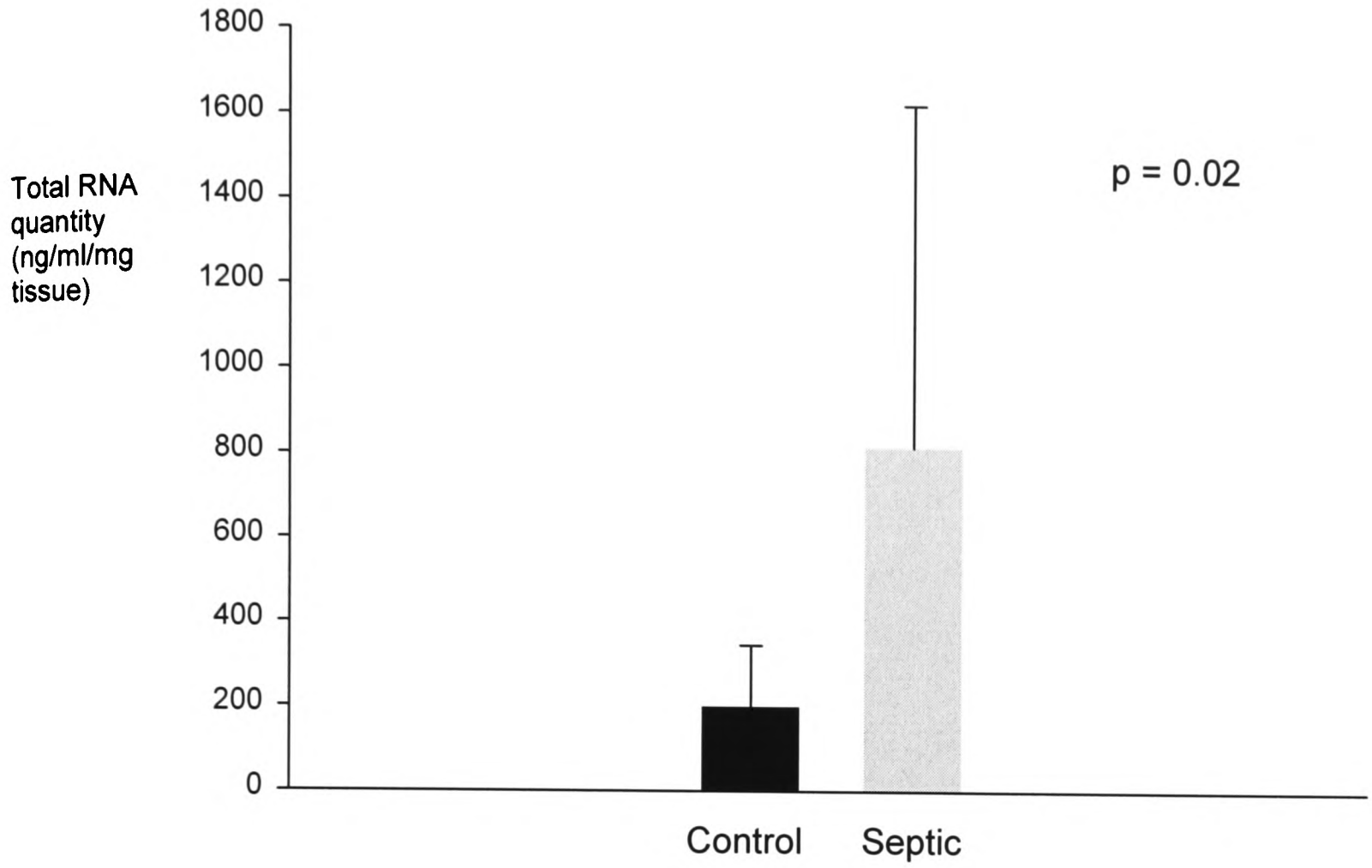


Figure 6.3a
Total RNA quantity isolated from arterial smooth muscle from septic patients and healthy controls, normalised to the weight of the tissue. Error bars represent standard deviation.

Chapter 7:

Arginine transporter and nitric oxide synthase protein quantity: Western blotting

7.1 Western blot: Method

PBMCs and arterial smooth muscle were stored frozen in AEBSF as described in Chapter 2. Samples were processed without any further adjustment of their concentration. However one aliquot of cells from each patient was used to estimate the protein concentration of the samples, as described in Appendix III. This value was used to standardise total protein loading between samples (see discussion, below); blots were also analysed without this standardisation.

A 1:10 dilution of protease inhibitor cocktail (containing AEBSF, aprotinin, leupeptin, bestatin, pepstatin A and E-64)(Sigma)(see Appendix II for details) was added to each aliquot of sample, which was then thawed on ice. To each sample was added an equal volume of Laemmli sample buffer with DTT (composition: see Appendix II). The samples were then mechanically homogenised by 20 passages through a 23 SWG needle and hypodermic syringe to ensure complete cell lysis, and to shear the DNA strands sufficiently to allow later aspiration into a fine pipette tip. The tubes containing the samples were heated to 95°C for 5 minutes and centrifuged at 15000g for 5 minutes to dissolve membrane associated proteins and pellet any insoluble protein.

10ul of each sample was loaded into the stacking gel wells above a 7.5% Tris HCl SDS polyacrilamide gel. Into positive control lanes were loaded with lysate from RAW 264.7 cells which had been activated with LPS and PMA. Kaleidoscope prestained molecular weight standards (BioRad) were used to identify the molecular weights of the protein bands. Gel electrophoresis (200V, Tris/Glycine/SDS buffer) was followed by transfer of proteins to nitrocellulose membranes (submerged blotting, Tris/glycine/methanol, 100V, 1hr). The membranes were blocked by agitating in 5% w/v low-fat dried milk in TBS with 0.1% Tween 20 (TBS-T) for 1 hour at room temperature. Membranes were rinsed with TBS-T, then incubated overnight at 4°C in the appropriate primary antibody solution, the optimal concentration of which had been determined in a series of preliminary experiments using positive control tissue. For iNOS this was 1:2000 anti-iNOS (Santa Cruz, rabbit anti-human) in TBS-T but no milk; for eNOS this was 1:500 anti-eNOS (Santa Cruz, rabbit anti-human) in TBS-T + milk. After further rinses with TBS-T, the membranes were incubated in 1:2000 anti-rabbit peroxidase linked donkey secondary antibody (Amersham), which allowed immunoreactive protein bands to be visualised using the ECL detection system (Amersham), according to the manufacturer's instructions.

So as to preserve the available biological samples for other procedures, eNOS protein was quantified by stripping the antibodies from the membrane which had been probed for the lower molecular weight heme oxygenase (see Chapter 9), and reprobing with eNOS antibody, rather than running a third gel. The membranes were immersed in the stripping buffer described in Appendix II for 30 minutes at 50°C, then washed thoroughly with TBS-T. After re-blocking (following exactly the same procedure as above), the membranes were incubated with the eNOS primary antibody.

Immunodetection was as described above. Though there was no positive control for eNOS included on these membranes, eNOS had been identified in a preliminary experiment at the expected molecular weight in human placenta extract using exactly these conditions (figure 7.1b).

The specificity of the antibody / protein interaction was confirmed in a series of preliminary experiments involving both omission of the primary antibodies, and pre-absorption of the primary antibodies using the peptides against which they had been raised. (figures 7.1a and b). All preliminary blots show specific staining at the expected molecular weights of the proteins, though there is also some staining of protein at other molecular weights. Only the intensity of the bands at the expected molecular weight has been used to determine protein concentration in the PBMCs and arterial smooth muscle studied.

The amount of protein detected by immunostaining of the nitrocellulose membranes was quantified by computerised analysis of the photographic images using the GDS8000 video capture gel documentation system (UVP) incorporating GrabIt and GelWorks software.

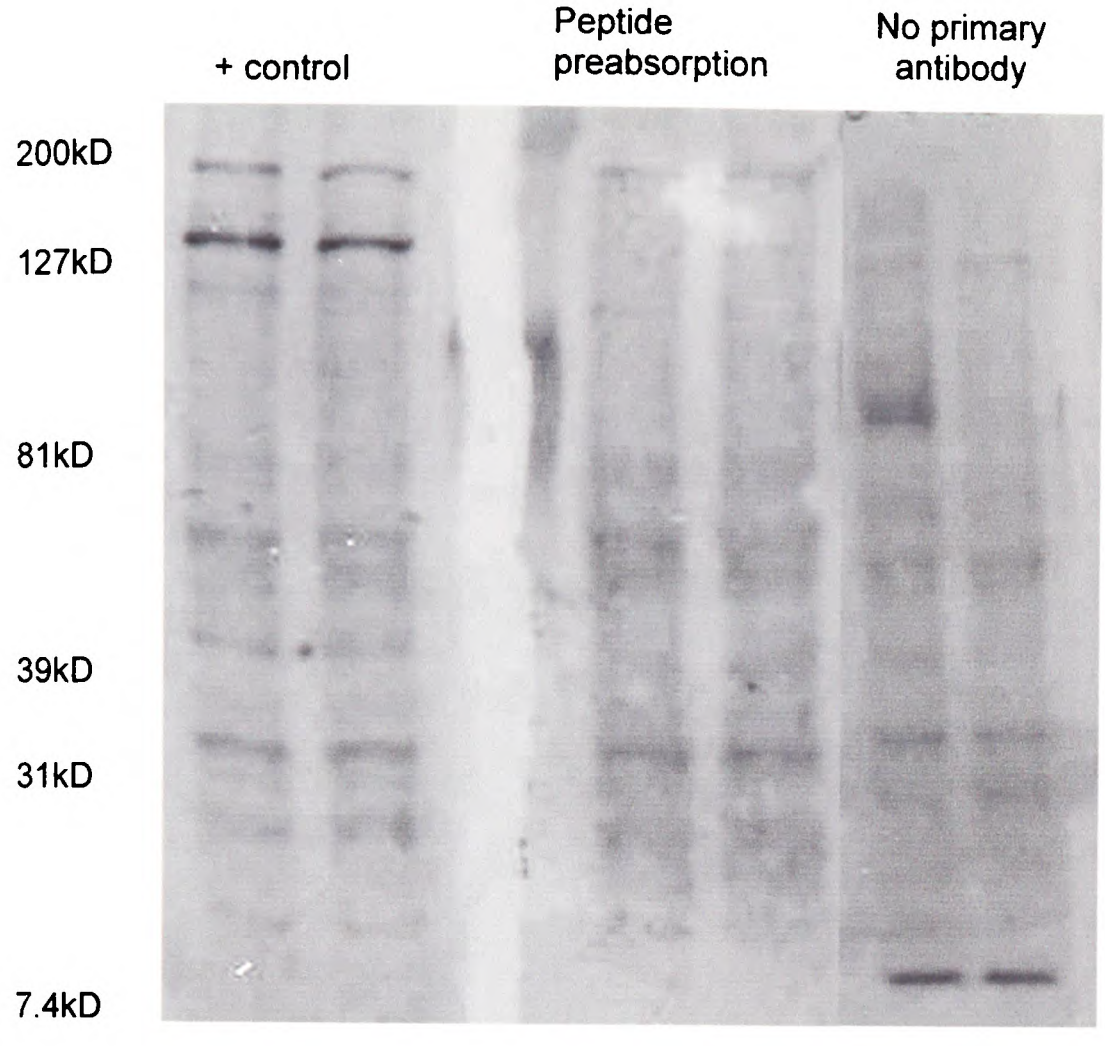


Figure 7.1a
No primary antibody and peptide pre-absorption control Western blots for iNOS protein. Coloured molecular weight markers were not detected by the primary and secondary antibodies, and so were marked on the photographic image of the blot by hand. The expected molecular weight of iNOS is 130-135 kD.

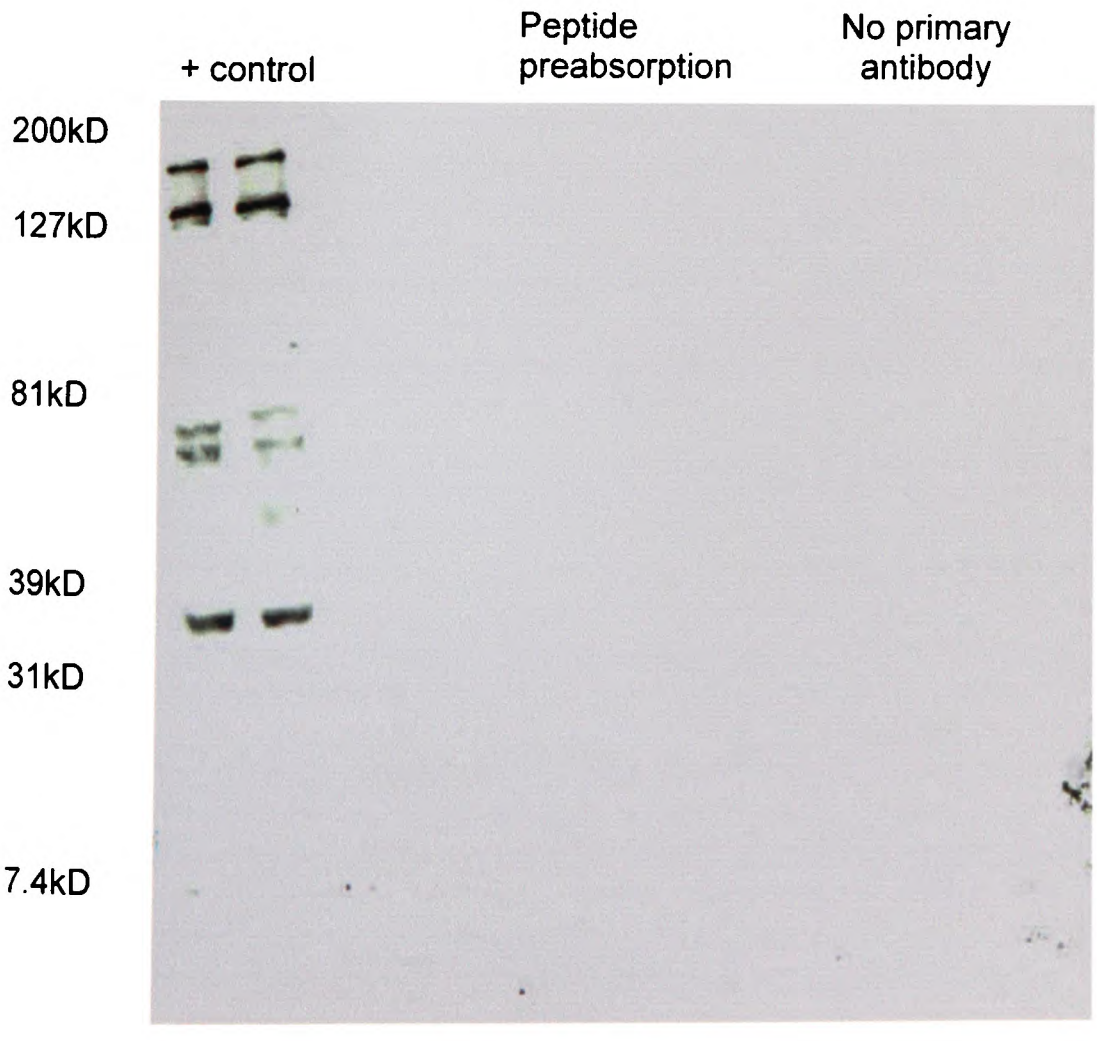


Figure 7.1b
No primary antibody and peptide pre-absorption control Western blots for eNOS protein. Molecular weight markers have been appended, as in figure 7.1a. The expected molecular weight of eNOS is 120 kD.

Double bands for both iNOS and eNOS

There are two apparently specific bands at approximately the expected molecular weight in these blots, for both iNOS and eNOS. In addition there are bands of much smaller sizes, which perhaps represent breakdown products of the NOS proteins; especially those at approximately half the expected molecular weight, as NOS is known to exist as a dimer of equal-sized proteins. Similar double bands have been observed for both iNOS (Purcell *et al.* 1997) and eNOS (Belhassen *et al.* 1997). In the case of eNOS in rat ventricular myocytes, a 150kD isoform was identified which was processed to a functional 135kD isoform prior to insertion in the sarcolemma. 135kD and 124kD isoforms of iNOS were identified in rat placenta, though only the 135kD isoform was present in rat cervix. The functional significance of the two isoforms, and indeed whether both were truly iNOS, was unclear from this study.

In the analysis of the blots in Chapter 7.2 and 7.3, it was intended to include both specific bands of approximately the expected molecular weight in the densitometry anaylsis, and also to analyse each separately. However, while in some samples there are clearly two bands, in others these are incompletely resolved. The analysis has thus only been performed with the two grouped together as one. It was also hoped that the possible NOS breakdown products (of much less molecular weight) could also be quantified. However, it was not possible in some samples to resolve these bands from others nearby which most likely represented non-specific staining. Ideally each patient sample would be performed with a pre-absorption control to identify the

specific lower molecular weight bands. Unfortunately this was not possible due to the limited availability of protein sample.

Correction for different concentrations of experimental sample protein loaded onto each lane

The use of measured total protein concentration in the samples prior to loading onto the gel to correct for differences in total protein loading is a reasonable technique. An alternative and perhaps superior method would have been to dilute the protein samples to a standard concentration, and include in the analysis of the blots some check of total protein loading; for example Coomassie blue staining of the residual protein bands on the gel following transfer, or use of a primary antibody raised against a 'housekeeping' protein such as GAPDH, in a manner similar to that used in the RT-PCR experiments. This was not done in these experiments because it was unclear how much total protein from the cell types in question would need to be loaded onto each gel to allow an adequate signal from the specific proteins in question to be measured. There was an insufficient quantity of many samples to allow for preliminary experiments to be performed to determine this amount. Rather than dilute all samples to match that with the least concentration, it was decided to run all samples at the concentration at which they were stored, after preliminary experiments with experimental control tissue and cells had demonstrated that most were likely to be at roughly an appropriate concentration. It would still have been possible to quantitate total protein loading by the methods descried above, but this was not done

as it was unclear whether this would have provided a more accurate correction factor than the highly reproducible protein concentration assay described in Appendix III.

7.2
Nitric oxide synthase protein in peripheral blood mononuclear cells

iNOS protein was detectable in PBMCs from all 5 healthy controls, but was absent or nearly absent in PBMCs from the 5 septic patients (figure 7.2a)(mean intensity of bands ± standard deviation (arbitrary units): a. when not corrected for total protein loading: 12.7± 7.0 in controls, 0.7 ± 1.7 in sepsis, p = 0.007; b. when corrected for total protein loading: 6.2 ± 3.2 in controls, 0.2 ± 0.4 in sepsis, p = 0.003).

eNOS protein was also significantly reduced in PBMCs from septic patients (figure 7.2b). (mean intensity of bands ± standard deviation (arbitrary units): a. when not corrected for total protein loading: 14.6 ± 8.8 in controls, 0.5 ± 1.1 in sepsis, p = 0.007; b. when corrected for total protein loading: 7.3 ± 4.6 in controls, 0.1 ± 0.3 in sepsis, p = 0.009).

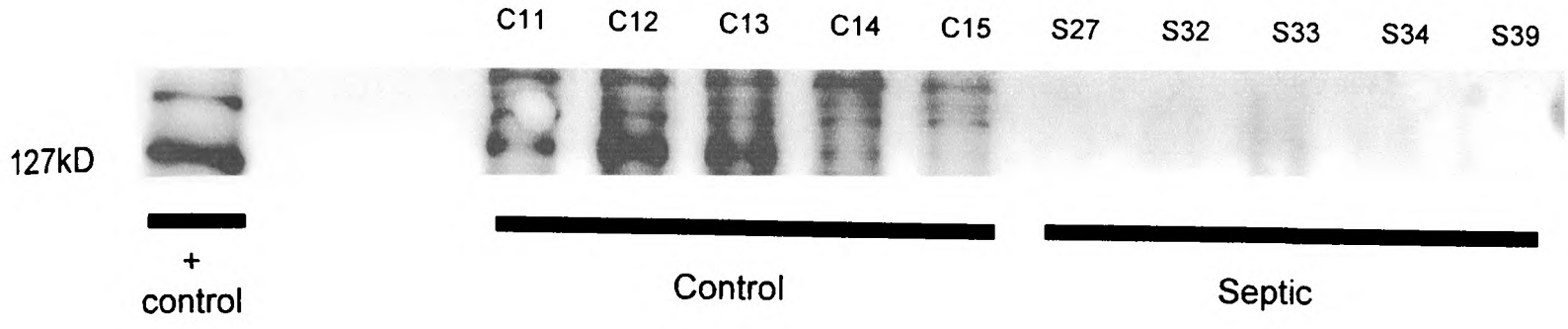


Figure 7.2a
iNOS protein detected by Western blotting of PBMCs from 5 septic patients and 5 controls.

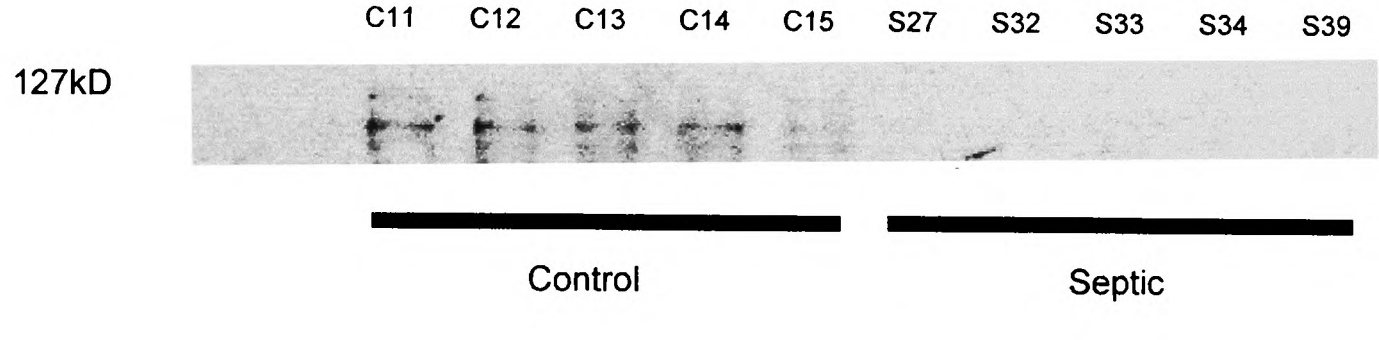


Figure 7.2b
eNOS protein detected by Western blotting of PBMCs from 5 septic patients and 5 controls. This blot was the stripped and reprobed membrane used in the heme oxygenase experiments described in Chapter 9; the positive control on this membrane was purified recombinant HO-1 protein, which does not bind the anti-eNOS used here. There is thus no positive control band visible on this gel; however, the bands do appear at the same molecular weight as those on the eNOS positive control blot (figure 7.1b)

There was a 3-fold variation in the quantity of protein loaded onto each lane, as shown in Table 7.2a, though analysis with and without correction for these differences did not affect the interpretation of the results.

	[protein] mg/ml		[protein] mg/ml
Control 11	1.92	Septic 27	3.25
Control 12	2.07	Septic 32	1.39
Control 13	2.44	Septic 33	4.63
Control 14	1.43	Septic 34	1.27
Control 15	2.46	Septic 39	3.55

Table 7.2a

Protein concentration of each of the samples loaded onto the Western blot gel, as determined by the bicinchoninic acid method described in Appendix III. These values were used to adjust the results of densitometry analysis of the images above.

The quality of these blots is relatively poor. Especially in the case of iNOS, it appears that the protein had not been adequately dissolved in the buffer, and hence has not resolved into clear bands. Unfortunately there was an insufficient quantity of some of these samples with which to repeat the experiment. While the results of this experiment must therefore be interpreted with caution, it is worth noting that protein levels for iNOS and eNOS appear to mirror the changes seen in mRNA in Chapter 6. Additionally, these cells do display some of the expected changes of PBMCs in sepsis. Notably, the protein blot which produced figure 7.2, when probed with anti-heme oxygenase (see Chapter 9), displayed the expected increase in HO-1 in sepsis.

7.3
Nitric oxide synthase protein in arterial smooth muscle

There was no increase in expression of iNOS; indeed iNOS protein was only barely detectable in these arterial smooth muscle samples (figure 7.3a).

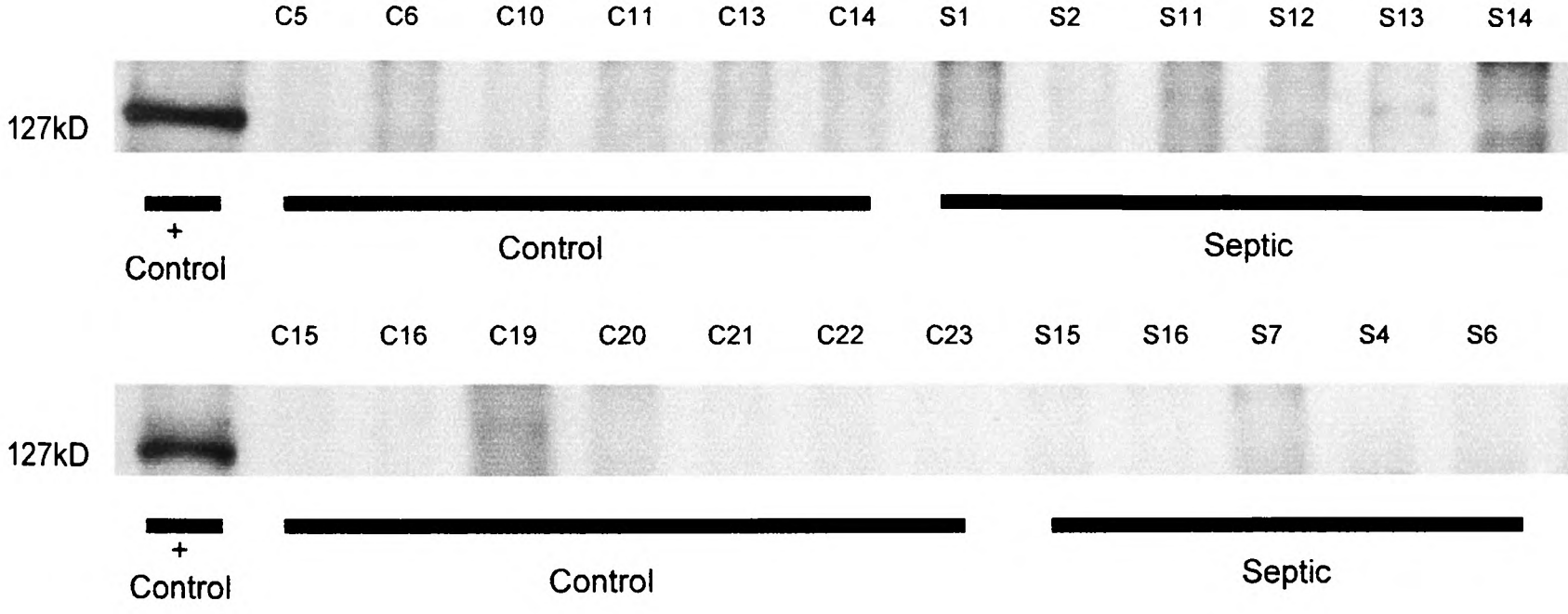


Figure 7.3a

iNOS protein detected by Western blotting in arterial smooth muscle from 13 control and 11 septic patients. iNOS is not detectable in most of these samples. There is no significant difference between septic and control samples when these bands are analysed by densitometry, with or without correction for differences in total amount of protein loaded. Note that subject designations (C5, S1, etc) are specific to arterial smooth muscle, and do not relate to those for the PBMC samples. Some subjects contributed tissue to both studies; this is described in detail in Appendix I.

In contrast, the expression of eNOS was significantly increased in arterial smooth muscle from septic patients compared to healthy controls (3.9 ± 5.3 units/mg control; 9.6 ± 7.1 units/mg septic)(both specific eNOS protein bands measured together, corrected for total protein loading (mg) per lane; mean ± standard deviation; n=13 controls, 11 septics, p=0.04, Student's unpaired *t* test)(figure 7.3b).

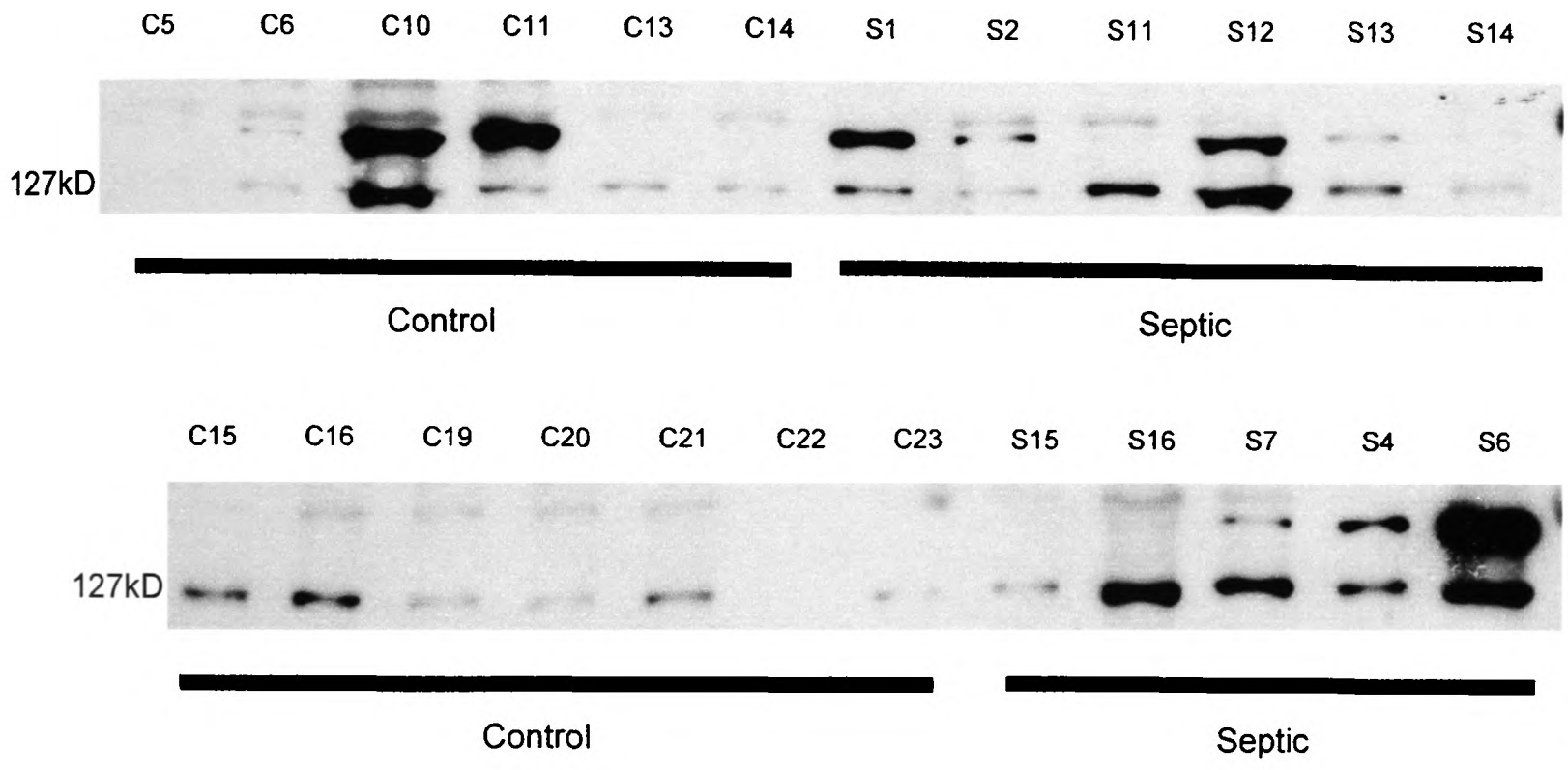


Figure 7.3b

eNOS protein detected by Western blotting in arterial smooth muscle from 13 control and 11 septic patients. There are two specific bands for eNOS, which is consistent with some previously published studies (see chapter 7.1 for discussion). As both bands disappear with preabsorption of the anti-eNOS antibody by purified eNOS, both were used in the calculation of total eNOS amount by densitometry.
As was the case for eNOS PBMC Western blots, this blot was the stripped and reprobed membrane used in the heme oxygenase experiments described in Chapter 9; the positive control on this membrane was purified recombinant HO-1 protein, which does not bind the anti-eNOS used here. There is thus no positive control band visible on this gel; however, the bands do appear at the same molecular weight as those on the eNOS positive control blot (figure 7.1b)

The amounts of total protein from different samples loaded onto each well were broadly comparable, as shown in Table 7.3a.

	[protein] mg/ml		**[protein] mg/ml**
Control 5	1.18	Septic 1	1.24
Control 6	1.35	Septic 2	1.54
Control 10	1.90	Septic 4	1.35
Control 11	2.61	Septic 6	3.08
Control 13	0.99	Septic 7	1.77
Control 14	2.81	Septic 11	1.38
Control 15	1.05	Septic 12	2.79
Control 16	1.82	Septic 13	1.18
Control 20	1.14	Septic 14	1.72
Control 21	1.33	Septic 15	0.87
Control 22	1.25	Septic 16	2.20
Control 23	2.33		

Table 7.3a

Protein concentration of each of the samples loaded onto the Western blot gel, as determined by the bicinchoninic acid method described in Appendix III. These values were used to adjust the results of densitometry analysis of the images above. There is no significant difference between septic and control tissues, which would be expected as similar sized vessels were collected from both septic and control patients.

A number of the samples on the image of the eNOS blot (especially C10, C11 and S6) (figure 7.3b) show an atypical pattern compared to the rest of the membrane. This pattern is not repeated on the iNOS blot, though when the membranes are probed for inducible heme oxygenase (figure 8.2b), HO-1 in C11 and S6 appear to have run to a lower molecular weight. C11 and S6 (but not C10) were at particularly high total protein concentrations when loaded onto the gel (table 7.3a), and it may be that there was inadequate dissolution of the protein at such a high concentration. However, S16 (for example) was also present at a high concentration, but has produced a more

typical pattern. While certain samples do not appear to fit the general trend, then, there appears no good reason to exclude them from the overall analysis of the blots.

The apparent increase in eNOS protein is interesting, in that eNOS mRNA was decreased in these cells. Total NOS activity, as reported in Chapter 3.3, was increased in arterial smooth muscle from septic patients, but this increase appeared to be due to iNOS rather than eNOS. The results of the NOS activity study are, however, limited by the signal to noise ratio of the assay and the small numbers of samples studied. There was a trend to increased eNOS activity observed in sepsis, which agrees with this eNOS Western blot result. That eNOS protein remains elevated while its mRNA is reduced is not impossible, as mRNA is generally considered more labile than protein.

As was the case for the Western blots of iNOS and eNOS in PBMCs, the technical quality of these blots is relatively poor. In particular, there was substantial binding of antibody to the membrane at lower molecular weights. This may reflect proteolysis of the sample, or non-specific binding of the antibody to other proteins in the homogenate. Proteolysis my have resulted from the relatively prolonged procedure undertaken to dissolve the protein, though it would appear that even this may have been insufficient in the PBMC samples, as noted in Chapter 7.2. The control tissue used in the development of the assay produced relatively specific bands (Chapter 7.1). Unfortunately there was insufficient human arterial smooth muscle and septic PBMCs with which to develop the technique further in these specific tissues. Use of animal tissues and a range of different tissue preparation methods might allow the development of a better assay of these proteins in these human tissues.

The technical limitations of these blots imply that the results must be interpreted with caution. A more sensitive assay for iNOS protein may indeed detect a difference in septic arterial smooth muscle. Better quality blots might add more certainty to the conclusion that eNOS is increased in sepsis. However, as was the case for PBMCs, it is worth noting that heme oxygenase protein (which would be expected to be increased in arterial smooth muscle in sepsis) does indeed appear to be increased in these samples (Chapter 9). Moreover, the inability to demonstrate the increase in iNOS protein seen in many animal models of sepsis agrees with the finding of reduced iNOS mRNA in these tissues (Chapter 6). iNOS protein is not seen in healthy arterial smooth muscle in other studies (Chester *et al.* 1998), so if its amount is decreased in sepsis, as is suggested from the mRNA results, it is not surprising that virtually no iNOS was detected by Western blotting in these tissues. Interestingly, the same primary antibody used in these Western blotting experiments was able to detect iNOS in the media of control vessels analysed by immunohistochemistry (Chapter 9).

Chapter 8:

Heme oxygenase mRNA and protein quantity: Real-time reverse transcription polymerase chain reaction and Western blotting

8.1 Real-time reverse transcription polymerase chain reaction and Western blotting for inducible heme oxygenase mRNA and protein: Method

These studies of heme oxygenase used the same cDNA and protein homogenate prepared for the studies of arginine transporters and nitric oxide synthase enzymes described in Chapter 6 and 7.

Real-time RT-PCR: heme oxygenase

The experiments were performed in exactly the same way as those described in Chapter 6, using the primers and probe shown in table 8.1a. The concentration of forward and reverse primer was optimised in a similar preliminary experiment to those described in Chapter 6; the optimal primer concentrations were 50nM and 900nM (forward and reverse, respectively).

Target sequence	GenBank identification		Sequence	Position	Amplicon length
HO-1	4504436	Forward	AGGCCAAGACTGCGTTCCT	685-704	139bp
		Reverse	GCAGAATCTTGCACTTTGTTGCT	823-800	
		Probe	CTCAACATCCAGCTCTTTGAGGAGTTGCAG	705-735	

Table 8.1a.
Sequences of the primers and probes used in this study of heme oxygenase.

The identity of the PCR products was confirmed by visualisation of fragments of the expected size by agarose gel electrophoresis (figure 8.1a).

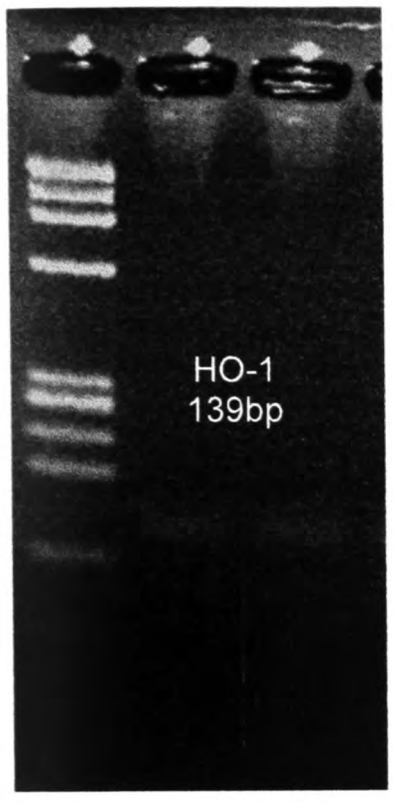


Figure 8.1a
Agarose gel stained with ethidium bromide, demonstrating a PCR amplicon of the expected size for the mRNA of HO-1.

Western blotting: heme oxygenase

Western blots of protein homogenate were prepared from PBMCs and arterial smooth muscle exactly as described in Chapter 7. Membranes were rinsed with TBS-T, then incubated overnight at 4°C in 1:1000 anti-heme oxygenase (StressGen, rabbit anti-human) in TBS-T + milk (the optimal concentration having been determined in a preliminary experiment using positive control purified recombinant HO-1 protein; figure 8.1b). Hybridisation of secondary antibody, protein band visualisation and correction for total protein loading were also exactly as previously described.

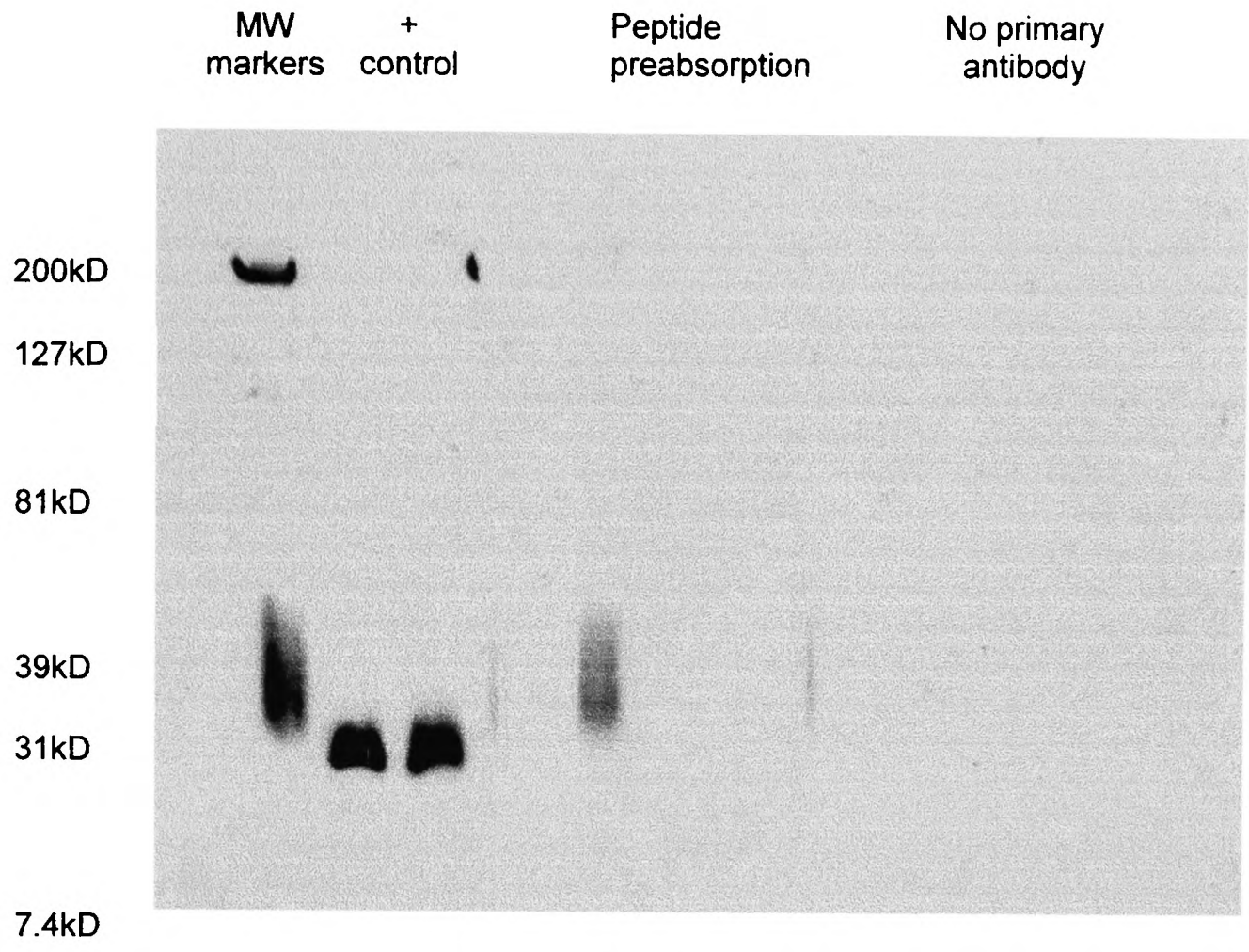


Figure 8.1b

No primary antibody and peptide pre-absorption control Western blots for HO-1, using purified recombinant HO-1 protein. Most of the coloured molecular weight markers were not detected by the primary and secondary antibody, and so were marked on the photographic image of the blot by hand – though unexpectedly there was a relatively non-specific interaction of the primary antibody and the 200kD and 39kD markers. The expected molecular weight of HO-1 is 32kD.

8.2
Inducible heme oxygenase mRNA and protein in peripheral blood mononuclear cells and arterial smooth muscle

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Inducible heme oxygenase mRNA

mRNA for heme oxygenase 1 was significantly increased in both peripheral blood mononuclear cells and arterial smooth muscle from septic patients compared to controls (table 8.2a).

Note that in the iNOS and eNOS experiments, samples from 8 septic patients and 8 controls were analysed. These same samples were used for the HO-1 experiments, though analysis of these results showed a difference which was not quite statistically significant; hence another 3 controls and 1 septic patient were added to the study of HO-1.

	Control	Septic	
PBMC HO-1	252 ± 149 (n=7)	424 ± 159 (n=8)	p = 0.05
ASM HO-1	158 ± 138 (n=11)	280 ± 166 (n=9)	p = 0.01

Table 8.2a

Inducible heme oxygenase mRNA expression in PBMCs and arterial smooth muscle, expressed as mean % ± standard deviation of that found in placental reference tissue. HO-1 mRNA was significantly increased in both tissue types in sepsis.

Inducible heme oxygenase protein

HO-1 protein was also significantly increased in both PBMCs and arterial smooth muscle from septic patients (table 8.2b; figures 8.2a and b).

	Control	**Septic**	
PBMC HO-1 (corrected for protein amount)	3.6 ± 3.6 (n=5)	11.8 ± 7.1 (n=5)	p = 0.05
PBMC HO-1 (not corrected for protein amount)	7.7 ± 8.2 (n=5)	28.1 ± 15.4 (n=5)	p = 0.03
ASM HO-1 (corrected for protein amount)	2.2 ± 5.5 (n=10)	46 ± 51 (n=11)	p = 0.015
ASM HO-1 (not corrected for protein amount)	4.6 ± 10.7 (n=10)	64 ± 58 (n=11)	p = 0.005

Table 8.2b

Inducible heme oxygenase protein expression in PBMCs and arterial smooth muscle, expressed as mean arbitrary density units ± standard deviation, either corrected or not corrected for total protein loading on the gel. HO-1 protein was significantly increased in both tissue types in sepsis, by either analysis.

The protein concentrations used to correct for small differences in protein loading between difference samples are the same as those shown in tables 7.2a and 7.3a.

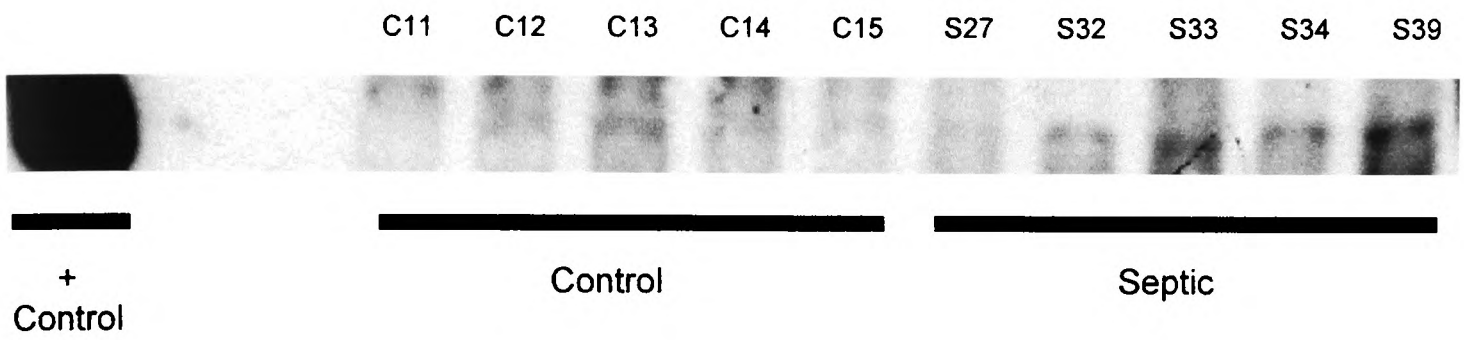


Figure 8.2a
HO-1 protein as determined by Western blotting of PBMCs from 5 septic patients and 5 controls.

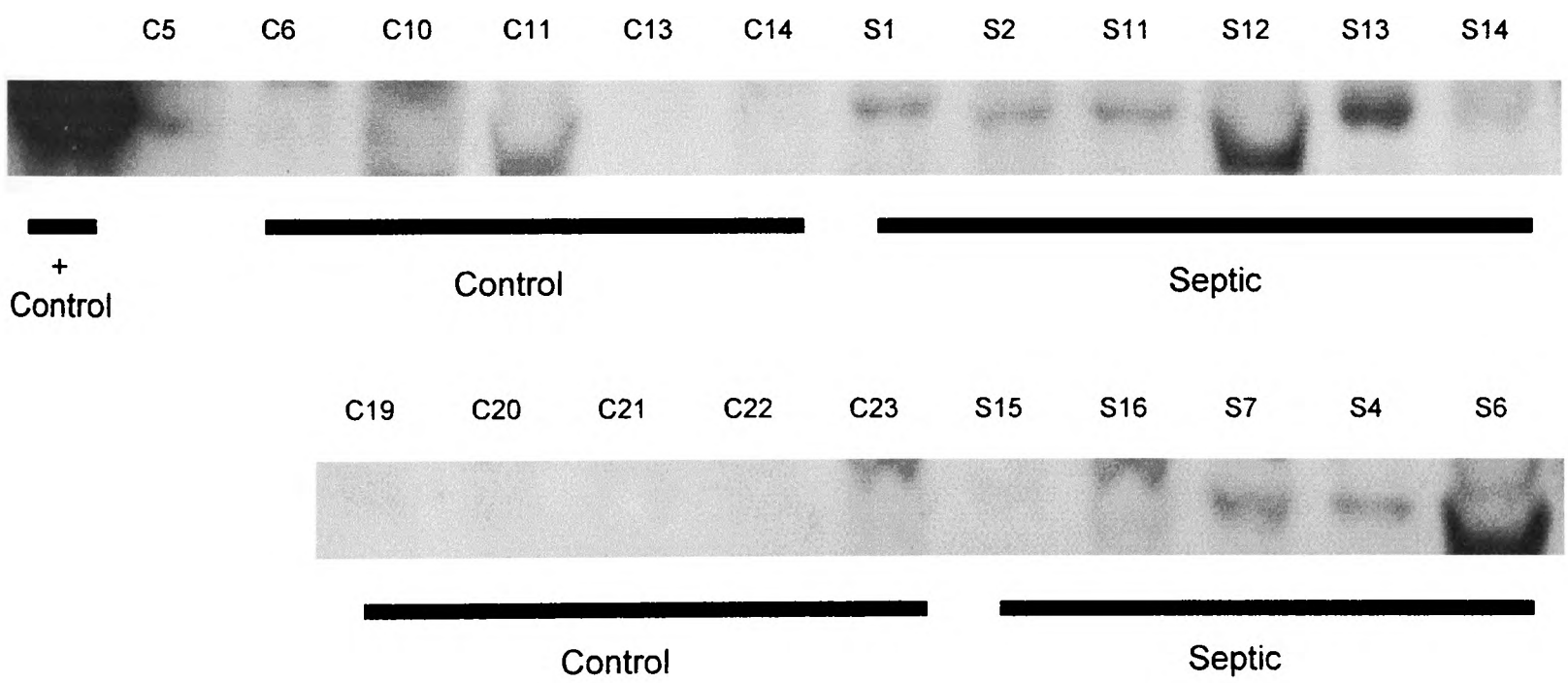


Figure 8.2b
Heme oxygenase 1 in arterial smooth muscle from 10 control and 11 septic patients. Note C5 was excluded from the analysis as it appeared the band present in this lane had spilled over from the adjacent positive control lane.

The increase in HO-1 mRNA and protein seen here in sepsis is entirely consistent with results using animal and in vitro models. This suggests that these clinical samples are indeed 'activated' cells. Chapters 6 and 7 present the first study of NOS mRNA and protein in PBMCs and arterial smooth muscle from patients with clinical sepsis, using the same tissues as were used here. No animal or in vitro study purporting to model sepsis has ever found a decrease in NOS mRNAs; virtually all report an increase. That HO-1 is increased in the tissues with decreased NOS expression improves the degree of confidence in this unexpected result. These tissues appear similar to the various experimental models with respect to heme oxygenase regulation, but are for some reason markedly dissimilar with respect to nitric oxide biology. The implications of these results are explored in detail at the end of this thesis.

Chapter 9:

Location of nitric oxide synthase and heme oxygenase protein in vessel wall: Immunohistochemistry

9.1 Localisation of nitric oxide synthase and heme oxygnase in arterial vessel wall by immunocytochemistry: Method

The protein and mRNA results described in Chapters 6, 7 and 8 are based on experiments using the mechanically isolated medial layer of mesenteric artery. It has been assumed that the cell population in this tissue is exclusively arterial smooth muscle; this is supported by the H&E tissue images presented in Chapter 2. However, it is possible that other cells (in particular, inflammatory cells) may be present in the media. Moreover, it may be that other layers of the arterial wall produce nitric oxide in sepsis. This chapter address both of these questions by immunocytochemical localisation of iNOS, eNOS and HO-1 protein in complete sections of mesenteric artery.

The experiments presented in this chapter have been performed in collaboration with Dr J.L. Millo of the Nuffield Department of Anaesthetics, University of Oxford. I contributed to the collection of tissue samples and the design of the experiments; however Dr Millo prepared all of the tissue samples presented here.

Rings of mesenteric artery (with the endothelium and adventitia intact) were fixed in 4% paraformaldehyde, permeated with 20% sucrose, suspended in mounting medium (Tissue-Tek OCT compound, Sakura Finetek), frozen slowly over liquid nitrogen and stored at –80°C until 8uM sections were cut and laid onto glass slides. Tissues were

permeabilized in 0.2% Triton X and blocked with 10% goat serum in 2% bovine serum albumin before overnight incubation at 4°C with the appropriate dilution of primary antibody in 2% BSA. Antibody dilutions were 1:200 for anti-iNOS and anti-eNOS (the same antibodies were used as in the Western blotting experiments). Tissues were rinsed in 1% hydrogen peroxide to quench endogenous peroxidase activity before incubation with 1:200 biotinylated secondary antibody and detection using the Avidin/Biotinylated Peroxidase Complex system with 3,3 diaminobenzidine as the chromogenic substrate (Vector Laboratories). Haematoxlin was used as a counterstain. Some sections from each patient were also stained with haematoxylin and eosin, to assess the preservation of their morphology.

9.2
Location of nitric oxide synthase and heme oxygenase in arterial vessel wall

Control experiments in each of the tissues (in which the primary antibody was omitted) confirmed the specificity of the immunostaining (figure 9.2a).

A chronic inflammatory cell infiltrate was noted in the adventitia of the 4 septic vessels studied, but not in that of the 3 controls.

The endothelial layer stained strongly for eNOS in each of the 3 vessels from control patients. The intensity of this staining was reduced or non-existent in the 4 septic vessels. Representative sections are shown in figures 9.2b and c. There was a low intensity staining of eNOS in the media of both septic and control vessels.

HO-1 and iNOS protein were located principally in the medial layer of the vessel wall in both septic and control vessels, as demonstrated in the representative photomicrographs in figures 9.2 d/e and f/g. The inflammatory cell infiltrate in the adventitia of the septic vessels also stained for iNOS and HO-1.

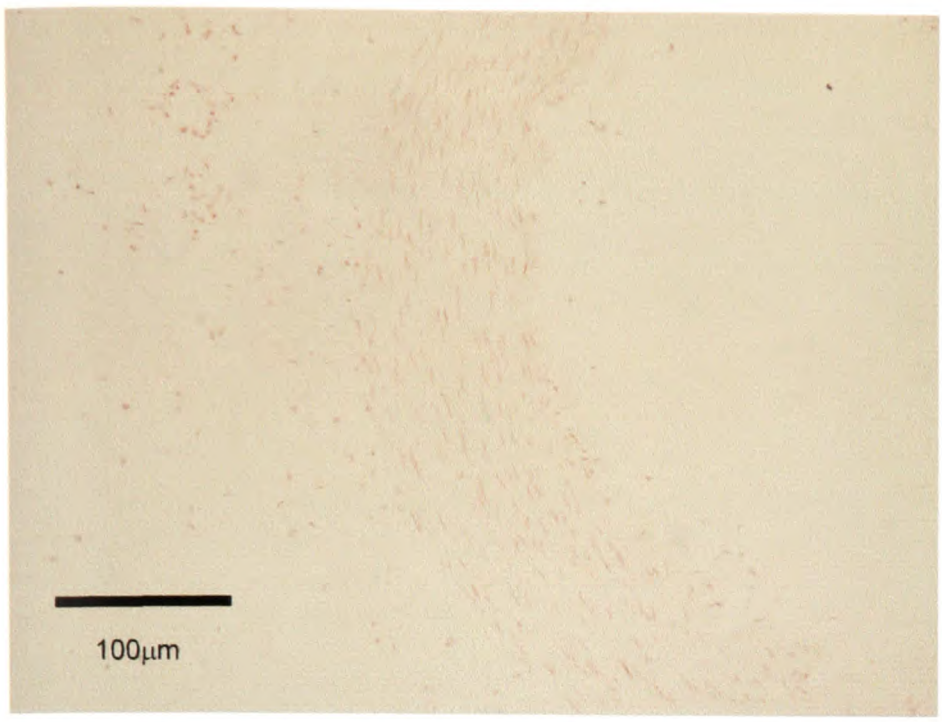


Figure 9.2a
Experimental control tissue stained with secondary, but no primary, antibody

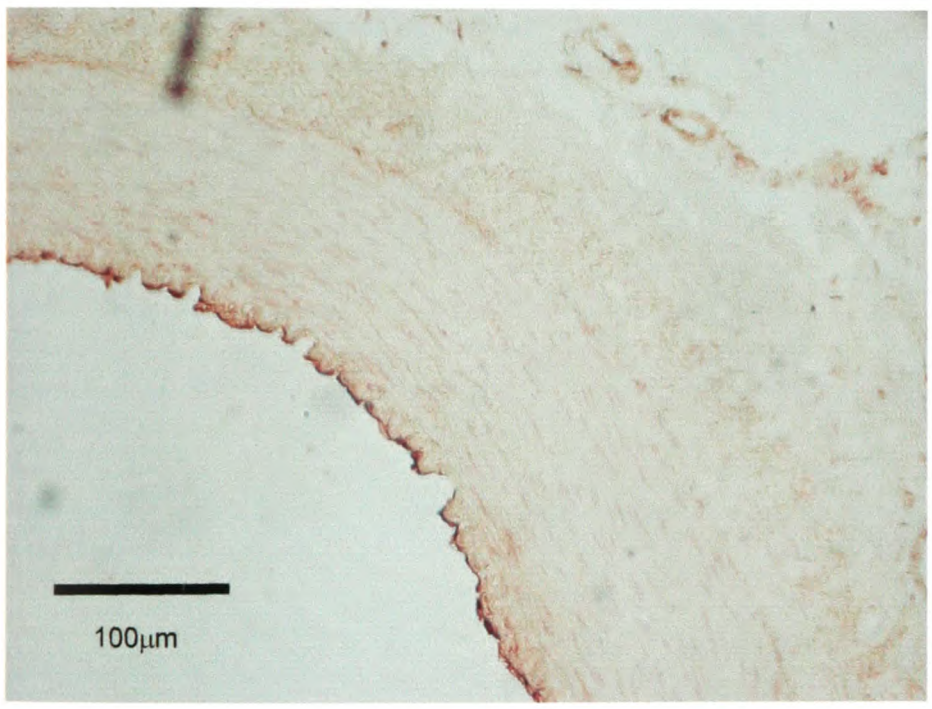


Figure 9.2b
Control tissue stained with anti-eNOS. As expected, eNOS is present principally in the endothelium.

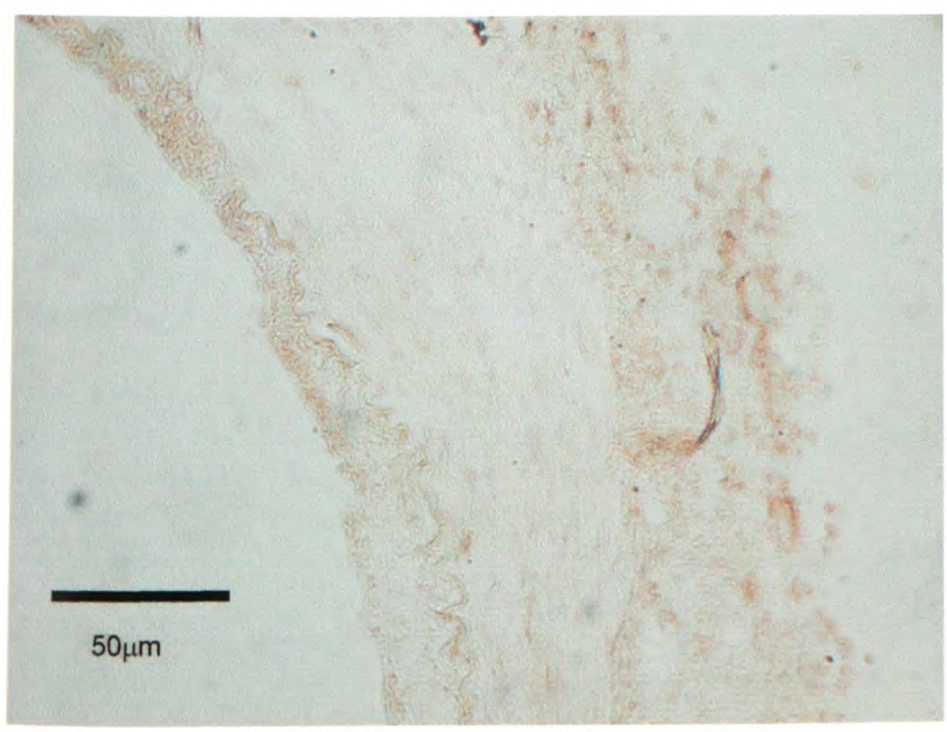


Figure 9.2c
Septic tissue stained with anti-eNOS. The endothelial eNOS of control tissue has been lost.

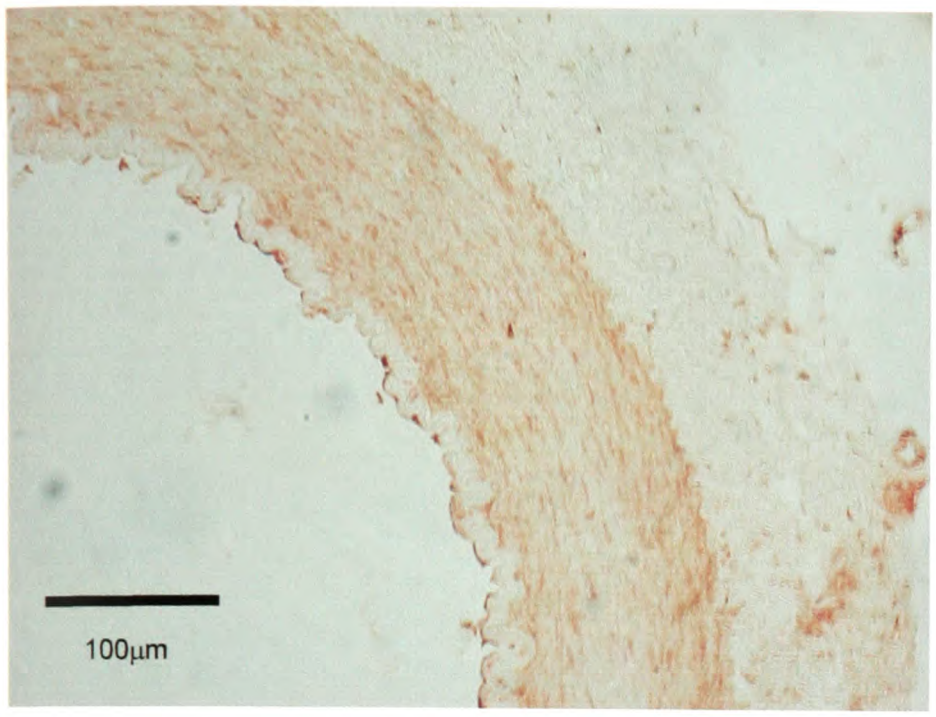


Figure 9.2d
Control tissue stained with anti-iNOS. iNOS is present principally in the medial layer.

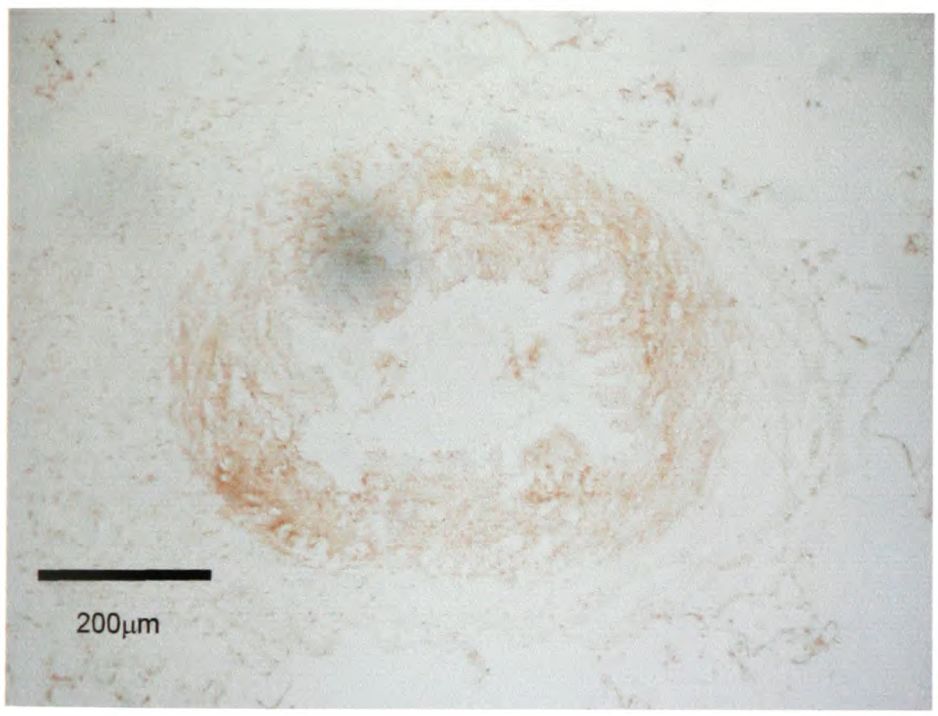


Figure 9.2e
Septic tissue stained with anti-iNOS. There appears to be less iNOS in the medial layer than in control tissue.

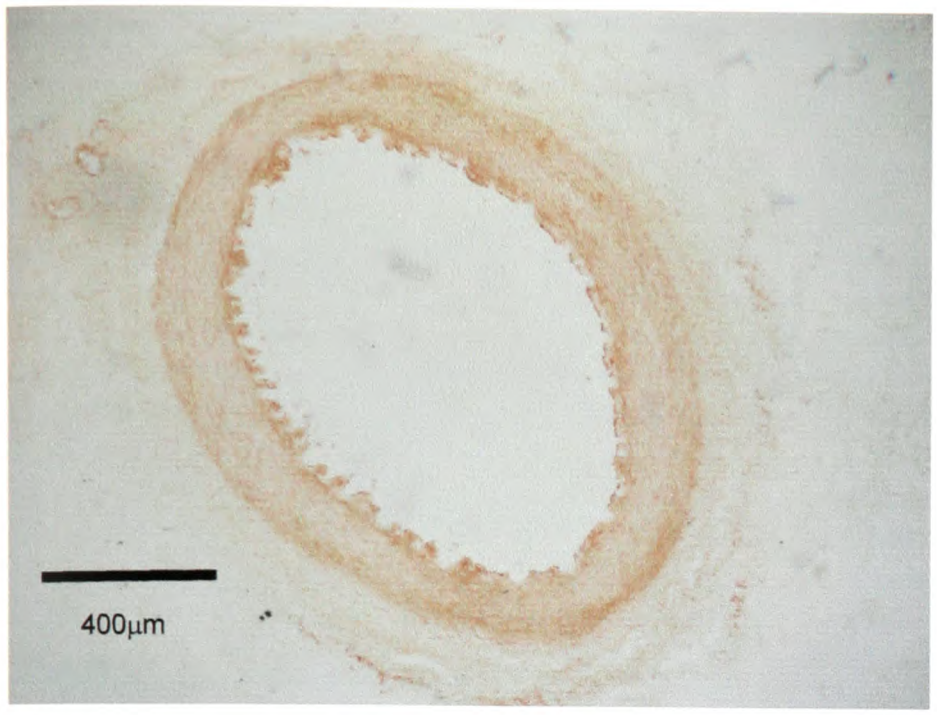


Figure 9.2f
Control tissue stained with anti-HO-1. HO-1 is present throughout the intimia, media and adventitia.

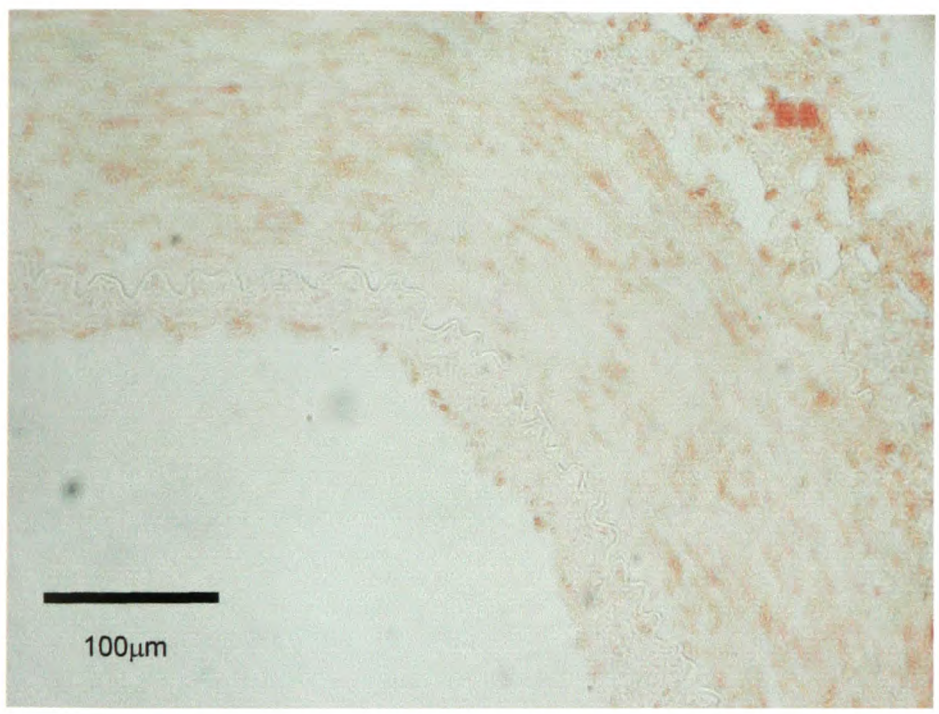


Figure 9.2g
Septic tissue stained with anti-HO-1. The medial layer still contains HO-1.

The above images (and those from the other 3 septic patients and 2 control patients studied) show that the media is the main site of expression of iNOS and HO-1 protein, thus validating the decision to study this segment of the vessel wall in detail in the preceding experiments. The disappearance of eNOS from the intima in vessels from patients with septic shock agrees with the results of a number of previous studies (MacNaul & Hutchinson 1993) (Zhou *et al.* 1997) (Wang *et al.* 1996). As Western

blotting was felt to be a more accurate quantitative measure of protein expression, these images have not been quantitatively analysed.

Chapter 10:

Summary, discussion and conclusion

10.1
Summary of Results

The experiments described in this thesis, excluding those with equivocal results presented in the Appendices, are summarised in table 10.1a.

	PBMCs	**Arterial smooth muscle**
NO production (DAF)	Increased in sepsis	Decreased in sepsis
NOS activity (rate of arginine to citrulline conversion)	No change (trend towards increase in sepsis)	Increased in sepsis (increased iNOS fraction)
Arginine transport (radiolabelled arginine uptake)	Increased in sepsis, though newly expressed y^+	--
Arginine transporter mRNA (real-time RT-PCR)	CAT2B increased in sepsis All others: no change	CAT1 decreased in sepsis All others: no change
iNOS/eNOS mRNA (real-time RT-PCR)	Decreased in sepsis	Decreased in sepsis
HO-1 mRNA (real-time RT-PCR)	Increased in sepsis	Increased in sepsis
iNOS protein (Western blot)	Decreased in sepsis	No change
eNOS protein (Western blot)	Decreased in sepsis	Increased in sepsis
HO-1 protein (Western blot)	Increased in sepsis	Increased in sepsis
Location of iNOS protein (immunocytochemistry)	--	In medial layer
Location of eNOS protein (immunocytochemistry)	--	Decreased in endothelium in sepsis. Present in medial layer
Location of HO-1 protein (immunocytochemistry)	--	In medial layer

Table 10.1a
Summary of all results

10.2
Discussion

PBMCs

The research described here demonstrates for the first time that nitric oxide production is increased in peripheral blood mononuclear cells from patients with septic shock. This is the first time any cell type from clinically septic patients has been directly shown to produce increased nitric oxide.

Increased PBMC nitric oxide production in sepsis is perhaps an expected result if one believes that human inflammatory cells mediate cytotoxicity in part by release of nitric oxide. However, as noted in the introduction, while this is certainly true for animal inflammatory cells, to date the evidence from human studies has been much less clear. Many in vitro studies have failed to stimulate nitric oxide production in human PBMCs. These in vitro studies used lipopolysaccharide and cytokine mixtures which remain essentially constant during the incubations, while in clinical sepsis the concentrations of the various cytokines are known to vary markedly (Martinez *et al.* 1999). Additionally, in clinical sepsis the population of PBMCs is constantly being renewed as progenitor cells differentiate in response to the septic stimulus. Thus the results of studies using human and animal PBMCs activated in vitro have always had

questionable applicability to the rapidly changing cytokine and cellular environment of clinical sepsis.

DAF-2T fluorescence reflects NO production by intact cells, the rate of which may be influenced by the quantity of NOS enzyme, the availability of NOS cofactors, and the substrate supply to the enzyme. In contrast, the labelled arginine to citrulline conversion assays presented in Chapter 4 measure the activity of NOS in PBMCs in the presence of excess enzyme cofactors, and without the influence of variable substrate supply. As such they specifically quantify only the amount and intrinsic activity of the NOS enzymes present in these cells. There was no difference in the NOS activity of septic and control PBMCs, which possibly reflects the poor sensitivity of this assay, as discussed in Chapter 4. However, it may be that the intrinsic NOS activity of these cells really is unchanged in sepsis, while the increased NO production observed is due to either increased substrate supply or increased cofactor availability.

In support of this substrate supply hypothesis, arginine transport is increased in the peripheral blood mononuclear cells from these patients. The increased transport occurs through a newly expressed, functionally distinct transporter, y^+, whose contribution increases from approximately 7% of total arginine transport in health to 36% of the total in septic shock. Again, this agrees with most (but not all (Kiemer & Vollmar 2001)) results in animal macrophages (Bogle *et al.* 1992) (Chou *et al.* 1998) (Kakuda *et al.* 1999) and human PBMC in vitro studies (Boyd & Crawford 1992) (Chen *et al.* 1996) (Crawford *et al.* 1994). It would appear logical that cells producing extra nitric oxide would increase their rate of transport of its precursor. As

noted in Chapter 1, the availability of arginine from the extracellular space is rate limiting to the production of NO, in spite of intracellular arginine concentrations which would appear to be more than sufficient to saturate the NOS enzymes. This 'arginine paradox' is thought to be due to the localisation of NOS enzymes in membrane-bound caveloae, which have access to arginine in the extracellular space but not the cytoplasm (McDonald *et al.* 1997).

There are a number of possible mechanisms by which arginine transport through y^+ might be increased. These include an increase in the activity of the transporter under the influence of a regulatory signal or an increase in the amount of one or more of the y^+ proteins. Such an increase in protein amount could be due to increased mRNA transcription or translation; increased mRNA stability; movement of intracellular stores of protein to the cell membrane, or decreased breakdown of the transporter protein. While none of these possibilities can yet be discounted, it has been demonstrated in this study that mRNA encoding CAT2 is increased in these cells. None of the other known arginine transporter mRNAs are affected. CAT2 protein has the kinetic properties of a y^+ transporter. The selective increase in CAT2 mRNA is also consistent with most, but not all, studies of animal macrophages. Rat macrophages exposed to lipopolysaccharide and interferon produced more CAT2 mRNA (Caivano 1998). However, mouse macrophages stimulated with LPS increased not only their CAT2, but also CAT1 (Closs *et al.* 2000). Confusingly, other studies of mouse macrophages activated in vitro show decreased CAT1 mRNA (Kakuda *et al.* 1998) (Kakuda *et al.* 1999). In its finding that CAT1 mRNA is not changed in clinical human sepsis, the current study highlights the limitations of these animal models of human disease. There are no studies of transporter mRNA in either

humans or animal PBMCs activated either in vitro or in vivo with which to directly compare the results of the current study.

New evidence has emerged regarding CAT3 and CAT4 transporters since the experiments described in this thesis were performed. It has become apparent that CAT3 is expressed in human tissue, and that in contrast to rat and mouse CAT3, human CAT3 is expressed outside the nervous system. The kinetic properties of human CAT3 are different to that in rat and mouse, instead closely resembling human CAT2B (Vekony *et al.* 2001). It will be interesting to determine the expression of CAT3 in PBMCs from patients with sepsis; however, there were insufficient samples left in this experimental series to allow this experiment to be performed.

Also of interest is the recent demonstration that CAT4 is probably not a functioning amino acid transporter. CAT4 had been identified as a potential amino acid transporter solely on the basis of the similarity of its DNA sequence to that of the other CATs. However, its kinetic properties had not been defined at the time the experiments designed in this thesis were performed. It has now been clearly demonstrated that CAT4 expression in the plasma membrane is not sufficient to induce amino acid transport activity in Xenopus laevis oocytes or human cells (Wolf *et al.* 2002). Thus CAT4 is either not an amino acid transporter, or it needs an additional factor not present in these experiments to become functionally active. No other putative function of the CAT4 sequence has been proposed.

The heterogeneity observed in the fluorescence of DAF loaded cells (figure 3.1c) suggests that some PBMC subsets are producing more nitric oxide than others, or

perhaps that different cell types have different viability over the time course of the assay. If there is only a subset of cells which have increased NO production, presumably this is also the subset with increased y^+ transport. The current study did not distinguish between PBMC subsets. However, studies of in vitro activated PBMC y^+ transport found it was the T cell component which was much more affected by PHA stimulation (Boyd & Crawford 1992), and of these T cells the $CD8^+$ $CD45RA^+$ subpopulation had the largest increase in y^+ transport (Crawford *et al.* 1994). Whether different cell types were making different amounts of nitric oxide could be resolved by labelling the cells with antibodies to specific CD markers; dying cells could be stained using markers of apoptosis. This has not been done as the number of cells available from each septic patient was limited by the amount of blood it was ethically possible to obtain. This is the only demonstration of any heterogeneity in the PBMC response to sepsis among all of the experiments described in this thesis. In all other experiments the whole PBMC fraction has been processed together. As such this experiment is the only indication that separate characterisation of each of the PBMC subsets might be warranted.

The increase in CAT2 mRNA in these human cells is encouraging from a therapeutic point of view. A CAT2 knockout mouse model of sepsis has demonstrated that CAT2 is required for 92% of the increase NO production and 95% of the increase in arginine uptake when the cells from these animals are activated using IFNγ and LPS (Nicholson *et al.* 2001). A number of other studies have begun to report potential modulation of nitric oxide production by alteration of arginine transport (Zharikov *et al.* 2001) (Colton *et al.* 2001).

However, there is also reason for caution in pursuing arginine transport as a means to limit nitric oxide production. An elegant series of papers has reported that two of the isoforms of NOS (nNOS (Xia *et al.* 1996) and iNOS (Xia & Zweier 1997), but intriguingly not eNOS (Xia *et al.* 1998)) make the even more toxic mediator superoxide (and subsequently peroxynitrite) when deprived of adequate arginine. Increased arginine transport in these cells in this context could be seen as a protective effect. These studies counsel caution, but do not rule out a role for arginine transport inhibitors in the treatment of septic shock, as the relative quantitative detrimental effects of nitric oxide and superoxide have not been established. It may be that, as with most drugs, a small amount of arginine inhibitor will be helpful, while a large amount will be toxic.

mRNA and protein for both iNOS and eNOS were, surprisingly, decreased in PBMCs from patients with sepsis, despite increased nitric oxide production. This is in contrast to the results of a published study of human monocytes, where iNOS mRNA and protein were increased after activation with LPS ± IFNγ (Weinberg *et al.* 1995). Surprisingly, though, their production of nitrite/nitrate and their NOS activity were very much lower than that of mouse macrophages, and not significantly affected by LPS and IFNγ. The explanation for the apparent discrepancy between these results and those of the current study may lie in the concentration of NO in the medium surrounding the cells. If nitric oxide feeds back on its own production (Buga *et al.* 1993), it may be that the increased NO in the plasma of septic patients has reduced PBMC NOS expression. If there was no NO formed in the in vitro culture medium or if it was scavenged, this would not have occurred.

Heme oxygenase mRNA and protein expression was increased in septic PBMCs. This is reassuring, as essentially all studies of cells subjected to oxidative stress (such as that which occurs in sepsis) show increased HO-1 amount and activity (Yet *et al.* 1997) (Otani *et al.* 2000) (Camhi *et al.* 1995) (Suzuki *et al.* 2000) (Tsukiji *et al.* 2000) (Pellacani *et al.* 1998). The studies of nitric oxide and arginine transport discussed above are thus of truly 'activated' cells. Increased heme oxygenase activity is considered an adaptive response to oxidative stress, as the bilirubin formed in the breakdown of heme is a powerful antioxidant. Carbon monoxide, also formed by heme oxygenase, is now also recognised as having anti-inflammatory properties, probably involving modulation of the p38 MAP kinase pathway (as reviewed by Otterbein, 2002). However, increased carbon monoxide production by these cells may also have detrimental effects (notably, vasodilation by activation of cGMP) and may interact with nitric oxide production. The possible significance of the increased heme oxygenase expression observed in PBMCs is discussed in detail below (in the 'proposed unified explanation for the results found').

Arterial smooth muscle

It had always been assumed that vascular smooth muscle acts merely as the target of nitric oxide released by other cells. However, there has for some time been a recognition that vascular smooth muscle cells from both animals (Baydoun *et al.* 1999) (Gill *et al.* 1996) and humans (MacNaul & Hutchinson 1993) (Stoclet *et al.* 1999) (Chester *et al.* 1998) can be made to induce the enzymatic machinery (identified at an mRNA or protein level) to produce nitric oxide. Indeed there may be

a low level of constitutive expression of iNOS even in healthy vascular smooth muscle (Buchwalow *et al.* 2002). Many studies have shown rat aortic smooth muscle cells stimulated in culture produce nitric oxide (Baydoun *et al.* 1999) (Durante *et al.* 1995) (Gill *et al.* 1996) (Hattori *et al.* 1999) (Kojima *et al.* 1998a), as does bovine (Brophy *et al.* 2000) and human (Chester *et al.* 1998) arterial smooth muscle. The physiological relevance of these in vitro cell culture experiments could be questioned. There has been indirect evidence (mainly from observation of the effects of NOS inhibitors on isolated smooth muscle preparations) that animal vascular smooth muscle stimulated in vivo does produce nitric oxide (Julou-Schaeffer *et al.* 1990) (Schwarz *et al.* 1999) (Biguad *et al.* 1990) (Wakabayashi *et al.* 1987) (McKenna *et al.* 1986) (Chen *et al.* 1994). The current finding is the first direct demonstration that *human* arterial smooth muscle cells not artificially stimulated in vitro do produce a basal level of nitric oxide.

In contrast to the results in PBMCs, nitric oxide production by these arterial smooth muscle cells is decreased in septic shock. This is the opposite of what would be predicted from all of the previous studies of animal cells activated in vitro and in vivo, and of human cells activated in vitro (reviewed in detail in Chapter 1). There are some methodological concerns with this study, which combined with the small number of tissues studied suggest the result must be interpreted with caution. The conversion of DAF-2 to DAF-2T is known to be highly specifically dependent on nitric oxide. However, the fluorescence of DAF-2T is known to be affected by $[H^+]$ below a pH of approximately 9: the fluorescence increasing modestly down to pH 7.5, then decreasing markedly to virtually nothing at a pH of 5 (as was noted in passing by Kojima *et al.* 1998a). Additionally, the presence of various reducing agents in a non-

cell based reaction of nitric oxide donor and DAF-2 attenuated the intensity of the fluorescence produced (Nagata *et al.* 1999). The intracellular space of arterial smooth muscle from septic patients might well be more acidotic than controls if the cells have been hypoperfused in vivo. They might also be subject to greater oxidative stress. The effects of these two potential abnormalities would work in opposite directions on the fluorescence of the DAF-2 loaded cells, though whether one or the other is quantitatively more important is not known. It is theoretically possible to measure the redox potential and pH of the intracellular environment using specific dyes; however this was not possible in these experiments due to the very limited amount of tissue available. Perhaps the best argument in favour of the validity of these results is that nitric oxide production measured in a very similar way in PBMCs from septic patients was increased (Chapter 3.2) – an entirely expected result.

If the DAF-2 signal is NO-specific, it is surprising that L-NAME did not reduce fluorescence more than it did, in both the PBMC and arterial smooth muscle experiments. However, wells (containing no tissue) with the NO donor sodium nitroprusside, L-NAME and DAF-2 had a greater fluorescence than those without L-NAME (Table 3.2a). The effect of L-NAME in attenuating the fluorescence due to nitric oxide production may well have been obscured by this non-cell dependent interaction of L-NAME with nitric oxide and DAF.

Notwithstanding these methodological concerns, confirmation that this surprising decrease in nitric oxide production may be correct comes from the finding that expression of mRNA for both the constitutive and inducible forms of nitric oxide synthase are simultaneously decreased in these cells. These differences highlight the

limitations of the various models in replicating clinical human disease. That the NOS mRNAs are decreased is by itself a surprising result, and is best understood in terms of the theory of negative feedback described below.

Western blots of arterial smooth muscle for iNOS and eNOS initially seem to conflict with findings of decreased mRNA for both these enzymes. The technical limitations of these experiments have been discussed in Chapter 7. That iNOS mRNA is reduced while the protein remains unchanged is however quite plausible, as mRNA might be expected to be more labile than protein once formed. This could potentially also be true of eNOS. That eNOS protein is elevated accords with the increased total NOS activity (measured by rate of arginine to citrulline conversion) seen in arterial smooth muscle. That eNOS (calcium-dependent) NOS activity was not significantly increased in septic arterial smooth muscle probably reflects the limited numbers of tissues studied, and the only barely adequate sensitivity of the technique (as discussed in Chapter 4). That NO production (measured by DAF-2 fluorescence) is nonetheless decreased may be because the necessary NOS cofactors (such as BH_4) are more labile than the NOS proteins themselves. It should be possible to study BH_4 levels in these cells in future experiments.

The only 'in vivo' study of human tissue prior to this is that of Bhagat *et al.* (1999), where a segment of human vein was isolated from the circulation but remained in place while infused with IL-1B. On subsequent excision, these vessels had increased expression of eNOS mRNA, but not iNOS mRNA. After excision the vessels were hyporesponsive to noradrenaline, and this was reversed by an inhibitor of nitric oxide synthase. This study used vein rather than artery, and the stimulus for activation was

far from physiological. However this was the first human in vivo study investigating NO regulation, and it produced the first results to suggest iNOS was not induced in human vascular smooth muscle when activated in vivo, in contrast to the situation in the human in vitro models of sepsis described above.

CAT1 mRNA expression was decreased in arterial smooth muscle from patients with septic shock. This is consistent with this tissue type producing less, not more, nitric oxide at this time point in sepsis. There would be no need to increase substrate supply to NOS if NOS activity was not increased. In contrast, many of the studies of rat arterial smooth muscle cells demonstrating increased NO production after stimulation in culture also often found increased CAT1 mRNA (Baydoun *et al.* 1999) (Hattori *et al.* 1999) (Low & Grigor 1995). However, it was more typically their CAT2B mRNA which was increased (in all the studies mentioned; also that of (Gill *et al.* 1996)), along with their y^+ activity. In the current study of human septic arterial smooth muscle, control tissue had a relatively high expression of CAT2 and CAT2B (certainly compared to that in PBMCs), but there was no evidence that this was altered in sepsis. As is the case for the PBMC experiments described above, it would be interesting to study the expression of CAT3 in human sepsis; however this was unfortunately not possible in this experimental series as there was insufficient tissue remaining at the time the CAT3 sequence was described.

The present study sheds new light on the findings of Tsuneyoshi et al. (Tsuneyoshi *et al.* 1996) and Stoclet et al. (Stoclet *et al.* 1999), who conducted the only published functional studies of mesenteric arterial rings from patients with sepsis. The vessels from the three septic patients Tsuneyoshi et al. studied had a reduced contractile

response to noradrenaline compared to their four controls. This hyporesponsiveness was reversed by an inhibitor of nitric oxide synthase. Stoclet et al. drew the same conclusions from their similar study. These observations do imply that the hyporeactivity must have been due to overproduction of nitric oxide by a component of the vessel wall, but whether this was the media cannot be determined. In that the present study shows a decreased production of nitric oxide by arterial smooth muscle, it might be that Tsuneyoshi's and Stoclet's tissue preparations contained other NO producing cell types (such as inflammatory cells in the adventitia). They may have been taken from a patient population in an earlier stage of disease, prior to the induction of the negative feedback response proposed below. Alternatively, there may have been another mediator (such as carbon monoxide) responsible for the observed hyporesponsiveness, while at the same time there was sufficient residual nitric oxide to result in vasoconstriction when its production was blocked.

As was the case in PBMCs, heme oxygenase mRNA and protein expression were increased in arterial smooth muscle in sepsis. For the same reasons as outlined above, this is both expected and reassuring in terms of the interpretation of the nitric oxide experiments in this tissue.

Proposed unified explanation for the results found

The haemodynamic abnormalities observed in septic shock result from a complex interplay of pathological insult, inflammatory reaction and overreaction to that insult, and the failure of compensatory feedback mechanisms which would normally act to

limit inflammation and restore vascular tone. It would be most unusual if there were no evidence of these feedback mechanisms in these studies. Negative feedback of nitric oxide upon its own production is well described in endothelial cells (Zhou *et al.* 1997) (Buga *et al.* 1993).

A likely explanation for these results is that, in the early phase of the development of sepsis, both PBMCs and arterial smooth muscle increase their nitric oxide production, by increasing the transcription and protein synthesis of iNOS and CAT2. The first phase of the negative feedback involves a decrease in iNOS and eNOS mRNA transcription, which is seen in both PBMCs and arterial smooth muscle. Interestingly CAT2 mRNA remains higher in septic PBMCs at the time when NOS mRNAs are reduced – perhaps it is more stable.

Whereas PBMCs are still overproducing nitric oxide at the time point at which they were sampled in this study, arterial smooth muscle is at a different stage in the process of negative feedback. This may be because the PBMCs are not a static cell population, but are being continuously replenished; hence the cells studied have not all been exposed to the septic stimulus for the same amount of time. Arterial smooth muscle NO production is decreased at the time point studied. This appears to be because the NOS cofactors have been downregulated, as while the iNOS protein has returned to normal levels, the eNOS protein remains raised, and this has kept the NOS activity (independent of cofactors) higher in the septic cells.

The regulation of NO production in PBMCs appears to be different to that in arterial smooth muscle. Rather than reduce the supply of NOS cofactors, the negative

feedback in PBMCs has reduced the protein levels of both iNOS and eNOS. That PBMC NOS production remains elevated at this time point probably reflects the greater importance of cofactor availability compared to NOS enzyme quantity in determining NO output. Indeed this has been shown in other cells (Bhagat *et al.* 1999).

The observation that heme oxygenase mRNA and protein are increased in both of these cell types adds another layer of complexity to this explanation. Carbon monoxide interacts with nitric oxide in many ways. NO is a strong stimulus to the induction of HO-1: it appears to increase HO-1 mRNA stability, and possibly increase the DNA binding activity of transcriptional factor activator protein-1. NO may do this by increasing cGMP, or by causing oxidative stress through peroxynitrite formation (as summarised by Hartsfield (2002)). Conversely, there are several mechanisms whereby HO-1 directly, or through carbon monoxide, can reduce NO production (Maines 1997). HO-1, by metabolising heme, destroys an essential component of the NOS enzyme structure, though whether heme availability is limiting to NOS activity has not been described. Fe^{2+} formed from the Fe^{3+} released in the catabolism of heme is a direct inhibitor of NOS. Heme oxygenase and NOS compete for NADPH. Gaseous CO inhibits rat macrophage NOS activity (White & Marletta 1992), and a variety of inducers of HO-1 suppress the induction of iNOS mRNA and activity by cytokines (Cavicchi *et al.* 2000). Inhibition of HO activity increases NO production by mouse macrophages exposed to endotoxin (Turcanu *et al.* 1998). Thus, in addition to the direct negative feedback on its own production, the various components of the NOS system may be being downregulated by the upregulated heme oxygenase/carbon monoxide system.

10.3
Conclusion and potential therapeutic implications

The purpose of these experiments was to find a cellular abnormality linking sepsis with hypotension which might be amenable to therapeutic manipulation. As explained above, if indeed nitric oxide production by PBMCs (and probably also marginated and tissue infiltrated monocytes/macrophages) is quantitatively significant, reducing arginine transport by interfering with the upregulation of CAT2B expression is a very attractive proposition.

However, mRNA and protein levels in PBMCs, as well as studies of arterial smooth muscle (a tissue likely to be physiologically much more relevant cell in terms of blood pressure regulation), strongly suggest the body has its own counter-regulatory processes in place. These reduce NO production by arterial smooth muscle in the subacute phase of sepsis. Strategies to further reduce this NO production are unlikely reduce the mortality of septic shock. Indeed this has been the experience in early clinical trials.

These experiments have suggested, however, that the vasodilation which undoubtedly exists at this point in sepsis may be in part due to an overproduction of carbon monoxide by the upregulated heme oxygenase enzyme system. Inhibition of heme

oxygenase does improve blood pressure in septic shock (Yet *et al.* 1997). It is true that there are a number of studies showing either non-selective inhibition of all heme oxygenase enzymes (Downard *et al.* 1997) or HO-1 gene deletion (Poss & Tonegawa 1997) is detrimental to the survival of septic or endotoxic animals. This may be due to loss of the antioxidant effect of bilirubin or the reduced inhibition of nitric oxide synthesis. However, the experiments performed to date have made no attempt to titrate dose of heme oxygenase inhibitor to effect, and no specific inhibitors of HO-1 have been studied. These strategies warrant further investigation, and in the light of the results of this thesis, perhaps more than current strenuous efforts to develop inhibitors of the nitric oxide synthetic pathway.

Chapter 11:

Future Work

11.1
Future Work

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1. Gene expression abnormalities in PBMCs during septic shock, and on recovery, using microarray technology and possibly proteomic analysis.

The studies presented in this thesis have demonstrated abnormalities in the heme oxygenase and nitric oxide synthase pathways. These genes have a number of regulatory sites; which are important in human sepsis is not known. There are likely to be a number of other metabolic pathways altered in septic shock (such as those controlling energy utilisation, cytokine production, and defence to oxidative stress); very little is known about how these are affected by sepsis. RNA microarray and proteomic approaches are likely to be the most efficient first step in defining these changes.

In order to overcome the problem of genetic diversity between individuals, these experiments would be best performed using PBMCs from patients during episodes of septic shock and subsequently some months after their complete recovery. The number of samples required would be relatively small, as between subject variability would be eliminated by studying changes within the same patient.

2. Development of an in vitro model which reflects the changes seen in clinical sepsis

All in vitro studies reported to date (using either human or animal tissue) use only pro-inflammatory cytokines to mimic the septic state. The expression of iNOS is increased in these cells, whereas it is decreased in clinical sepsis. Presumably there are other fundamental shortcomings in the existing in vitro models. This study would attempt to stimulate cells in a manner which replicates the reduction in NOS expression, and increase in HO-1 expression, observed in clinical sepsis.

Such experiments would most easily be done using a cell line to which commercially available anti-inflammatory as well as pro-inflammatory cytokines would be added. The appropriate combination and concentrations of cytokines could only be determined by trial and error.

Additionally, cells could be cultured in the presence of plasma from patients with clinical sepsis.

An expression profile of iNOS and HO-1 mRNA which matched that in these studies of clinical sepsis (as determined by real-time RT-PCR) would be the desired end point of these in vitro stimulation experiments.

Once a satisfactory cytokine combination had been determined (or if the incubation with septic plasma proves successful), this could be used with a commercially available human smooth muscle cell line.

It may also be possible to analyse appropriately activated cells using the RNA microarray and proteomic techniques listed above.

3. Investigation of the inter-relationship of heme oxygenase and nitric oxide synthase systems in human cells

Most of the evidence concerning the ability of NO to upregulate HO-1 expression, and carbon monoxide and heme oxygenase to downregulate NOS expression and function, is derived from animal cells. Even if an appropriate in vitro model of sepsis can be developed, it is possible that the culture conditions will not accurately reflect the concentrations of NO and CO found in vivo. This study would activate normal human arterial smooth muscle rings obtained during cardiac surgery using an established technique (Chester *et al.* 1998), and investigate the effects of different environmental CO and NO concentrations on the NO and CO/heme oxygenase systems.

Human blood vessels would be cultured in the presence of pro-inflammatory cytokines and varying concentrations of CO and NO. It may also be possible to over-express HO-1 in these cells, as has been recently reported in animal cells (Juan *et al.* 2001).

The contractile responsiveness of these vessel rings could be assessed using a standard organ bath technique. At the conclusion of these functional studies, analysis

of iNOS and HO-1 expression could be performed by real-time PCR and Western blotting, as was done in the experiments described in this thesis.

4. Effect of selectively reducing the activity of inducible heme oxygenase activity

The only studies of heme oxygenase inhibition in sepsis or endotoxin exposure have used either non-selective heme oxygenase inhibitors, or HO-1 deficient mice. These studies showed a detrimental effect of heme oxygenase inhibition. However, nothing is known of the effects of lesser degrees of selective HO-1 inhibition. No selective HO-1 inhibitor currently exists, but it should be possible to reduce HO-1 activity in a dose-dependent manner using anti-sense oligonucleotides. Antisense oligonucleotides which work successfully in vitro have recently been described (Lee & Chau 2002).

The effect of HO-1 antisense oligonucleotides in the cell lines described above could be investigated. End points would be markers of oxidative stress (using commercially available redox-sensitive dyes), cell viability, and iNOS mRNA expression by real-time RT-PCR. If the antisense technique is successful, the procedure could be repeated with tissue from human volunteers and possibly also in whole animals.

Appendices

Appendix I

Patient data and detailed experimental results for each subject

1. Septic patients

PBMC study No	VSM study No	Age	Classification	Reason for exclusion	PBMC studies: NO production (DAF) (arbitrary fluorescence units) No L-NMMA / L-NMMA	PBMC studies: Arginine to citrulline conversion (fmol/mg/min)	PBMC studies: Arginine transport: total (pmol/10⁷ cells/min)	PBMC studies: Arginine transport: v+ (pmol/10⁷ cells/min)	PBMC studies: mRNA by RT-PCR CAT1	CAT2	CAT4	CD98	y+LAT1	y+LAT2	iNOS	eNOS	HO-1	PBMC studies: Protein (Western blot) iNOS	eNOS	HO-1	ASM studies: NO production (DAF) (arbitrary fluorescence units)	ASM studies: NO production (DAF) (fmol/mg/min) total / iNOS / eNOS	ASM studies: Arginine to c mRNA by RT-PCR CAT1	CAT2	CAT2B	CAT4	CD98	y+LAT1	y+LAT2	iNOS	eNOS	HO-1	Protein by Western blotting iNOS	eNOS	HO-1
1		43	Septic shock						0.3	0	0	2.3	17.6	5.8	0	0.1	5.2																			
2		43	Septic shock	Consumed in preliminary experiments																																
3		25	Septic shock	Consumed in preliminary experiments																																
4		64	Septic shock	Consumed in preliminary experiments																																
5		56	Septic shock	Consumed in preliminary experiments																																
6		73	Septic shock						1.2	5.18E-06	0	1.553	16.73	2.96	0.19	0.08	4.99																			
7		76	Septic shock				1.31	0.86	1.078	8.10E-04	0	1.056	8.77	1.9	0.3	0.03	3.12																			
8		67	Septic shock	Same as septic 12																																
9		61	Septic shock						1.117	2.90E-06	0	1.42	17.26	4.29	0.175	0.1	6.13																			
10	5	59	Septic shock				0.57	0.11																1.24	1.63	2.87	0.03	0.67	5.63	10.85	0.18	0	2.84			
11		74	Septic shock						0.79	3.80E-04	2.95E-04	0.51	8.09	1.17	0	0.04	1.62																			
12		67	Septic shock				0.98	0.45																												
13		60	Septic shock				1.12	0.4	0.52	0	0	1.26	15.7	2.94	0	0.06	6.34																			
14		70	Septic shock	Had had small dose prednisone																																
15		67	Not analysed	On inhaled NO																																
16		60	Not analysed	Same as septic 13																																
17		67	Septic shock	On inhaled NO, same as septic 15																																
18	7	54	Septic shock																					0.56	0.41	0.74	0.17	0.44	5.45	14.51	0.15	0.22	1.66	0	10372689	477288.8
19		81	Not analysed	Consumed in preliminary experiments																																
20		64	Septic shock				1.51	0.44	0.914	1.55E-04	5.17E-05	1.776	8.76	3.19	0	0.03	3.31																			
21		35	Not analysed	Consumed in preliminary experiments																																
22		69	Not analysed	Consumed in preliminary experiments																																
23		19	Not analysed	Consumed in preliminary experiments																																
24		64	Not analysed	Consumed in preliminary experiments																																
25	12	75	Septic shock		1198 / -																		437 / .262 / .184	0.91	0.88	1.82	0.39	0.78	19.41	52.02	0.39	0.18	4.63	93184.59	1121154	140540.9
26		78	Septic shock																																	
27		78	Septic shock		1116.6 / -	0.1898												0	725.4	2607.52															
28		17	Septic shock		910 / 785																															
29		77	Septic shock		1299 / 969																															
30		61	Septic shock	Consumed in preliminary experiments																																
31		46	Septic shock		1169 / 687																															
32		80	Septic shock		1263 / 1017	0.06522												0	0	11815.25															
33		67	Septic shock			0.07702												80314.73	0	9061.56															
34		55	Septic shock			0.6218												0	0	22612.76															
35	13	80	Septic shock			0.3658															539417													0	437534.7	1825631
36		66	Septic shock	Consumed in preliminary experiments																																
37		70	Septic shock	Consumed in preliminary experiments																																
38	14	78	Septic shock																		1148372	.352 / 1.3 / 0	0.67	1.68	2.82	0	0.87	7.99	16.65	0.3	0	1.85	0	69890.74	276974.5
39		74	Septic shock															0	0	12638.82															
	1	70	Septic Shock																															0	1543063	686236.3
	2	89	Septic Shock																			2.36 / .214 / 2.16	0.84	0.84	0.98	0.14	0.84	16.49	29.55	1.54	0.14	2.6	0	263211.7	370762.3
	3	65	Faecal peritonitis	Hepatitis C infection risk																																
	4	77	Sepsis																															79531.01	1349504	496517.4
	8	76	Severe Sepsis																			49 / .597 / 0											6.88	0	2477829	83021.39
	8	49	Septic Shock	Consumed in preliminary experiments																																
	9	72	Chronic infection	Did not meet study criteria																																
	10	58	Septic Shock																					1.66	0.67	1.43	0	1.37	9.73	13.22	2.91	0.25	6.66			
	11	85	Septic Shock																															0	699909.4	605087
	15	69	Severe Sepsis																		1258413			0.8	0.34	0.51	1.71	0.51	38.89	177.86	0.17	0.09	2.82	0	223206.4	615536.96
	16		Septic Shock																			.685 / .47 / .0217	1.55	2.62	5.35	0	1.55	35.08	101.07	1.19	0.12	3.57	20733.85	997356.11	2631.651

2. Healthy controls, or controls with bowel malignancy

PBMC study No	VSM study No	Age	Classification	Reason for exclusion	PBMC studies: NO production (DAF) (arbitrary fluorescence units)	PBMC studies: Arginine to citrulline conversion (fmol/mg/min)	PBMC studies: Arginine transport: total (pmol/10⁷ cells/min)	PBMC studies: Arginine transport: v+ (pmol/10⁷ cells/min)	PBMC studies: mRNA by RT-PCR CAT1	CAT2	CAT4	CD98	y+LAT1	y+LAT2	iNOS	eNOS	HO-1	PBMC studies: Protein (Western blot) iNOS	eNOS	HO-1	ASM studies: NO production (DAF) (arbitrary fluorescence units)	ASM studies: NO production (DAF) (fmol/mg/min)	ASM studies: Arginine to citrulline conversion	ASM studies: mRNA by RT-PCR CAT1	CAT2	CAT2B	CAT4	CD98	y+LAT1	y+LAT2	iNOS	eNOS	HO-1	Protein by Western blotting iNOS	eNOS	HO-1
1		56	Control						0.368	0	7.70E-03	0.631	11.27	4.49	0.26	0.14	2.41																			
2		30	Control						1.09	0	0.0462	1.299	13.06	4.61	1.09	0.13	2.87																			
3		47	Control						1	0	0.0158	0.935	10.68	5.5	0.37	0.18	2.02																			
4	NR		Control	Unsuccessful RNA extraction																																
5		55	Control						0.82	0	2.84E-03	0.658	8.32	2.66	0.27	0.13	1.89																			
6	NR		Control	Unsuccessful RNA extraction																																
7		68	Control						1.676	0	0.011	1.66	27.6	10.7	0.7	0.2	6.03																			
8		55	Control						0.47	0	4.44E-03	0.871	13.46	3.77	0.69	0.11	1.99																			
9		42	Control						0.69	0	0.29	0.494	6.6	4.7	0.76	0.09	1.56																			
10		36	Control						0.86	0	0.14	0.596	7.28	4.97	0.24	0.1	1.38																			
11		30	Control			0.03193												879010.6	0	0																
12		43	Control			0.1489												868255.7	11015.94	0																
13		36	Control			0.284												771789.3	8234.67	8171.07																
14		34	Control			0.3405												455106.3	11127.55	6046.99																
15		29	Control			0.07887												134509.1	5890.16	4071.86																
	1	84	Control																				0.35	0.66	0.92	0.04	0.44	7.35	14.76	0.88	0.35	1.28			
	2	40	Control																				2.31	1.24	0.2	0.08	1.57	23.97	49.34	2.56	1.49	4.55			
	3	NR	Control	Unsuccessful RNA extraction																																
	4	70	Control	Oesophageal mesentery, not bowel																																
	5	81	Control																															0	0	
	6	85	Control																				2.76	0.78	0.84	0.26	1.81	14.14	21.03	2.84	0.17	3.53	29239.26	81660.74	0
	7	72	Control	Unsuccessful RNA extraction																																
	8	63	Control	Unsuccessful RNA extraction																																
	9	NR	Control																				1.02	0.51	0.66	0	0.49	5.98	15.45	0.36	0.69	0.49			
	10	NR	Control																			0 / 0 / 0	1.45	0.71	1.32	0.06	0.82	10.19	20.79	2.3	0.16	1.62	232148.9	1897237	172530
	11	69	Control																			0 / 0 / .132											0	986940.2	516993.87
	12	NR	Control																		1697797		3.99	1.82	2.38	0	1.4	10.91	18.11	2.66	0.35	1.54			
	13	33	Control																		2763411												0	135920	0
	14	67	Control																		2632429	0 / 0 / 0											0	28629.56	0
	15	73	Control																				1.44	2.37	4.85	0.1	1.19	21.08	48.92	1.88	0.36	20.6	0	547437.4	
	16	NR	Control																			0 / 0 / .0253											0	496296.4	
	17	NR	Control	Active diverticulitis																			2.19	1.13	1.78	1.6	0.89	29.82	72.66	2.66	0.3	1.42			
	18	36	Control																															0	313774.9	0
	19	NR	Control																															0	204012.3	0
	20	78	Control																															0	320796.4	0
	21	NR	Control																															0.16	0	0
	22	63	Control																															0.4	0	0
	23	71	Control																			0 / 0 / .0618												0.33	0	97910.58	0
DAF1		68	Control		503 / -																															
DAF2		33	Control		671.8 / -																															
DAF3		23	Control		731 / 583																															
DAF4		30	Control		857 / 671																															
DAF5		47	Control		535 / 516																															
DAF6		42	Control		616 / 359																															
UPT 1		42	Control				0.92	0.1																												
UPT 2		56	Control				0.55	0.03																												
UPT 3		30	Control				0.42	0																												
UPT 4		56	Control				0.84	0.04																												
UPT 5		30	Control				0.96	0.1																												

Appendix II

Reagent suppliers and details of solutions

Ringer's physiological salt solutions

Standard	
NaCl	110 mM
KCl	5 mM
$MgSO_4$	1.25 mM
$CaCl_2$	1 mM
Na_2HPO_4	16 mM
NaH_2PO_4	4 mM
D-Glucose	5 mM

pH adjusted to 7.4 with Na_2HPO_4 or NaH_2PO_4; aerated with filtered room air.

With K^+ replacing Na^+:

KCl	115 mM
$MgSO_4$	1.25 mM
$CaCl_2$	1 mM
K_2HPO_4	16 mM
KH_2PO_4	4 mM
D-Glucose	5 mM

pH adjusted to 7.4 with K_2HPO_4 or KH_2PO_4; aerated with filtered room air.

Western blot nitrocellulose protein stripping buffer

Tris HCl	62.5 mM
SDS	2% w/v
β mercaptoethanol	100 mM

Arginine to citrulline assay 'reagent mix'

50 mM Tris buffered saline	pH 7.4
$MgCl_2$	6.67 mM
$CaCl_2$,	1.33 mM
FAD	26.5 uM
FMN	26.5 uM
NADPH	6.6 mM
BH_4	66.2 uM
calmodulin	66.2 uM
valine	334 mM
^3H arginine	5.56×10^{-3} mCi/ml

Sigma protease inhibitor cocktail

AEBSF	104 mM
Aprotinin	80 uM
Leupeptin	2.1 mM
Bestatin	3.6 mM
Pepstatin A	1.5 mM
E-64	1.4 mM

Laemmli sample buffer

Tris	62.5 mM
SDS	2% (w/v)
glycerol	10% (w/v)
bromophenol blue	0.1% (w/v)
DTT	50 mM
pH 6.8	

Tris-HCl electrophoresis gel 7.5%

water	4.85 ml
1.5M Tris-HCl pH 8.8	2.5 ml
10% SDS	100 ul
Acrylamide/Bis (30%)	2.5 ml
10% ammonium persulfate	50 ul
TEMED	5 ul

Tris-HCL stacking electrophoresis gel 4%

water	6.1 ml
0.5M Tris-HCl pH 6.8	2.5 ml
10% SDS	100 ul
Acrylamide/Bis (30%)	1.33 ml
10% ammonium persulfate	50 ul
TEMED	10 ul

Suppliers of reagents and equipment

		City	Country
Heparin	CP Pharmaceuticals	Wrexham	Wales
EDTA	Vacutainer, BD UK	Cowley, Oxfordshire	England
PBS (molecular grade)	Gibco (Invitrogen)	Carlsbad, CA	USA
RNA Extraction kit	Gentra	Minneapolis, MN	USA
Antibodies	Autogen Bioclear (Santa Cruz)	Calne, Wiltshire	England
Antibodies	StressGen	Victoria, BC	Canada
Labelled amino acids	NEN Life Sciences	Boston, MA	USA
Immunochemistry reagents	Vector Laboratories	Burlingame, CA	USA
Oligonucleotides	Sigma-Genosys	Pampisford, Cambridgeshire	England
Labelled oligonucleotides	MWG-Biotech AG	Ebersberg	Germany
RNA reagents	Ambion	Huntigndon, Cambridgeshire	England
Western blotting reagents	Amersham Pharmacia Biotech	Little Chalfont, Buckinghamshire	England
Human placenta protein and RNA extracts	Clontech	Palo Alto, CA	USA
Western blotting reagents	Bio-Rad	Hemel Hempstead	England
Real-time PCR reagents	Applied Biosystems	Warrington	England
PBMC isolation gradient	ICN / Cappel	Aurora, OH	USA
DAF-2DA	Calbiochem	La Jolla, CA	USA
Formaldehyde	Electron Microscopy Sciences	Fort Washington, PA	USA
Mounting medium	Sakura Finetek	Zoeterwoude	Netherlands
Protein concentration reagents	Pierce	Rockford, IL	USA
Scintillation counter	Beckman Coulter	High Wycombe, Buckinghamshire	England
Confocal Microscope	Olympus		Japan
Gel imaging system	UVP	Upland, CA	USA
Miscellaneous reagents and those not otherwise mentioned	Sigma-Aldrich	Poole, Dorset	England

Appendix III

Quantitation of protein concentration: method

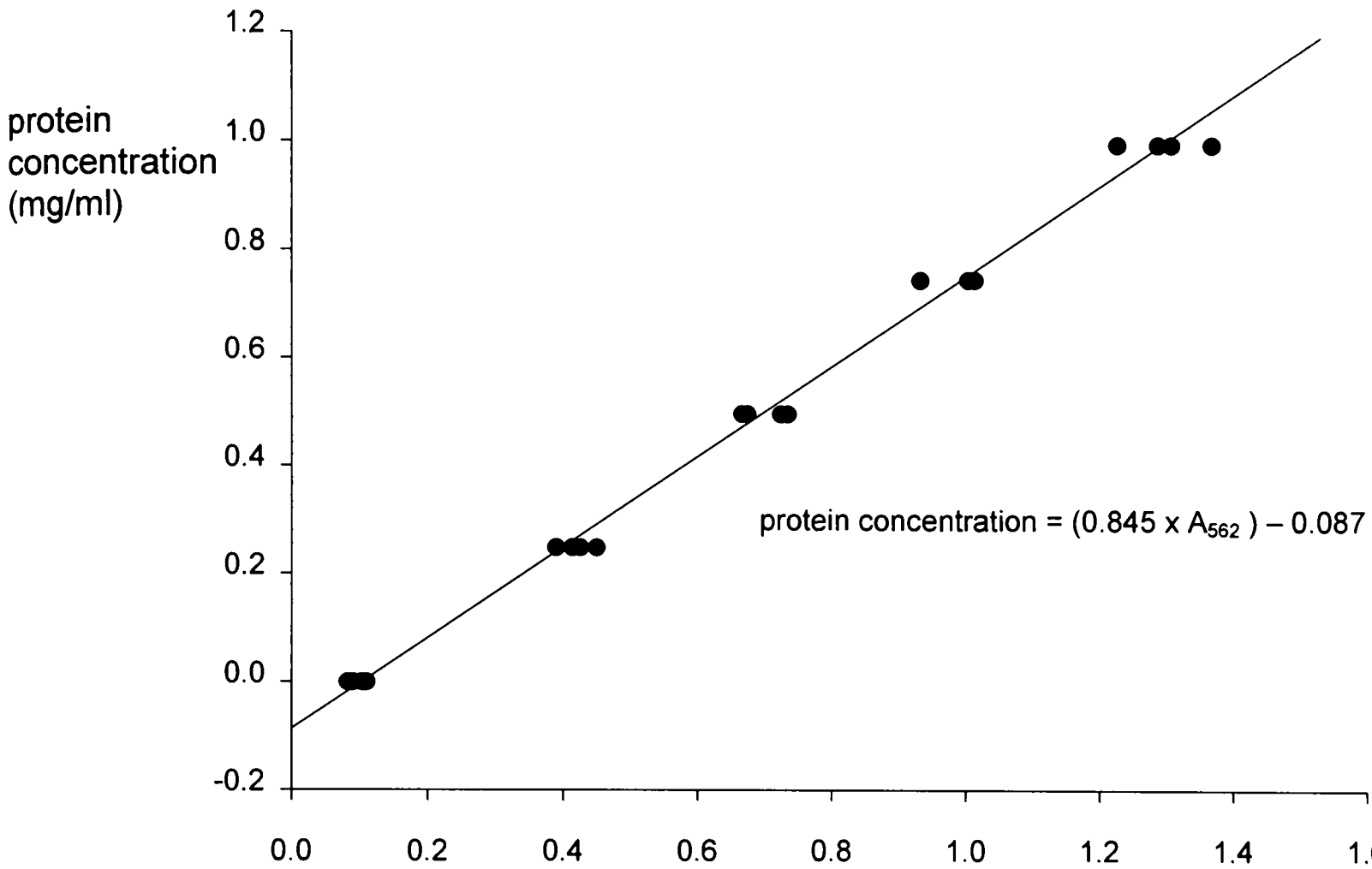
This method was used to determine the protein concentration of solutions of PBMCs and homogenised arterial smooth muscle.

50ul of protein solution was added to a test tube containing 1ml bicinchoninic acid + divalent copper ion reagent solution (50 parts 'solution A', 1 part 'solution B', Pierce Chemical Company). The solution was incubated at 37°C for 30 minutes, then its absorbance at 562nm measured using a spectrophotometer. Protein reduces Cu^{2+} to Cu^{+}, which reacts with bicinchoninic acid to form a purple coloured solution. The intensity of this colour (measured at 562nm) is proportional to the concentration of protein added.

A standard curve was constructed using serial dilutions of a 2mg/ml albumin solution, with each point performed in quadruplicate.

All samples were assayed in duplicate, and the mean of these two readings taken as the protein concentration of the sample.

An example of the results of an experiment to determine protein concentration (in this example, some of the arterial smooth muscle samples stored in AEBSF) is shown in figure A-IIIa.



	Mean A_{562}	**Protein concentration (mg/ml)**
Control 6	1.704	1.35
Control 5	1.499	1.18
Septic 1	1.565	1.24
Septic 2	1.921	1.54
Septic 4	1.699	1.35
Septic 6	3.750	3.08
Septic 7	2.199	1.77

Figure A-IIIa

Example of an experiment to determine the protein concentration of samples, in this case arterial smooth muscle samples stored in AEBSF. The standard curve (constructed using dilutions of a known concentration of albumin) is shown to provide an indication of the small variability in the technique, along with a sample table showing protein concentrations in experimental samples, which have been read from the standard curve.

Appendix IV

Investigation of a potential modulator of y^+ function: Resveratrol

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Work done by another member of this laboratory (Clark 1998) had shown that PBMCs from ovine blood exposed to endotoxin for 14 hours demonstrated a similar increase in y^+ activity and total arginine transport to that of PBMCs from patients with clinical sepsis (chapter 5 and Reade *et al.* (2002)). The study by Clark attempted to discover the signalling pathway from endotoxin receptor to y^+ function by use of inhibitors of intracellular second messengers. If applied prior to endotoxin exposure, cyclohexamide, thimerosal, dexamethasone and the non-selective cyclo-oxygenase inhibitor (COX) ibuprofen all prevented the increase in y^+ activity. The selective COX 2 inhibitor nimesulide and the COX 1 inhibitor resveratrol were also both able to prevent the increase in y^+ activity (Clark 1998). However, resveratrol was able to not only prevent, but reverse the upregulation of y^+ transport when added at the end of the endotoxin incubation, immediately prior to separation of the PBMC fraction and assay of transporter function. This was potentially a most important result: a non-toxic drug which could return upregulated y^+ function to normal *after the onset of sepsis* could possibly limit the substrate supply to nitric oxide synthase, and hence reduce nitric oxide production.

Resveratrol (3, 4', 5-trihydroxystilbene) is a non-toxic phytoalexin found in red wine. With anti-inflammatory and anti-oxidant properties, beneficial effects on lipid profiles, and the ability to reduce platelet aggregation (Fremont 2000), resveratrol it thought to contribute to the 'French Paradox', whereby the consumption of red wine is thought to reduce the incidence of cardiovascular disease in a population at high risk for atherogenesis. There is conflicting evidence that resveratrol might increase (Hsieh *et al.* 1999) or decrease (Tsai *et al.* 1999) (Kawada *et al.* 1998) nitric oxide production, and indeed might additionally act as a direct vasodilator (Chen & Pace-Asciak 1996) (El-Mowafy 2002). The possibility that at least part of this effect might be via arginine transport in human cells warranted investigation.

20ml whole blood was drawn from 3 septic patients and 3 healthy controls. To half of this volume resveratrol was immediately added at a concentration of 50 umol/L whole blood. To the other half of the sample, 2.5 ul 16% DMSO in ethanol was added; this was the vehicle in which the resveratrol was dissolved. This was the same protocol used by Clark in 1998. Separation of the PBMC fraction commenced 30 minutes after collection. Two fractions were prepared for each experimental condition: one using standard Ringer's physiological salt solution containing Na^+, and the other where Na^+ had been replaced by K^+(Appendix II). Rate of arginine uptake was determined using the oil separation method described in detail in Chapter 5 and in Reade *et al.* (2002) (Appendix VII). The results are shown in figure A-IVa.

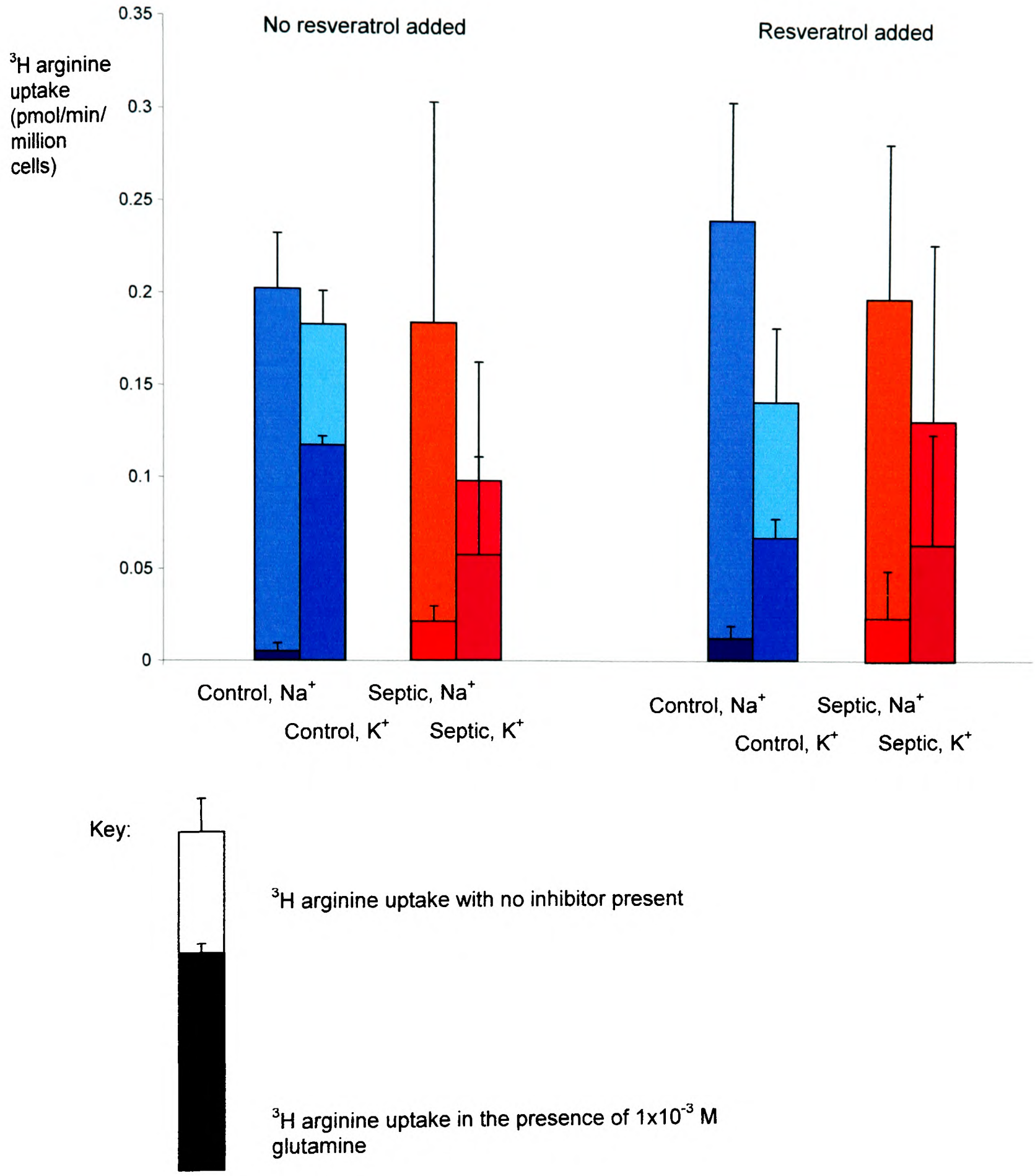


Figure A-IVa

^{3}H arginine uptake by PBMCs from 3 septic patients and 3 healthy controls, in solutions containing Na^+, or where Na^+ has been replaced with K^+. Each bar represents the mean total uptake (in the presence of no inhibitor) or the mean uptake when y^+L transport has been maximally inhibited by $1x10^{-3}$M glutamine. On the right are measurements in PBMCs which were exposed to resveratrol; on the left no resveratrol was used. Error bars represent standard deviation.

A number of conclusions can be drawn from figure A-IVa:

Firstly, analysis of the experiments without resveratrol reveals a number of trends, but few significant differences because of the small sample sizes. However, each confirms the conclusion of chapter 1.4 (all units are % or pmol/min/million cells, ± SD):

- Transport through y^+ (when sodium is present in the medium) is a greater proportion of total transport in PBMCs from septic patients (16.9 ± 14.3%) compared to healthy controls (2.4 ± 2.1%), though this effect is quantitatively less marked than in the experimental series reported in Chapter 5, and just fails to reach statistical significance (p=0.16, Student's unpaired t test).
- When Na^+ is removed from the medium, glutamine inhibits a substantially smaller proportion of total transport than when Na^+ is present, in PBMCs from both septic patients (residual uptake 50.0 ± 26.8%)(p=0.13, Student's unpaired t test) and healthy controls (residual uptake 64.4 ± 3.7%)(p=0.001, Student's unpaired t test). This is consistent with the known Na^+ dependence of glutamine transport through y^+L.
- When suspended in medium containing K^+ instead of Na^+, there is a more marked fall in total (uninhibited) transport in PBMCs from septic patients (from 0.18 ± 0.12 to 0.10 ± 0.06, such that 52 ± 18% of the uptake remains) than controls (from 0.20 ± 0.03 to 0.18 ± 0.02, such that 87 ± 21% of the uptake remains)(p=0.14, Student's unpaired t test). This is also similar to the results of chapter 5: PBMCs from septic patients have a higher proportion of their total transport through y^+, and the (electrogenic) transport of arginine through y^+ is opposed when the cell is depolarised in the high K^+ medium.

- Total transport in medium containing Na^+ is not increased in PBMCs from septic patients (0.18 ± 0.12; control 0.20 ± 0.03), in contrast to the results of Chapter 5. This may be due to an insufficient number of samples studied.

Secondly, comparing these results to those from cells exposed to resveratrol:

- Resveratrol has no significant effect on the total arginine uptake of either septic or control PBMCs in media containing Na^+, or when Na^+ is replaced by K^+.

These results thus largely confirm those of Chapter 5, but do not replicate the effect of resveratrol observed by Clark in 1998. This may be because of species differences, differences between clinical sepsis and in vitro endotoxin exposure, or possibly small variations in experimental technique (including time to process whole blood after addition of resveratrol, which was not recorded in the studies of ovine PBMCs). In any case, it would appear that resveratrol lacks promise as an inhibitor of upregulated arginine transport in human sepsis.

Appendix V

Attempts to develop antibodies recognising human proteins with y^+ activity

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The sequences of the proteins with y^+ function have been known for some time, and there is now general agreement on the likely tertiary structure of these proteins, though none has been crystallised. Despite this no antibody has been successfully prepared which recognises human CAT1, CAT2A, CAT2B or CAT4. The studies reported in this thesis have shown an increase in CAT2B mRNA in PBMCs and a decrease in CAT1 mRNA in arterial smooth muscle from patients with sepsis. We hoped to design antibodies which might allow the quantification of these proteins by either Western blotting or immunocytochemistry. This work is ongoing, and only preliminary results are presented here. The immunocytochemistry images in this appendix were prepared by Dr J.L. Millo, and the selection of antibody sequences was performed under the direction of Dr C.A.R. Boyd. All other work is my own.

Antibodies were synthesised using peptides based on the published human sequences of CAT1, CAT2A, CAT2 (non-specific) and CAT4. These peptides were selected from regions which showed no differences in the various published descriptions of each gene (derived from both mRNA and genomic DNA data). The putative structure of the CAT proteins has been described (based on animal data) (Deves & Boyd 1998), and the sequences selected were in the extracellular domains of the proteins. Sequences were thought more likely to be immunogenic if they contained proline, and

did not contain repeated elements or areas of low amino acid complexity. Possible structures identified in this way were tested using the Profile Fed Neural Network Systems From Heidelberg (PHD) computer program, which used different algorithms to analyse for likely secondary structure (helix, extended region or loop). The sequence was also checked for spurious matches against the human genome. Once a satisfactory sequence had been identified for each protein, the peptides were synthesised and conjugated to keyhole limpet hemocyanin (KLH) to enhance antigenicity. This work was performed by a commercial organisation (Research Genetics). 0.1mg peptide was then injected subcutaneously (with complete Freund's adjuvant) into two New Zealand White rabbits (for CAT1, CAT2A and CAT4) or two rats (for CAT2 non-specific). In the case of the antibodies raised in rabbit, boosters of antigen + complete Freund's adjuvant were given 2, 6 and 8 weeks later; serum was collected after week 4 and 8. At 10 weeks the animals were humanely sacrificed and terminally bled. For the antibody raised in rat, boosters of antigen (0.05mg in incomplete Freund's adjuvant) were given 2, 6 and 8 weeks later with a small test bleed taken at week 7 and the terminal bleed at week 10, at which time the animals were humanely sacrificed. The peptide sequences used are given in table A-Va.

	Species used	**Peptide sequence used as an immunogen**
CAT1	rabbit	CEEASLDADQARTPDGNLD
CAT2 (non specific)	rat	CEGHLRDENNEEDAYPD
CAT2A specific	rabbit	CRFLARVSKRQSPVAATLT
CAT4	rabbit	CEETVQAMQPPSQAPAQD

Table A-Va
Peptide sequences used for antibody synthesis

All three peptides injected into rabbits proved to be highly immunogenic, producing ELISA titres of >100 000 at 10 weeks, while the peptide used in rats produced a mean ELISA titre of approximately 5000 at the time of the test bleed, which is high for antibodies raised in this animal. All ELISA tests were performed by the manufacturer.

Multiple attempts have been made using these antibodies in Western blotting of tissue thought to express CAT proteins at sufficiently high levels: commercially produced human placental cell homogenate, and the PBMC and arterial smooth muscle homogenates used in other experiments described in this thesis. The general method used was that described in chapter 7. Multiple variations of primary antibody concentration, duration and temperature of exposure, and blocking solution composition, concentration, duration, time and temperature were used. Despite this, other than for CAT1, it has not been possible to identify bands at the expected molecular weights of any of these proteins which are not also present in negative control lanes probed either with pre-immune serum, or with serum which has been theoretically neutralised by incubation with the immunogenic peptide. The most successful examples of these are shown in figures A-V a, b and c.

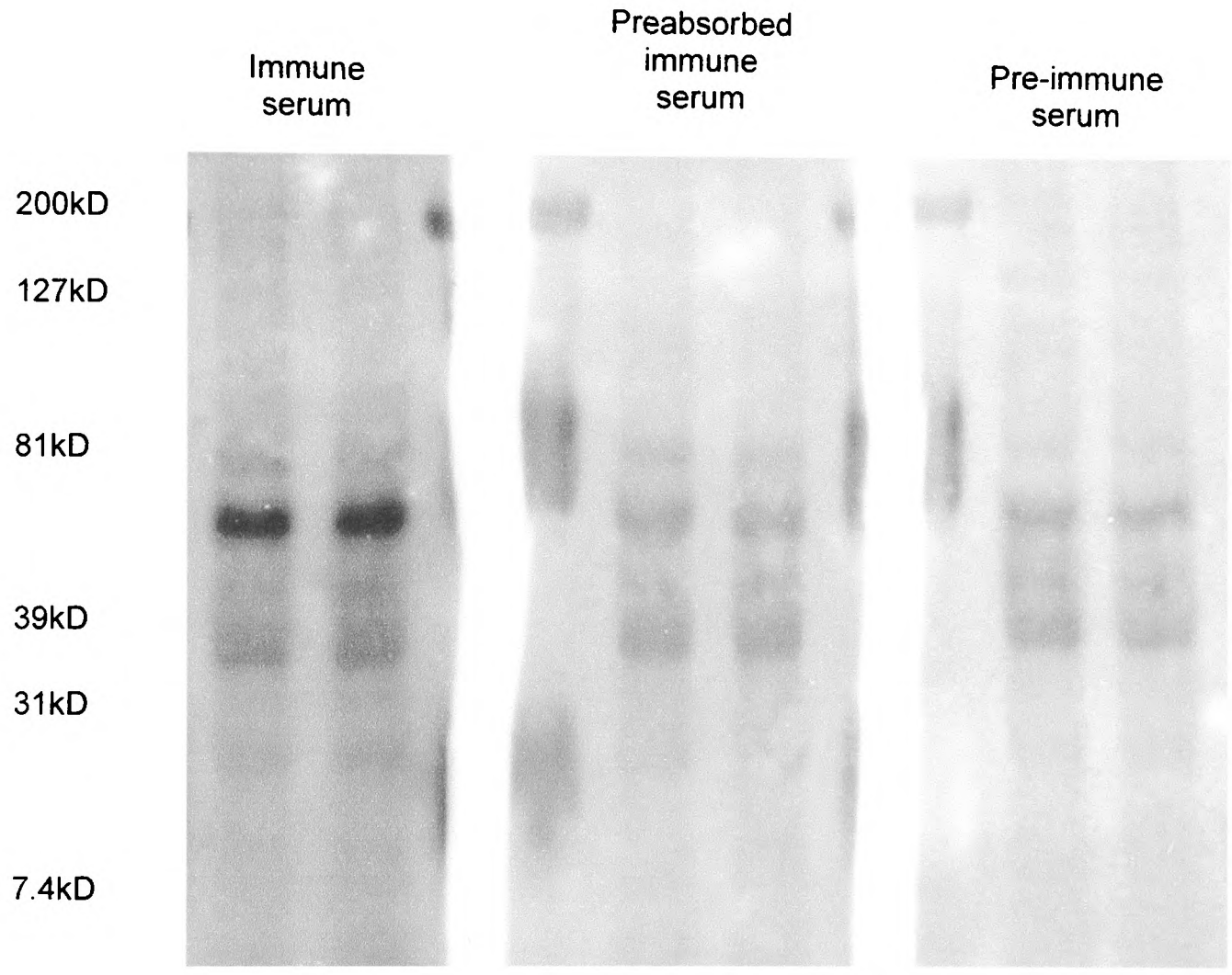


Figure A-Va
Western blot of human placenta homogenate probed with antibody raised against CAT1 (expected molecular weight 67kD). Coloured molecular weight markers were not detected by the primary and secondary antibodies, and so were marked on the photographic image of the blot by hand.

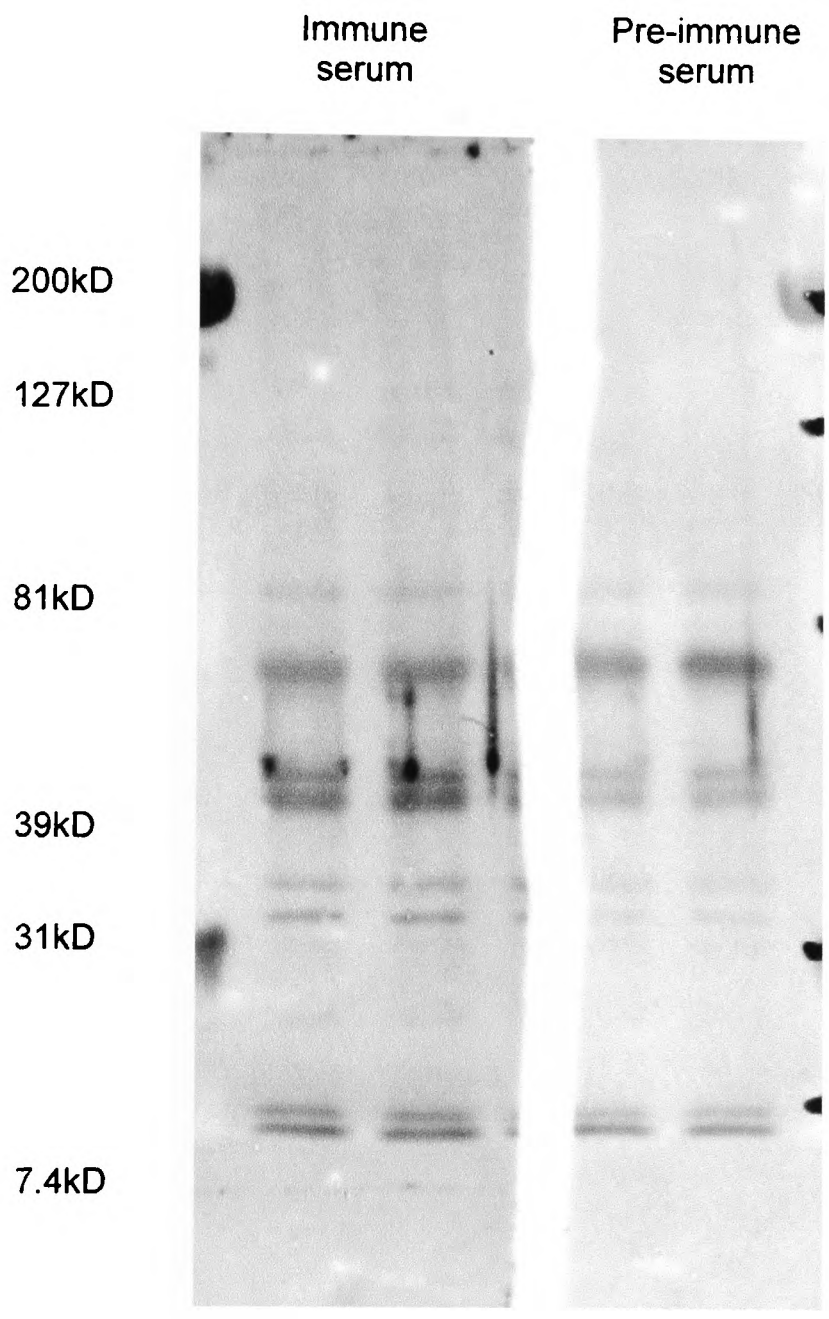


Figure A-Vb
Western blot of human placenta homogenate probed with antibody raised against CAT2 (expected molecular weight 72kD).

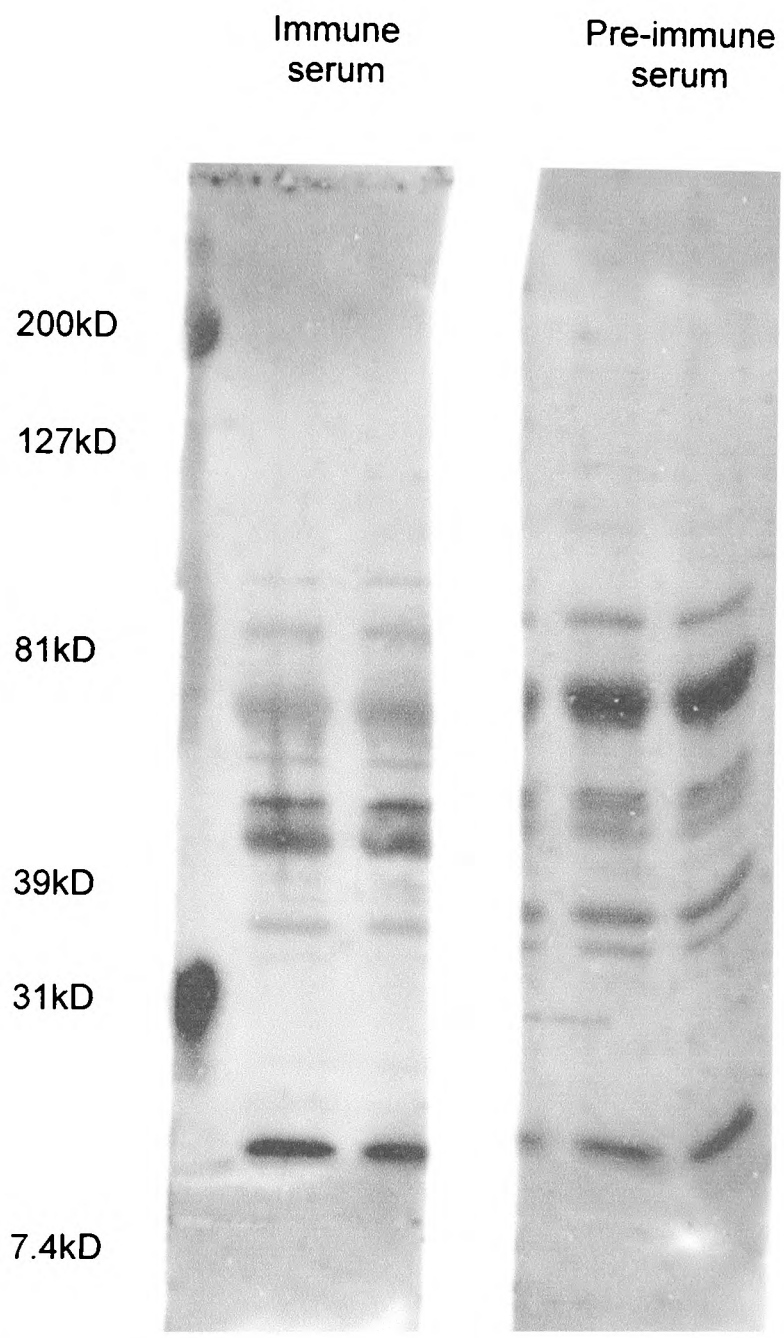


Figure A-Vc
Western blot of human placenta homogenate probed with antibody raised against CAT4 (expected molecular weight not known)

The lack of success of these negative controls may be related to the very high titres of antibody produced in these animals. It may be that the earlier bleeds (in the case of the serum from rabbits) will produce more specific bands. It might also be necessary to affinity purify these antibodies from the whole serum in which they are supplied. This is being done in collaboration with a group in Italy, but as yet the results of experiments by this group using our antibodies are not available. This line of investigation is undoubtedly worth pursuing, as there are still no successful anti-CAT antibodies described, and we have succeeded in possibly the most difficult step: designing sufficiently antigenic peptides.

Immunocytochemistry images of arterial smooth muscle stained with CAT2 (non-specific) have also been obtained (figure A-Vd; using an FITC labelled secondary antibody rather than the peroxidase-linked secondary antibody technique used in Chapter 9). There is a high amount of non-specific staining in the experimental control sections stained with either no antibody or secondary antibody only, which must be reduced before these antibodies can be used in useful experiments.

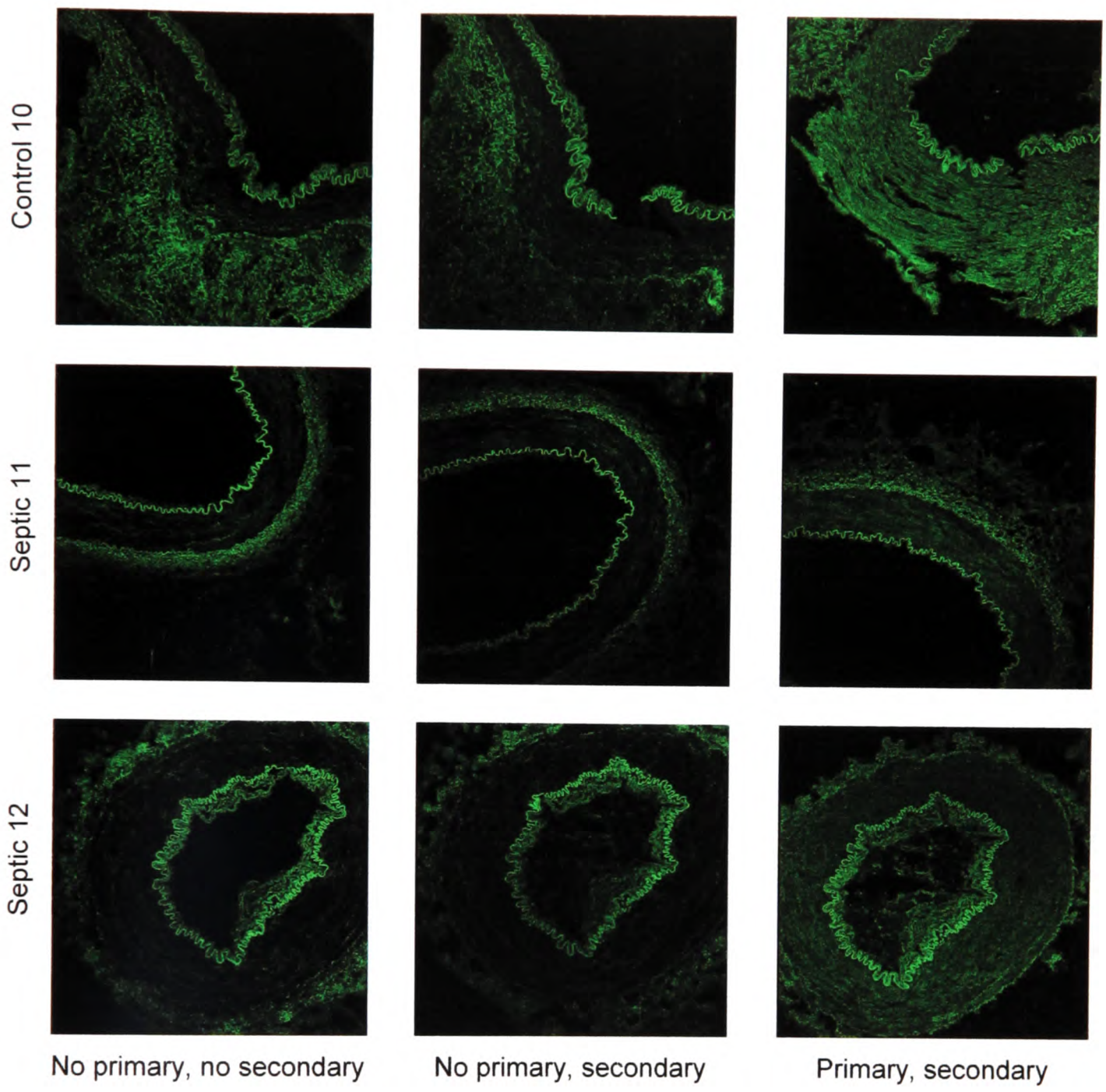


Figure A-Vd

Human arterial smooth muscle stained with antibody raised against CAT2 (non specific) protein. (L to R): no primary and no secondary antibody; no primary but secondary antibody; primary and secondary antibody. There appears to be primary antibody-dependent staining in the medial layer of the vessel, but it was not possible to reduce the non-specific staining in the other layers.

Appendix VI

Attempted functional study of heme oxygenase activity: Rate of bilirubin formation measured by spectrophotometry

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Heme oxygenase (HO) activity can be detected using a heme degradation assay (protocol modified from Maines *et al.* and Tenhunen *et al.* 1969). HO converts heme to biliverdin, which is subsequently reduced to bilirubin by biliverdin reductase. The complete conversion of heme to bilirubin also requires the cofactors NADPH and cytochrome *c* reductase (CCR). Bilirubin can be readily quantified using a spectrophotometer by measuring absorbance at 450nm.

Published protocols for the assay of HO activity in this manner suggest the microsomal fraction of the tissue studied should be prepared. This proved impossible in these studies of arterial smooth muscle and PBMCs, as there was insufficient material available. Thus the HO activity of the total cell lysate was measured instead. Rat spleen microsomes (prepared as outlined below), a tissue with known high HO activity, was used as a positive control. Fresh rat spleens were obtained from the unused portion of animals killed for another purpose; this procedure was performed according to UK Home Office animal welfare guidelines.

PBMC and arterial smooth muscle cell lysate was prepared as described in Chapter 2. The protein was diluted to 1mg/ml with an amount of PBS determined by the previously assayed protein concentration of each sample. Triton X-100 (final concentration 0.04%) was added to disrupt the cell membranes, and to prevent the aggregation of the enzyme with heme.

Rat spleen microsomes were prepared from 2.2g fresh tissue, which was homogenised using a high speed liquidiser in 9 volumes (v/w) homogenisation buffer (0.01 M Tris-Cl, pH 7.5, 85.58 g/L sucrose). The homogenate was centrifuged at 10 000g for 20 minutes at 4°C to remove the particulate debris. The supernatant was further spun at 100 000g for 1 hour at 4°C. The pellet formed by this step contained concentrated microsomes, which were resuspended in buffer containing 0.02 M Tris-Cl, pH 7.5, 20% glycerol, 1 mM EDTA and 0.4% Triton X-100.

The assay itself was performed as follows. A stock reagent solution was prepared, containing 108ul 1mM hemin / albumin (6.25mg hemin, 250ul 0.1M NaOH, 1g Tris HCl, 132mg bovine serum albumin, 0.2ml 0.1M HCl, made up to 10ml in water), 72ul 10mg/ml cytochrome c reductase, 6.42ml assay buffer (containing 3.402 g KH_2PO_4, 13.064 g K_2HPO_4, 2 ml 0.5 M EDTA, in 1L water, pH 7.4) and 810ul 2.75mM NADPH. To this was added 600ul of a biliverdin reductase solution, prepared from rat kidney in a manner similar to that for the spleen microsomal extract, but where the cytosolic supernatant (rather than the microsomal pellet) at the end of the 100 000g spin was used. For each sample, 177.5ul of this mixture was added to 2 wells of a 96 well plate. To one of these wells was added 22.5ul of the enzyme preparation; to the other was added its diluent, forming a 'no-enzyme' blank. All reagents were brought

to 37°C prior to mixing, as was the plate, removing any possibility that the reaction would be artifactually slowed in the first few minutes as the reagents warmed. The plate was incubated on a plate reader at 37°C, agitating every 3 minutes. The absorbance at 450nm (the nearest wavelength to the theoretical absorption maximum of bilirubin, 468nm, fitted to the machine) was read every 4 minutes for 6 hours.

The A_{450} of the enzyme (less no-enzyme wells) were plotted against time. The heme oxygenase activity in each sample was estimated from the initial slope of this plot. Representative graphs of the change in A_{450} with time for neat spleen microsomal extract are shown in Figures A – VIa and b.

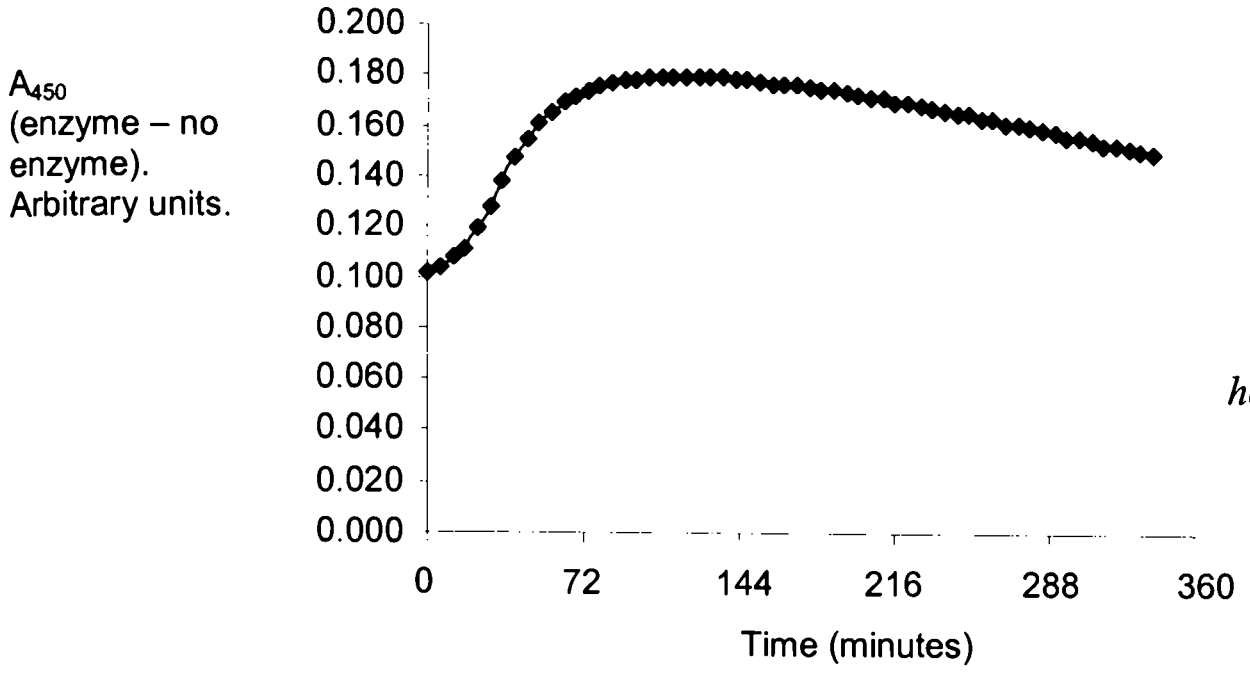


Figure A – VIa
Change in A_{450} (enzyme less no enzyme) over the entire 6 hour time course of the study.

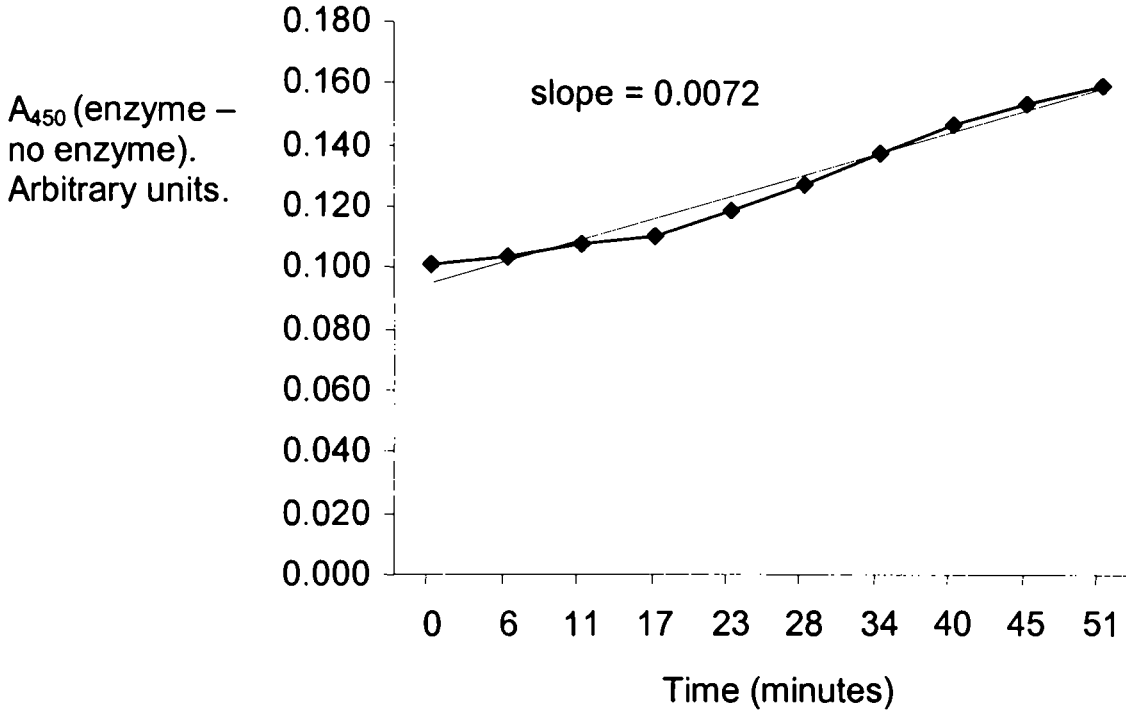
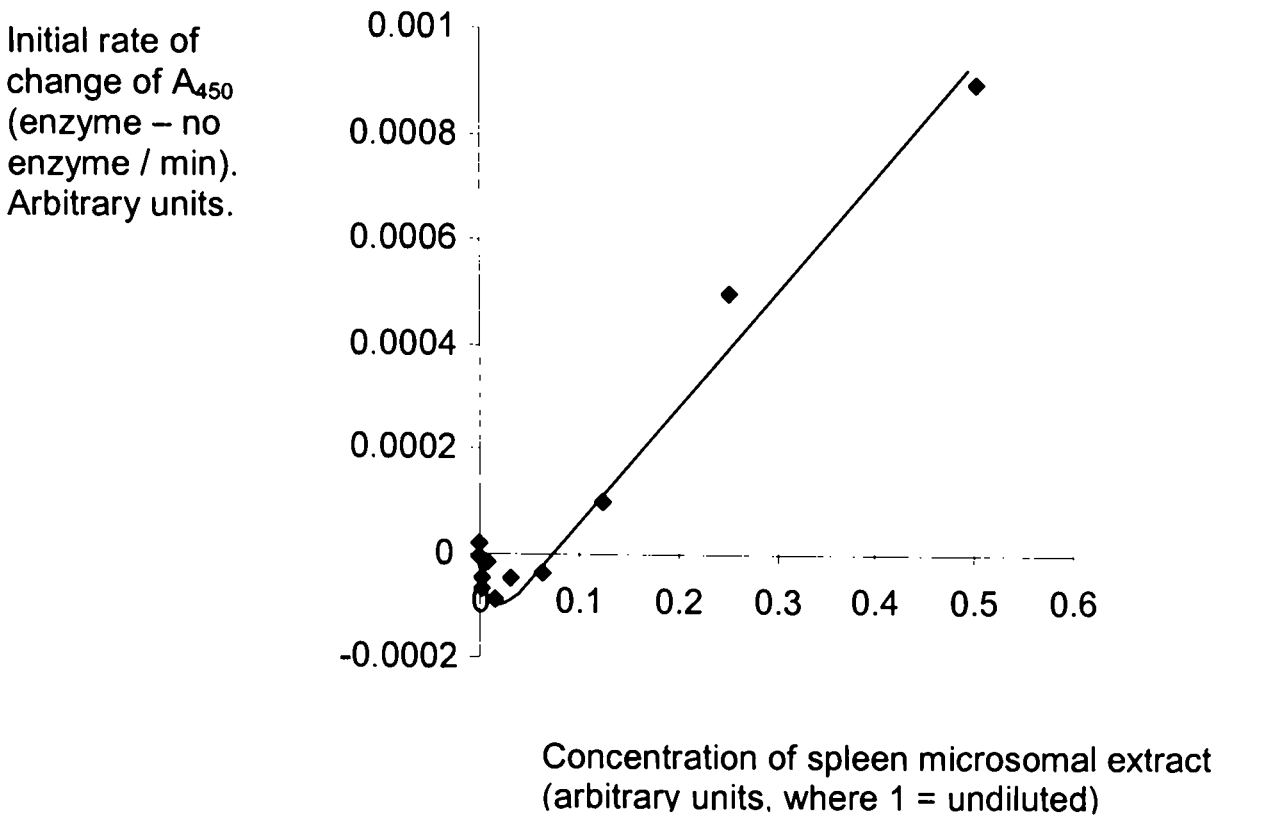


Figure A – VIb
Change in A_{450} (enzyme less no enzyme) over the period of linear change vs. time.

The change in A_{450} with time does not fit any simple predicted model of enzyme kinetics. Rather than reaching a plateau, the A_{450} declines with time. This must either be because the reaction product is being degraded, or there is some other coloured component of the reaction mix, the A_{450} of which decreases with time. A serial dilution of microsomal enzyme was performed to determine which of these two possibilities was the case, as well as to demonstrate the limit of sensitivity of the assay (figures A-VI c and d).



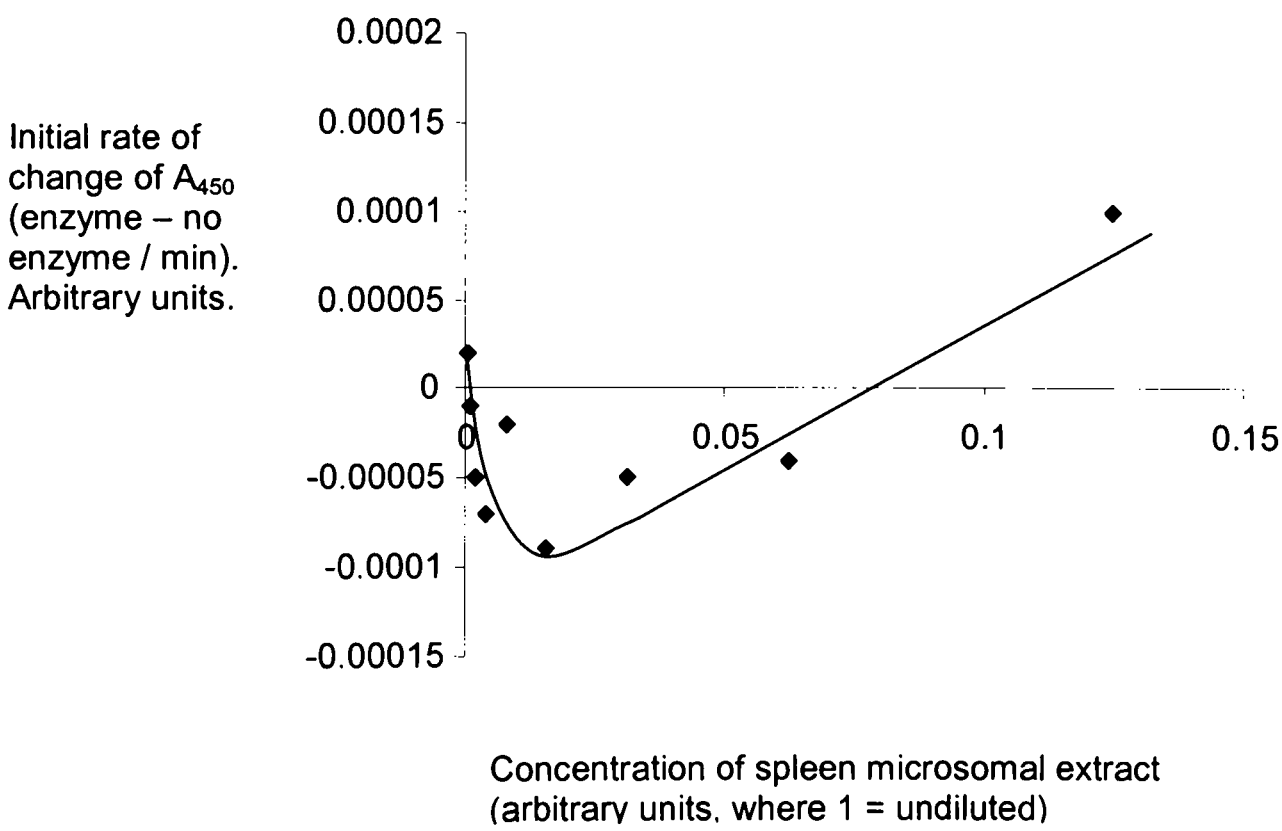


Figure A – VIc and d

Initial rate of change of A_{450} (enzyme less no enzyme / min) vs. concentration of spleen microsomal extract. At higher dilutions (seen most clearly on the expanded scale in figure A-IX d), the breakdown of other coloured components of the microsomal enzyme mix becomes a greater influence on the A_{450} of the solution than does the formation of bilirubin: hence the A_{450} decreases, rather than increases. As the concentration approaches zero, the microsomal enzyme mix approaches the composition of the no enzyme blank, so subtracting this blank value gives a value of zero.

As can be clearly seen, below a certain protein concentration, the rate of change of A_{450} becomes negative: that is, the A_{450} reduces rather than increases with time at lower enzyme concentrations. This is likely to be because the other coloured components of the microsomal enzyme mix with absorption spectra encompassing 450nm are degrading faster than bilirubin is being formed by the low heme oxygenase concentration. Cytochrome c reductase is particularly labile, and has a large absorbance in this part of the spectrum. As enzyme concentration reduces to approach zero, the rate of change of A_{450} returns to zero, as the value recorded is that of enzyme – no enzyme. As [enzyme] approaches zero this value must also approach zero. It can be clearly seen, then, that this assay has a minimum level of sensitivity for the detection of heme oxygenase activity.

When the PBMC or arterial smooth muscle tissue homogenate was assayed in this manner, the rate of change of A_{450} was always negative, though similarly approached zero as the dilution of protein became infinitely large (as seen in figure A - VIe).

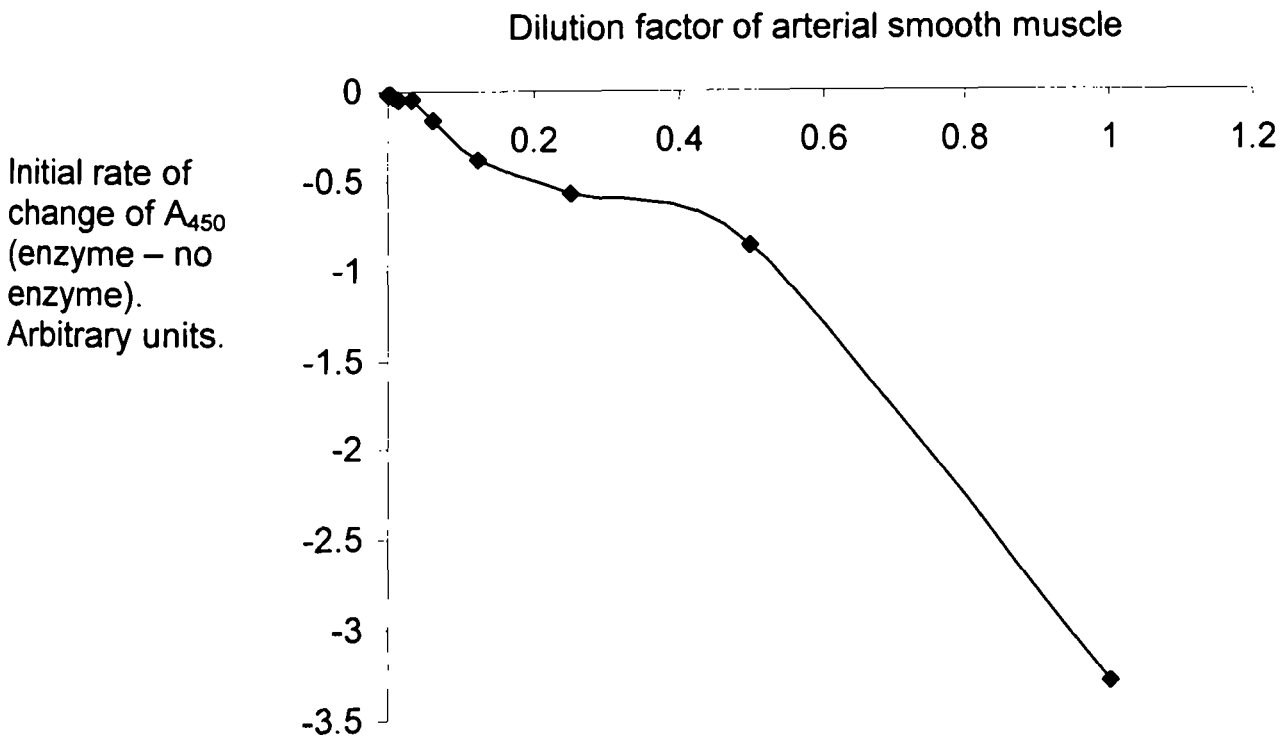


Figure A – VIe

Initial rate of change of A_{450} (enzyme less no enzyme) vs. dilution factor of arterial smooth muscle homogenate. The activity of heme oxygenase, even at the highest concentration of enzyme, is insufficient to make enough bilirubin to offset the reduction in A_{450} due to breakdown in the other reaction components.

Though the decline in A_{450} with time is dependent on the concentration of protein in the well, this is most unlikely to be an action of heme oxygenase alone. Only the formation of bilirubin, as indicated by a rise in A_{450}, can be considered heme oxygenase specific. As such, it must be concluded that this assay lacks the necessary sensitivity to detect the very low levels of heme oxygenase activity present in these tissue homogenates of PBMCs and arterial smooth muscle.

Samples of arterial smooth muscle and PBMCs had detectable HO-1 protein on Western blots. The amount of heme oxygenase protein required to give a detectable signal in this activity assay has not been determined. This could possibly be done by quantifying the amount of heme oxygenase protein in the rat spleen microsomes using Western blotting. However, this would need to be done for all three heme oxygenase isoforms. This would be best done using an antibody which recognised all heme oxygenase isoforms; unfortunately none has been described. If two or more antibodies were used, their relative affinities would have to be quantified to allow any meaningful addition of band staining intensity. The information gained by such an experiment was not thought to warrant such a complex protocol.

A variety of modifications of this technique were attempted, including decreasing the concentration of cytochrome c reductase, increasing the amount of enzyme solution added to the substrate mix, extracting the bilirubin from its aqueous phase using chloroform, using a bilirubin assay kit (based on the reaction of total bilitrubin with diazotized sulfanilic acid in the presence of DMSO to give the coloured product azobilirubin), and reading the absorbance at 468 nm after an incubation of 1 hour

using a scanning spectrophotometer: nothing was found to substantially improve the above result. The technique was therefore abandoned.

Appendix VII

Publications

Full papers:

READE, M.C., CLARK, M.F., YOUNG, J.D. & BOYD, C.A.R. (2002). Increased cationic amino acid flux through a newly expressed transporter in cells overproducing nitric oxide from patients with septic shock. *Clinical Science*, 102, 645-650.

READE, M.C. & YOUNG, J.D. (2003). Of mice and men (and rats): implications of species and stimulus differences for the interpretation of studies of nitric oxide in sepsis. *British Journal of Anaesthesia*, 90, 115-118.

Letters to professional journals:

READE, M.C., YOUNG, J.D. & BOYD, C.A.R. (2001). Abnormal y^+LAT1 L-arginine transport in lysinuric protein intolerance: intestinal epithelium is unlikely to be the only site affected. *Journal of Clinical Investigation*, online correspondence:
http://www.jci.org/cgi/eletters/108/5/717

Abstracts presented at international conferences:

READE, M.C., YOUNG, J.D. & BOYD, C.A.R. (2001). The effect of sepsis on arginine transport through y^+ in human peripheral blood mononuclear cells. *Journal of Physiology*, 531P, 116P

READE, M.C., BOYD, C.A.R. & YOUNG, J.D. (2001). Nitric oxide production and arginine transport are increased in peripheral blood mononuclear cells from patients with septic shock. *Anesthesiology*, 95: A347.

READE, M.C., YOUNG, J.D. & BOYD, C.A.R. (2001). Cationic amino acid transporter mRNA levels in peripheral blood mononuclear cells from patients with septic shock. *Journal of Physiology*, 535P, S033.

MILLO, J.L., READE, M.C., YOUNG, J.D. and BOYD, C.A.R. (2001). Inducible nitric oxide synthase in vascular smooth muscle from patients in septic shock. *Journal of Physiology*, 535P, S029.

READE, M.C., BOYD, C.A.R. & YOUNG, J.D. (2002). Arginine transporter abnormalities in cells producing nitric oxide in human septic shock. *Anaesthesia and Intensive Care*, 30, 513.

READE, M.C., MILLO, J., BOYD, C.A.R. & YOUNG, J.D. (2002). Heme oxygenase 1 mRNA is increased in human septic shock. *Critical Care Medicine*, 29 (12), 143/M32.

READE, M.C., MILLO, J., BOYD, C.A.R. & YOUNG, J.D. (2002). Reduction of nitric oxide synthesis and NOS mRNA in arterial smooth muscle from patients with septic shock. *American Journal of Respiratory and Critical Care Medicine*, A575.

READE, M.C., MILLO, J., BOYD, C.A.R. & YOUNG, J.D. (2002). Expression of heme oxygenase 1 and inducible nitric oxide synthase protein in human septic shock. *Journal of Physiology*, 543P, 39P. (winner of the Pfizer Prize)

READE, M.C., MILLO, J., BOYD, C.A.R. & YOUNG, J.D. (2002). Inducible and endothelial nitric oxide synthase protein in the smooth muscle of mesenteric arteries from patients with septic shock. *Intensive Care Medicine,* 28 (Suppl. 1), 361.
READE, M.C., BOYD, C.A.R. & YOUNG, J.D. (2002). Nitric oxide synthases are decreased in mononuclear cells from patients with septic shock. *Critical Care Medicine*, 31(2), 259/A52.

Abstracts presented at national conferences:

READE, M.C., MILLO, J., YOUNG, J.D. & BOYD, C.A.R. (2001). Arginine transport as a target for reducing nitric oxide production in human septic shock. *Science, Engineering and Technology Britain*, 8, E2-33.
READE, M.C., MILLO, J., YOUNG, J.D. & BOYD, C.A.R. (2001). Nitric oxide and arginine transport in human septic shock. *8th UK Intensive Care Medicine Conference*, Gloucester, UK.
READE, M.C., YOUNG, J.D. & BOYD, C.A.R. (2003). Nitric oxide and carbon monoxide: pathogenic factors in human septic shock? *Communications of the Medical Research Society*, M151, 55P.