Impaired Vascular Redox Signalling in the Vascular Complications of Obesity and Diabetes Mellitus

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Abstract

Significance: Oxidative stress, a crucial regulator of vascular disease pathogenesis, may be involved in the vascular complications of obesity, systemic insulin resistance and diabetes mellitus.

Recent advances: Excessive production of reactive oxygen species in the vascular wall has been linked with vascular disease pathogenesis. Recent evidence has revealed that vascular redox state is dysregulated in cases of obesity, systemic insulin resistance and diabetes mellitus, potentially participating to the well-known vascular complications of these disease entities.

Critical issues: The detrimental effects of obesity and the metabolic syndrome on vascular biology have been extensively described at a clinical level. Furthermore, vascular oxidative stress has often been associated with the presence of obesity and insulin resistance as well as a variety of detrimental vascular phenotypes. However, the mechanisms of vascular redox state regulation under conditions of obesity and systemic insulin resistance, as well as their clinical relevance, are not adequately explored. In addition, the notion of vascular insulin resistance, and its relationship with systemic parameters of obesity and systemic insulin resistance, is not fully understood. In this review, we present all the important components of vascular redox state and the evidence linking oxidative stress with obesity and insulin resistance.

Future directions: Future studies are required to describe the cellular effects and the translational potential of vascular redox state in the context of vascular disease. In addition, further elucidation of the direct vascular effects of obesity and insulin resistance is required for better management of the vascular complications of diabetes mellitus.

Key words: vascular disease; vascular redox signalling; oxidative stress; obesity; insulin resistance

List of abbreviations

AGE: Advanced glycation end-products

AMPKα: adenosine monophosphate activated kinase alpha

AngII: Angiotensin II

AP1: Activator protein 1

AT: Adipose tissue

ATP: Adenosine triphosphate

BH2: Dihydropterin

BH4: Tetrahydropterin

BMI: Body mass index

BNP: Brain natriuretic peptide

CaM: Calmodulin

CVD: Cardiovascular disease

DM: Diabetes mellitus

DPP4: Dipeptidyl peptidase-4

Duox: Dual oxidase proteins

eNOS: Endothelial nitric oxide synthase

FFA: Free fatty acid

GLP1: Glucagon-like peptide 1

GPx: Glutathione peroxidase

H_2O_2: Hydrogen peroxide

HDL: High density lipoprotein

IL-1β: Interleukin 1β

IL-10: Interleukin 10

IR: Insulin resistance

IRS: Insulin response substrate

LDL: Low density lipoprotein

LOX1: Lectin-like oxidised low-density lipoprotein receptor 1

MAPK: Mitogen-activated protein kinases

NADH: Nicotinamide adenine dinucleotide

NADPH: Nicotinamide adenine dinucleotide phosphate

NF-κB: Nuclear factor kappa beta

NO: Nitric oxide

NOS: Nitric oxide synthases

ONOO-: Peroxynitrite

oxLDL: Oxidised low-density lipoprotein

PI3K: Phosphoinositide 3-kinase

PKC: Protein kinase C

PON: Paraoxonase

PPARγ: Peroxisome proliferator-activated receptor gamma

PVAT: Perivascular adipose tissue

RAGE: Receptor for advanced glycation end-products

ROS: Reactive oxygen species

SGLT2: Sodium-glucose co-transporter 2

SOD: Superoxide dismutase

TNFα: Tumour necrosis factor alpha

VSMC: Vascular smooth muscle cells

1. Introduction

Low levels of reactive oxygen species (ROS) are involved in physiological cellular processes such as proliferation, apoptosis, hypertrophy, stress responses and survival (167). However, their excessive production in the vasculature is linked to vascular disease (40,112,136,212). The pathogenic properties of ROS may range from stimulation of vascular smooth muscle cell (VSMC) proliferation and migration to macrophage activation, propagation of local inflammation and impairment of endothelial function (112,125,136).

Obesity and diabetes mellitus (DM) are characterised by overlapping systemic biochemical and biological abnormalities including hyperlipidaemia, hyperglycaemia, insulin resistance (IR) and inflammation (96). Obesity, in particular, is characterised by increased body fat mass and, perhaps more importantly, by a dysregulated body fat distribution (4,8,109). DM, on the other hand, can be classified into many subtypes with differing pathophysiology which ultimately results in hyperglycaemia (14). All of the aforementioned metabolic abnormalities are able to induce profound changes in vascular cell phenotype with regards to inflammatory activation, haemostasis dysregulation, proliferation and migration capacity, and oxidative stress (166), leading to vascular complications.

In this review, we summarise the role of redox signalling in the vascular complications of obesity and diabetes. Importantly, we present recent advances in the pharmacological targeting of vascular disease and redox state in diabetes and obesity, and discuss about novel aspects of paracrine vascular redox state regulation.

2. Oxidative stress as a mechanism of vascular disease pathogenesis

Oxidative stress is characterised by an increased endogenous production of various ROS such as superoxide ($O_2^{\cdot-}$) radicals, hydrogen peroxide, hypochlorous acid and others (93). Endogenous ROS originate from various enzymatic systems such as NADPH-oxidases, indirectly from uncoupled nitric oxide synthases (NOS) and mitochondrial oxidase, and they can be increased in response to smoking and other exogenous oxidative stimuli (20,46,155). A multitude of experimental and pre-clinical studies have linked ROS with the pathogenesis of vascular diseases such as atherosclerosis (112), hypertension (135) and arterial aneurysm formation (68).

Excess production of ROS may contribute to vascular disease via a variety of mechanisms. Indeed, ROS induce direct DNA oxidative damage that initiates apoptotic pathways in endothelial cells and VSMC (10,64,125,152). Furthermore, ROS are able to oxidise low density lipoproteins (LDL), which are then internalised by macrophages and deposited in the subendothelial space at sites of dysfunctional endothelium, this being a key step for the initiation of atherogenesis (121,155). In addition, ROS are able to react with nitric oxide (NO) to form peroxynitrite ($ONOO^-$), a highly ROS itself (6). This results in direct loss of the vasoprotective effects of NO and further potentiation of the oxidative burst (112).

Recently, ROS have emerged as dynamic regulators of cellular homeostasis in in health and disease, in more complex ways than previously believed (184) (Fig. 1). Indeed, studies have demonstrated that ROS regulate many key redox sensitive transcriptional pathways (such as that of nuclear factor kappa beta (NF-κB) and activator protein 1 (AP-1)) interfering with endothelial activation, growth, survival and/or apoptosis, differentiation and inflammation (11). Interestingly, redox signalling is highly compartmentalised, as evidenced by the example of the AP-1 pathway, which depends upon oxidation for activation in the cytosol, but requires reduction prior interaction with DNA (89). In particular, both the NF-κB and AP-1 pathways are activated by a wide range of ROS such as hydrogen peroxide (H_2O_2), superoxide ($O_2^{\cdot-}$),

hypochlorous acid (HClO) and peroxynitrite ($ONOO^-$) (59,143). Consequently, prolonged exposure of the endothelium to increased levels of ROS can result into loss of redox compartmentalisation and cellular stress and, ultimately, to a detrimental endothelial phenotype (89,188). Apart from the endothelium, VSMC biology is also regulated by ROS-mediated signalling, with regards to differentiation, hypertrophy and growth, processes that are pivotal in the pathophysiology of diseases such as atherosclerosis, hypertension and aneurysmal disease (113).

A variety of enzymes and compounds in the human body act as endogenous antioxidants, neutralising ROS and maintaining redox balance under normal circumstances (48) (Fig. 2). Importantly, dysregulation of these enzymatic defence mechanisms has been associated with vascular disease (48). Such enzymes include superoxide dismutases (SODs), which catalyse the conversion of the highly reactive $O_2^{\cdot-}$ to oxygen and H_2O_2 (53), catalase, which converts H_2O_2 to water and oxygen (112), and the paraoxonase (PON) family of enzymes (51). Glutathione peroxidases (GPx) reduce a wide range of lipid peroxides to their corresponding alcohols, while they are also able to convert H_2O_2 to water and oxygen, thus also comprising important antioxidant enzymes (51). In addition to the aforementioned antioxidant enzymes, a number of small non-protein molecules can behave as endogenous antioxidants, including glutathione, uric acid, bilirubin and vitamin C, which behave as ROS scavengers while also potentially mediating the enzymatic production of ROS (48).

3. Obesity, diabetes mellitus and vascular disease

3.1. Relationship between obesity and vascular disease

Various studies have linked obesity, especially central obesity defined by an increased waist-to-hip ratio, with CVD progression (180). Several pathogenic mechanisms have been proposed

to justify this relationship. Indeed, obesity is characterised by marked adipose tissue (AT) inflammation (210). Inflamed AT, in turn, secretes high levels of pro-inflammatory molecules such as leptin, resistin, tumour necrosis factor (TNF) and interleukin 6 (IL-6) (42,66). Obesity may thus propagate vascular disease via induction of a systemic pro-inflammatory environment (207). Consistently, obesity has been associated by an increase in systemic oxidative stress evidenced by an increase in oxidised LDL levels and urinary F2-isoprostanes, which correlates with the circulating levels of inflammatory biomarkers such as monocyte chemoattractant protein 1 (MCP1) and TNF (180). Furthermore, obesity is characterised by nutrient overload and free fatty acid (FFA) overproduction, all of which can directly influence vascular biology (128). Importantly, there is growing evidence suggesting that the "dysfunctional adipocyte" drives significant changes in the AT secretome, shifting its metabolic profile (141). The microRNA content of AT–derived microvescicles is also shifted in obesity (81), and this leads to further changes in the endocrine cardiovascular effects of "dysfunctional" AT.

Central obesity is often observed in the context of the metabolic syndrome, namely a pathophysiological entity described by a number of characteristics including systemic low-grade inflammation, dyslipidaemia, nutrient overload, AT inflammation, insulin resistance, hypertension, hyper-coagulant state and endothelial dysfunction (96) (Fig. 3). The metabolic syndrome comprises an important public health problem, and is associated with both microvascular and macrovascular disease that accounts for major comorbidities in such patients (96). Although multiple studies have linked the metabolic syndrome and its individual risk factors with CVD, the specifics of how these concurrent risk factors collude to influence vascular function are unknown (203). Interestingly, metabolically healthy obese individuals with no other risk factors have no increased risk for CVD (71). This suggests that obesity is a heterogeneous entity, the direct effects of which on the vasculature are not easy to separate from the concomitant effects of co-exiting risk factors in clinical studies.

Although obesity, typically defined by BMI, has long been viewed as risk factor for CVD, recent advances have revealed that the quality of AT may be much more important than overall AT mass (8). The clinical observation that obese patients often have better cardiovascular outcomes, a finding termed "the obesity paradox", corroborate the fact that the relationship between AT biology and vascular disease is more complicated than previously thought (8). Importantly, distinct AT depots have opposing relationship with vascular disease, with visceral AT mass being detrimental and gluteal AT being protective (80). This anatomical variability in AT function may reflect region-specific differences in the secretome of AT (4).

3.2. Relationship between diabetes mellitus and vascular disease

The majority of DM cases can be classified as either type 1 or type 2 (181). Further to these two types, other, rarer, types of diabetes have been described based on specific aetiologies. These include genetic defects in pancreatic beta cell function (maturity onset diabetes of the young (MODY)), genetic defects in insulin function (such as in the context of leprechaunism and lipoatrophy), exocrine pancreatic pathologies, various endocrinopathies and certain drugs or infections (5). Depending on their underlying aetiology, different types of DM have differing risk for vascular complication. Thus, the fundamental cellular mechanisms of diabetic vascular injury may be similar, but the varying extent of peripheral insulin resistance and hyperglycaemia differentially determine the cardiovascular risk of the various types of diabetes (5,200).

Type 1 DM is an autoimmune disease characterised by primary pancreatic insufficiency and complete loss of insulin secretion (95). On the contrary, type 2 DM is primarily characterised by peripheral insulin resistance, which is often associated with hyperinsulinaemia in the early stages of the disease and prior to pancreatic failure (5). Type 2 DM appears in the context of a cluster of metabolic disturbances comprising the metabolic syndrome (181). Both types of DM

are associated with vascular complications, suggesting that hyperglycaemia has direct detrimental effects on the vasculature (17,18). In the case of type 2 DM, other factors such as obesity, hyperlipidaemia and abnormal insulin signalling may further contribute to vascular disease (17,18).

Several forms of non-type 1 diabetes are also associated with partial or complete insulin deficiency, including permanent neonatal diabetes mellitus (PNDM) and latent autoimmune diabetes in adults (LADA) (160,195). PNDM has been associated with a variety of genetic defects such as mutations of the *KCNJ11* and *ABCC8* genes, encoding for two subunits of the K_{ATP} channels involved in insulin secretion, as well as with mutations of the insulin gene (140). LADA, on the other hand, is an autoimmune disease typically associated with slower beta cell degeneration compared to type 1 DM (195). Both disease entities are characterised by failure of the pancreatic insulin release in response to hyperglycaemia. As such, LADA and PNDM share clinical similarities with type 1 DM, including the prevalence and pathophysiology of vascular complications (34), resulting from the detrimental vascular effects of hyperglycaemia (106).

MODY is a genetic, monogenic form of usually insulin-independent DM appearing in young adults below 25 years old (129). Mutations of the glucokinase (GCK) and the hepatocyte nuclear factors 1 and 4 alpha (HNF1A, HNF4A) gene account for ~80% of all MODY cases (55). GCK mutations (GCK-MODY) induce a delayed pancreatic response to glucose, resulting in minor fasting hyperglycaemia which however is usually still under some homeostatic control (129). The prevalence of vascular complications is relatively low in GCK-MODY patients (200). In contrast, HNF1A/4A mediate insulin synthesis and overall cell homeostasis, and thus mutations of their loci are associated with severe hyperglycaemia and high rates of macro- and micro-vascular complications (200). The discrepancy in vascular complications between GCK-MODY and HNF1A-MODY/HNF4A-MODY may reflect the differing severity of

hyperglycaemia (55). Furthermore, certain cases of GCK-MODY have been associated with various dyslipidaemias which may further contribute to vascular disease. In addition, the direct vascular effects of GCK versus HNF1A/4A mutations are unclear, and may be further involved in the vascular complications associated with MODY.

Circulating glucose levels and glycosylated haemoglobin (HbA1c) are regarded as crucial surrogate markers of DM severity, and their regulation has long been the primary goal for the management of the cardiovascular consequences of DM (116). On the other hand, it has recently been revealed that targeting glycaemic control alone is not sufficient to reverse the cardiovascular complications of DM (63,127,186), suggesting that the various agents used to control blood glucose may convey differential cardiovascular effects *in vivo*, which should be taken into account (116). Consequently, cardiovascular risk in diabetic patients is determined both by the quality of glycaemic control as well as the differing cardiovascular effects of the various agents used to achieve it. Essentially, each therapeutic strategy has a wider number of "pleiotropic" or "hypoglycaemia-independent" effects that may target vascular redox signalling directly, at a cellular level.

3.2.1. Vascular effects of glucose

Although normal levels of glucose are required for physiological cell metabolism, hyperglycaemia induces a number of cellular disturbances that propagate diabetic complications (18). These disturbances include increased activation of the polyol pathway, increased formation of advanced oxidation products (AGE), stimulation of protein kinase C (PKC) activity and induction of mitochondrial oxidative stress as explained in following sections (17,18).

The polyol pathway reduces toxic aldehydes to alcohols under physiological conditions (17). Aldose reductase, the first enzyme of the polyol pathway, has low affinity for glucose (17).

However, in conditions of hyperglycaemia, glucose levels are increased intracellularly and partially converted to sorbitol, which can then be oxidised via the polyol pathway to fructose with concomitant reduction of NADPH (18). Increased glucose flux via the polyol pathway may induce cellular damage and ultimately contribute to diabetic complications via a variety of mechanisms involving sorbitol-mediated osmotic stress, reduced Na^+-K^+ ATPase activity, dysregulation of the NADH/NAD^+ balance and reduction of cytosolic NADPH (17). Notably, NADH is crucial for maintenance of the intracellular bioavailability of glutathione, an important endogenous antioxidant (18). In particular, the intracellular NADH/NAD^+ ratio is a very sensitive regulator of intracellular redox balance, dysregulation of which results initially in reductive stress that eventually leads to oxidative stress and oxidative damage to macromolecules, including DNA, lipids, and proteins (213). Consequently, increased hyperglycaemia-induced flux via the polyol pathway is able to modify endogenous antioxidant defences, predisposing to cellular oxidative stress (18).

AGE are the end-products of non-enzymatic lipid and protein glycosylation (208). This can occur intracellularly, thus modifying cellular protein function and gene expression, while AGE precursors can also diffuse locally or in the circulation to further modify normal protein structures (18). AGE levels are increased in DM but also in obesity and hyperlipidaemia as well as with old age (60). AGE induce cellular injury via direct modification of protein function, dysregulation of cellular interactions with the extracellular matrix and by conveying specific signals via interaction with their receptors, RAGE, ubiquitous surface molecules of the immunoglobulin superfamily (17,60). It has been shown that via RAGE, AGE can regulate various cellular functions including growth, inflammation and ROS production in endothelial cells and VSMC as well as in stromal macrophages (18,60). Interestingly, chemical agents such as pyridoxamine that prevent the formation of AGE may reduce diabetic complications (85). Furthermore, the AGE inhibitor aminoguanidine was able to reduce renal complications in a

large, double-blind, placebo-controlled trial of patients with type 1 DM (17). However, larger clinical studies with focus on vascular effects are needed.

PKC can be found in multiple isoforms that are downstream targets of diacylglycerol, a molecule the intracellular synthesis of which is increased by hyperglycaemia via multiple enzymatic pathways (32). Thus, an increase of intracellular diacylglycerol in obesity and diabetes comprises a mechanistic link with PKC activation (84). Of all the PKC isoforms, which often have differing effects, PKCβ and PKCδ have been consistently linked with diabetes and obesity (134). Once activated, PKC can regulate the protein activity and gene expression of various targets, thus regulating processes such as inflammation, proliferation, apoptosis and ROS generation in a variety of cell types including VSMC and endothelial cells (32). In detail, PKC can regulate vascular oxidative stress, NO bioavailability, vascular permeability and intima thickening (32). PKC signalling is, in fact, tightly linked to ROS signalling. Indeed, PKC contains multiple cysteine residues in its zinc finger domains that are subject to direct oxidation by various ROS and, importantly, lead to activation of PKC similar to the phosphorylation of the enzyme (62). Furthermore, PKC activity is apparently regulated by endogenous glutathione, an important cellular redox sensor (62). In turn, PKC is able to directly stimulate ROS -generating enzymes such as NADPH-oxidases and mitochondrial oxidases (37), and activate downstream pathways similar to ROS, such as NF-κB and AP1 (90,133).

Hyperglycaemia has been associated with distinct epigenetic changes that persist even after hyperglycaemia is reversed *in vitro*, and this mechanism has been proposed to adversely affect vascular biology (69). Intriguingly, one such target was found to be $p66^{Shc}$, a redox sensor that regulates mitochondrial oxidative stress and promotes redox signalling (151). Redox signalling, in turn, is able to induce a variety of epigenetic changes via pathways such as that of NFκB (69).

3.2.2. Vascular effects of insulin and the notion of vascular insulin resistance

Systemic IR, a hallmark of obesity and T2DM, indicates the difficulty of the human body to preserve normal circulating glucose levels, and is accompanied by hyperinsulinaemia (91). At the cellular level, IR reflects the impairment of physiological insulin receptor downstream signalling in response to insulin (190). IR in key organs such as the liver and skeletal muscle is crucial for the handling of glucose and thus the establishment of systemic IR (91). Conversely, the specific consequences of peripheral IR in tissues such as the vasculature are less clear.

Insulin release is tightly regulated and as such can be considered as an integral initial part of insulin signalling (73). Glucose is a very potent stimulus for insulin secretion via various cellular mechanisms which include activation of K_{ATP} channels and increase in intracellular calcium (77). Interestingly, these pathways are amplified in response to excess hyperglycaemia, but this amplification potential is lost in diabetic patients (76). Insulin secretion is also further regulated by metabolic parameters such as free amino and fatty acids, catecholamines as well as endogenous hormones such as incretins, which are of clinical relevance as mentioned in later sections (172).

The insulin signalling pathway is initiated upon binding of insulin to its respective receptors (118). Insulin receptors exist as monomers in the plasma membrane of target cells, and become dimerized upon binding insulin (118). This dimerization allows for cross-phosphorylation of tyrosine residues to occur in the intracellular ends of the receptors, which subsequently phosphorylate a variety of post-receptor molecules including the insulin receptor substrates (IRSs such as IRS1) and Shc protein (118). These substrates can direct post-receptor insulin signalling towards two distinct pathways, namely the PI3K/Akt or the Ras/MAPK pathway, involved in multiple critical aspects of cellular biology (118). Ample evidence suggests that

abnormal phosphorylation of IRS1 at serine instead of tyrosine residues via TNFα and PKC signalling results in IR at a cellular level (174). Importantly, IR appears to selectively impair the responsiveness of the PI3K/Akt axis compared to the MAPK pathway, thus creating a crucial imbalance between the signalling axes (162).

Insulin signaling in the vasculature has multiple effects (162). Insulin via Akt is able to activate eNOS by Ser1177 phosphorylation and enhance L-arginine transport, thus resulting in increased NO bioavailability and enhanced vasorelaxation under physiological conditions (47,103,193). On the other hand, stimulation of the MAPK pathway by insulin is able to increase vascular ET1 in cell culture and animal models (162), thus being potentially able to induce activation of NADPH-oxidases as well as proliferation of VSMC (115,138). Therefore, the post-receptor balance between the two signaling axis may be crucial for vascular responses to insulin (Fig. 4). The fact that IR is characterized by relatively selective inhibition of the Akt pathway may be crucial for the direct effect on insulin on the vascular wall of patients with obesity and DM.

The net vascular effect of insulin appears to be predominantly mediated by the production of NO under physiological conditions, thus being considered protective for vessel homeostasis (26). Accordingly, the vasodilatory effects on the vessels is lost in individuals with obesity and systemic IR (87,211). This direct effect cannot be solely attributed to the systemic effects of obesity and T2DM, but rather to an immediate dysregulation of vascular response to insulin (94). It is postulated that selective inhibition of the PI3K/Akt axis of insulin signaling following post-receptor IR can result in an excessive activation of the MAPK pathway (79). Interestingly, ROS production at low levels, particularly via NOX4, appears to be important for preservation of cellular insulin sensitivity by inhibiting protein tyrosine phosphatase, enzymes that de-phosphorylate and deactivate insulin receptor (122). Therefore, ROS likely have a Janus-like

effect on insulin signaling, being required for its tight regulation but being potentially detrimental upon excessive production.

4. Regulation of vascular redox state in obesity and diabetes mellitus

As discussed previously, vascular oxidative stress contributes to vascular disease via direct cytotoxic damage, regulation of proliferation and apoptosis, propagation of downstream redox-sensitive proinflammatory signalling and reduction of NO bioavailability (184). Obesity and T2DM increase vascular oxidative stress by several mechanisms, potentiating those effects and facilitating vascular disease progression (166,194).

4.1. Sources of reactive oxygen species in the vasculature of obese and diabetic patients

4.1.1. NADPH-oxidases

NADPH-oxidases (alternatively known as NOX enzymes) are dedicated to $O_2^{\cdot-}$ production, comprising a major source of ROS in the vascular wall (102). Seven isoforms of NOX enzymes have been described, namely NOX 1-5 and dual oxidase DUOX proteins 1-2 (Duox1-2), which are expressed in a wide variety of cell types including endothelial cells and VSMC (149) (Fig. 5). The main NOX isoforms expressed in the endothelial cells are NOX2 and NOX4, whereas VSMC express mainly NOX4 and NOX1 (70). NADPH-oxidases are multi-subunit enzymes comprised of at least one catalytic membrane subunit, i.e., $p22^{phox}$ as well as $gp91^{phox}$ in the case of NOX2, the activity of which is often regulated by several cytosolic subunits such as the GTPase Rac1 as well as $p47^{phox}$, $p67^{phox}$ and $p40^{phox}$ in the case of NOX2 or NOXO1 and NOXA1 in the case of NOX1 (149). Evidence suggests that vascular NADPH-oxidases gene expression is upregulated in models of T2DM (104) and several factors can stimulate the activity of these enzymes in the presence of obesity and DM (54).

PKC is a ubiquitous enzyme participating in many signalling pathways, and its activity is reportedly upregulated in obesity and T2DM (44). FFA, which are often elevated in patients with the metabolic syndrome, are also activators of PKC (35). Once activated, PKC is able to propagate vascular disease via multiple mechanisms including regulation of endothelial activation and cell apoptosis (150,166) (Fig. 7). Importantly, PKC stimulates NADPH-oxidases activity via $p47^{phox}$ phosphorylation (111); PKC also facilitates endothelin-1 (ET-1) signalling, which can also lead to MAPK p38-mediated induction of NADPH-oxidases activity (166).

As mentioned earlier, AGE regulate various cellular processes including ROS production (148). Crucially, AGE-RAGE interaction results in upregulation of NADPH-oxidases catalytic subunits (175) and increased $O_2^{\cdot -}$ production by NADPH-oxidases (148). Indeed, increased NADPH-oxidases activity has been revealed as the underlying mechanism of the apoptotic effect of AGE on endothelial progenitor cells (24). Furthermore, AGE increases vascular permeability associated with diabetic nephropathy (209) and induces expression of heat shock factor-1 (HSF-1) and plasminogen activator inhibitor-1 (PAI-1) via NADPH-oxidases activation (176). Since glycation reactions are facilitated by ROS, the ability of AGE to stimulate oxidative stress allows for a vicious cycle to be established, whereby AGE induce oxidative stress which then further promotes AGE formation (60,148) (Fig. 7).

Angiotensin II (AngII) is another potent stimulus of NADPH-oxidases which is upregulated in obesity and T2DM (177) (Fig. 7). Indeed, upon binding to its membrane receptors, AngII increases NADPH-oxidases activity, especially NOX1 and NOX2, via multiple mechanisms including $p22^{phox}$ translocation and PKC activation (56). Activation of AngII signalling has been implicated in the development of diabetic retinopathy, diabetic nephropathy and aneurysm formation in obese mice (166). Consistently, clinical studies have supported the clinical benefit of angiotensin-converting enzyme inhibitors and angiotensin receptor blockers in diabetic nephropathy (166).

As mentioned previously, the metabolic syndrome is associated with low-grade systemic inflammation and the presence of pro-inflammatory cytokines such as tumour necrosis factor alpha (TNFα) in the circulation (202). Indeed, serum TNFα is reportedly elevated in obese individuals (42), and is established as a causal inducer of systemic and peripheral insulin resistance (78). Amongst its detrimental vascular actions, TNFα increases the activity of NADPH-oxidases in the vasculature (104). Intriguingly, the atypical ζ isoform of PKC has been shown as the mediator of NOX1 activation by TNFα in endothelial cells (52); in addition, TNFα is believed to regulate vascular cell apoptosis via regulation of NOX4 (13). Overall, systemic levels of TNFα in the context of the metabolic syndrome have direct stimulatory effects on vascular NADPH-oxidases activity (Fig. 7).

Vascular NOX enzymes may also use NADH as an alternative substrate (105). An increased cellular NADH/NAD^+ has been observed in cases of obesity and diabetes, presumably via excessive activation of the polyol metabolic pathway (166), which may constitute a biochemical parameter regulating vascular NOX activity in the presence of obesity or T2DM.

4.1.2. Endothelial nitric oxide synthase (eNOS)

eNOS is a homodimeric enzyme that catalyses the production of NO using L-arginine as a substrate under physiological conditions (110) (Fig. 6A). This enzymatic reaction depends on the presence of the critical co-factor tetrahydropterin (BH4) (110). Increased vascular oxidative stress induces significant oxidation of BH4, decreasing its bioavailability and resulting in "eNOS uncoupling", whereby the enzyme produces $O_2^{\cdot-}$ instead of NO (112) (Fig. 6B). The enzymatic activity of eNOS is delicately regulated by several phosphorylation sites such as Ser1177 and Thr495 (50). Phosphorylation at Ser1177 increases the activity of eNOS, contrary to the phosphorylation of Thr495 (50). Furthermore, the activity of eNOS is sensitive to intracellular calcium concentration (50).

Intact eNOS function has been revealed a major protective factor against vascular disease in obese and diabetic patients (126), something that comes to no surprise, considering the multitude of antioxidant, anti-inflammatory, vasodilatory and antithrombotic effects of NO (49). On the other hand, the proper function of eNOS is often compromised in obesity and DM (92). Excessive ROS production, irrespectively of its enzymatic origin, dramatically decreases BH4 bioavailability, leading to eNOS uncoupling and the transformation of eNOS to a $O_2^{\cdot -}$-producing enzyme (92). This ultimately results in a vicious cycle of eNOS uncoupling and oxidative stress in the vasculature.

Several signals in the presence of obesity and T2DM can modify eNOS activity apart from its coupling status (Fig. 7). In detail, PKC activation has been demonstrated to result in reduction of eNOS activity (32,150), possibly by phosphorylating the enzyme at Thr495 (154). In addition, Akt, an important stimulator of eNOS activity via phosphorylation at Ser1177, is O-GlcNAcylated in T2DM, resulting in marked inhibition of eNOS activity and endothelial dysfunction (150). Furthermore, AGE-RAGE interactions decreased both eNOS expression and activity in endothelial cells (83).

4.1.3. Mitochondrial oxidases

Enzymes of the mitochondrial respiratory electron transport chain may also be important sources of ROS in the cardiovascular system (112). More specifically, complexes I and III of the mitochondrial respiratory chain, i.e. the NADH dehydrogenase and the ubiquinone-cytochrome b-c_1 respectively, are constant cellular sources of $O_2^{\cdot -}$, influenced by the respiratory rate as well as the activity of SOD2 which is located in the mitochondrial matrix (48). Mitochondrial dysfunction has been associated with vascular disease (164), and experimental studies that mitochondrial oxidative stress may facilitate atherogenesis (146). However, the *in vivo* significance of mitochondrial ROS in human vascular disease remains to be explored.

Mitochondrial dysfunction has consistently been associated with the presence of obesity and T2DM (15,119). Notably, dysfunctional mitochondria produce excessive amounts of ROS (39). Indeed, increased cellular glucose influx impairs physiological respiration, thus promoting mitochondrial $O_2^{\cdot-}$ generation (166). Also, AGE-mediated NADPH-oxidase is able to stimulate mitochondrial oxidative stress (107). Furthermore, PKC activation as described in the metabolic syndrome has been shown to phosphorylate $p66^{Shc}$, a protein involved in redox signalling, consequently facilitating its translocation to the mitochondria and $O_2^{\cdot-}$ generation by these organelles (150). Consistently, mice lacking $p66^{Shc}$ were resistant to hyperglycaemia-induced oxidative stress and vascular disease progression (139), further supporting an important role for $p66^{Shc}$ in obesity- and DM-associated vascular disease regulation.

4.2. Vascular oxidative stress as a therapeutic target in obesity and diabetes

4.2.1. Targeting production of reactive oxygen species in the vasculature

Considering the crucial role of NADPH-oxidases in both vascular disease and diabetes (54), targeting of these enzymes (by targeting the activation and translocation of its key subunits such as $p47^{phox}$ and Rac1) may be of clinical relevance (102). A number of direct NADPH-oxidase inhibitors have been developed, which are able to reduce NADPH-oxidases derived superoxide with variable specificity (185). Agents such as apocynin, and the more specific inhibitors Vas2870 and Vas3947 are able to efficiently reduce the activity of all NOX isoforms (57,185). On the other hand, inhibitors such as gp91 dstat and GK-136901 have revealed increased specificity for NOX2 and NOX1/4 respectively (168,187). Most of these agents have been used in model experimental systems where they have proven to be beneficial against hyperglycaemia-related oxidative stress (185) but their clinical application has been very limited so far. Recently, water-soluble formulations of inhibitors of the Vas family have been successfully developed, broadening the translational potential of such inhibitors for use in *in vivo* settings (75). GKT137831, a specific NOX1/4 inhibitor, is the first agent in clinical

development, which has displayed satisfactory pharmacodynamics and pharmacokinetics (31). This novel inhibitor has, in fact, entered a phase II clinical trial targeting albuminuria in type 2 diabetic patients. Although GKT137831 failed to reduce diabetic kidney disease in that trial, its favourable *in vivo* tolerability and its ability to reduce systemic inflammation markers in those patients suggest that this inhibitor may be promising for treatment of diabetic complications and targeting of redox signalling (199).

Uncoupling of eNOS, resulting from excessive oxidation of BH4, further contributes to vascular oxidative stress, and thus rescue of eNOS coupling could be a promising target against vascular disease. This could be achieved by targeting ROS and thus preventing BH4 oxidation (185). In addition, supplementation with BH4 or sepiapterin have displayed some benefit in ameliorating eNOS uncoupling and endothelial dysfunction in humans (185). Interestingly, exogenous BH4 has been proposed to have protective effects on the cardiovascular system in a mouse model, associated with inhibition of inflammatory pathways independently of eNOS coupling (74). Folate, the active *in vivo* form of which is 5-methyltetrahydrofolate, is also able to inhibit peroxynitrite-mediated oxidation of BH4 and reverse eNOS uncoupling (6). However, such treatments face pharmacokinetic challenges and require more validation in larger clinical studies.

Xanthine oxidase is a source of ROS that may be associated with cardiovascular risk factors and the presence of metabolic syndrome in humans (45), thus warranting investigation regarding therapeutic targeting of this enzyme. Allopurinol is a purine analogue that mimics the substrate of xanthine oxidase, inhibiting the activity of the enzyme (135). Allopurinol is successfully used in diseases such as gout (135), but also displays a wide variety of roles that may be relevant in vascular disease associated with obesity and diabetes. Indeed, allopurinol has displayed direct antioxidant cardiovascular effects in a variety of animal models (136). Furthermore, xanthine oxidase by febuxostat, a non-purine inhibitor, resulted in less disease

509 burden in a mouse model of atherosclerosis *in vivo* (142). Interestingly, xanthine oxidase
510 inhibition by febuxostat has also shown the ability to improve glucose metabolism and reverse
511 diabetic kidney complications in mouse models (108,214). As such, xanthine oxidase inhibition
512 may be a target with pleiotropic implication in vascular and metabolic disease. An initial insight
513 to the cardiovascular effects of xanthine oxidase inhibitors such as febuxostat will be provided
514 by clinical trials such as the Febuxostat versus Allopurinol Streamlined Trial (FAST) currently
515 in progress (120).

516 Vascular disease is characterised by increased mitochondrial oxidative stress which is further
517 amplified by factors such as hyperglycaemia, oxidised LDL and triglycerides (197). Therefore,
518 specific targeting of mitochondrial ROS may be promising from a clinical point of view.
519 Specific targeting of mitochondria may be achieved by coupling an antioxidant factor with a
520 lipophilic cation, such as mitoquinone (mitoQ) and mitochondria-targeting TEMPOL
521 (mitoTEMPO) (197). These agents have displayed the ability to reduce oxidative stress and
522 inflammation in *in vivo* animal models, while mitoQ was also shown to reduce oxidative stress
523 and inflammatory activation in primary leucocytes isolated from diabetic patients (41).
524 Interestingly, hydrogen sulfide (H_2S) is an endogenous gaseous messenger shown to preserve
525 mitochondrial function and suppress mitochondrial oxidative stress (100). However,
526 harnessing the pharmacodynamics and pharmacokinetics of this molecule is challenging at
527 present, although SG1002, a novel H_2S prodrug has demonstrated the some favourable effects
528 *in vivo* such as increasing NO bioavailability in patients with heart failure (161). Whether H_2S
529 can be used to target the mitochondria *in vivo* is unknown.

530 Interestingly, several agents currently used in clinical practice for the management of vascular
531 disease have displayed direct antioxidant effects. Indeed, renin inhibitors, angiotensin
532 converting enzyme inhibitors, angiotensin receptor blockers, mineralocorticoid inhibitors and
533 statins have all variably displayed the ability to inhibit NADPH-oxidases activity via

preventing the membrane translocation of their key subunits, an effect accompanied by improved endothelial function and improvement of eNOS coupling (185), although statin treatment has been associated with a slightly increased risk for development of DM (179). However, to what extent such antioxidant properties contribute to the *in vivo* clinical benefit of these agents is unclear.

4.2.2. Targeting downstream vascular redox signalling

As mentioned earlier, PKC is activated in obesity and diabetes can both stimulate ROS production and be activated by ROS, thus propagating downstream redox signalling. Therefore, it would appear as an attractive therapeutic target. In fact, several small molecule PKC inhibitors have been developed, that are able to inhibit PKC activity by competing with its ATP-binding catalytic site or by binding its regulatory subunits (134). PKCβ inhibitors such as ruboxistaurin have demonstrated the ability to reduce diabetic complications in clinical trials (189). Furthermore, ruboxistaurin was shown to ameliorate diabetic cardiomyopathy severity in a rodent model of diabetes (28), while PKCβ inhibition by the inhibitor LY333531 reduced hyperglycaemia-induced endothelial dysfunction, at least partially by reducing NADPH-oxidases activity (67). On the other hand, activation of PKCε combined with inhibition of PKCδ synergistically reduced ischaemic heart injury in rats (82). This highlights the diversity of the role of the various PKC isoforms, and indicates that isoform-specific parameters should be accounted in order to successfully target PKC signalling.

AGE are crucial mediators of vascular complications in diabetes, and successful glycaemic control would be the most obvious way to prevent their formation. Furthermore, several agents have displayed the ability to directly inhibit the formation of AGE, reverse the AGE-protein links and prevent AGE-RAGE interactions (156). Such agents include pyridoxamine, glyoxal, aminoguanidine and others (208). Many of these agents have been associated with a decreased

risk of diabetic complications in small scale clinical studies, although larger studies are required to confirm the clinical benefit of such treatments (208). Interestingly, dietary regulation of exogenous AGE consumption has been shown to reduce neointima formation following arterial injury in mice (114) and improve glucose metabolism and reduced systemic oxidative stress in humans (27). In addition, commonly used medication such as metformin, statins and angiotensin converting enzyme inhibitors have shown direct AGE-lowering effects (156). Finally, exogenous administration of soluble RAGE may be beneficial to scavenge systemic AGE, although this would need validation in large studies (163).

Inflammation is a hallmark of vascular disease as well as both obesity and diabetes. Oxidised LDL (oxLDL) is a major mediator of vascular inflammation via stimulating the lectin-like oxLDL receptor 1 (LOX1) (158). Crucially, LOX1 signalling has also been implicated in diabetic complications such as renal disease in a rat model of diabetes (97). On the other hand, proprotein convertase subtilisin/kexin type 9 (PCSK9) is an enzyme that decreases the recycling of LDL receptors and positively regulates LOX1 signalling (38). Consequently, PCSK9 inhibitors, novel lipid-lowering drugs, may be of particular interest in vascular disease associated with obesity and diabetes, due to their effective inhibition of LOX1 signalling (30). In line with the previous considerations, the Canakinumab Anti-inflammatory Thrombosis Outcome Study (CANTOS) and the Randomized Evaluation of the Effects of Anacetrapib through Lipid Modification (REVEAL) trials further support the clinical importance of efficient targeting of inflammation and cholesterol respectively (65,169). Indeed, the CANTOS trial is the first trial to report improvement in cardiovascular outcome by directly targeting inflammation using Canakinumab, a monoclonal antibody against interleukin 1β (IL-1β); something that may reflect the crucial role of IL-1β in systemic and local inflammation and homeostasis (169). Anacetrapib, in turn, is an inhibitor of cholesteryl ester transfer protein (CETP) which increases HDL cholesterol while decreasing LDL cholesterol, and it is the first

583 drug of this class to significantly reduce cardiovascular events as reported very recently by the
584 REVEAL trial group (65).

585 Peroxisome proliferator-activated receptor gamma (PPARγ) is a master regulator of
586 adipogenesis which also has a multitude of tissue specific effects including stimulation of
587 insulin secretion, insulin sensitisation and prevention of inflammation and oxidative stress (86).
588 Interestingly, PPARγ appears to be redox-sensitive, being upregulated by lipid peroxidation
589 products such as 4-hydroxynonenal adducts (7). Furthermore, PPARγ may be activated by
590 established messengers and drugs such as statins, thiazolidinediones and hydrogen sulfide (4).
591 Despite the fact that thiazolidinediones such as pioglitazone (the most frequently used PPARγ
592 activator *in vivo*) have displayed often severe side effects in clinical trials (101), more selective
593 inhibition of PPARγ with the non-thiazolidinedione molecule INT131 improved insulin
594 sensitivity without the expected thiazolidinedione-related side effects (198). These
595 considerations identify PPARγ as an interesting, redox-sensitive link between obesity, diabetes
596 and vascular disease, which warrants research for its more selective targeting.

597 ***4.2.3. Scavenging of vascular reactive oxygen species***

598 A variety of antioxidant factors including vitamin C, vitamin E, flavonoids and polyphenols
599 have revealed the potential to scavenge ROS (191). Although pre-clinical studies have
600 consistently revealed the beneficial anti-oxidant effects of such agents, clinical studies have
601 been unexpectedly disappointing (191). The ability of large-scale randomised clinical trials to
602 detect beneficial effects of the aforementioned antioxidants may be compromised by the
603 generic nature of the primary endpoints studied, such as mortality of cardiovascular cause
604 (191). In contrast, small clinical studies have studied more isolated parameters such as blood
605 pressure and flow-mediated dilatation, where antioxidants showed significant effects (191). In
606 addition, unclear criteria for antioxidant regimen selection amongst different studies may also

account for the inconsistency of the respective results. Importantly, these contradicting results may reflect our lack of understanding regarding the heterogeneity of ROS sub-species and their overall biological functions (16), and suggest that research should be focused on specific enzymatic sources of ROS instead of non-specific ROS scavenging.

SOD is a major antioxidant enzyme that neutralises superoxide. Consequently, exogenous administration of SOD has been proposed to be of clinical use in oxidative stress-related diseases (21). Indeed, SOD injections have revealed beneficial effects in a variety of inflammatory diseases including, importantly, vascular disease such as hypertension in *in vivo* animal models (21). However, these have not been to humans yet, while the formulation of pharmacokinetically stable SOD forms is challenging, compromising the present translational potential of SOD in humans (29). Currently, intense work is attempting to introduce novel SOD formulations, such as SOD enzymosomes, that will improve the in vivo transport and bioavailability of the enzyme (29).

4.2.4. Effects of anti-obesity and anti-diabetes treatments on vascular redox state

Bariatric surgery is an established method for body reduction in severely obese patients (19). Clinical evidence suggests that, indeed, bariatric surgery is associated with improved glycaemic control and sustained weight loss (182), while it can also significantly reduce mortality associated with complications of obesity, DM and vascular disease (2). Interestingly, weight-loss surgery has been associated with a decrease in systemic oxidative stress (evaluated by a variety of markers such as malondialdehyde (MDA) levels and total antioxidant capacity) that parallels the decrease in body weight (58), although other studies have reported an increase in systemic oxidative stress parameters shortly after bariatric surgery (137). Considering that obesity is associated with increased ROS production, bariatric surgery may have a role in reducing oxidative stress by reducing AT mass and improving metabolic parameters such as

631 circulating lipid levels (159,206). One could presume that bariatric surgery would result in a
632 beneficial vascular phenotype, at least indirectly, via regulation of blood glucose, insulin and
633 AT inflammation. In support of this hypothesis, caloric restriction in mouse models has shown
634 the ability to improve endothelial function and reduce oxidative stress (170). In humans,
635 rigorous low-fat diet has been linked with both weight loss and increased nitric oxide
636 bioavailability (171). However, the specific effect of different weight loss treatment (such as
637 bariatric surgery versus certain types of diet) on vascular redox state is unknown.

638 Insulin has revealed the ability to increase NO bioavailability and reduce oxidative stress in
639 vascular cell culture models, animal models and healthy humans (162,216,217). However, the
640 protective and anti-contractile effects of insulin are abolished in obesity (4). Interestingly,
641 animal models have revealed that obesity is characterised by insulin resistance in the
642 vasculature, which is selective for the Akt-related branch of the insulin pathway (87). Indeed,
643 experimental studies have revealed that obesity is associated with vascular inflammation that
644 promotes vascular insulin resistance (98). Interestingly, in a mouse model of obesity, vascular
645 inflammation and insulin resistance preceded the onset of systemic disease (99). Importantly,
646 treatment with a variety of synthetic basal insulins has failed to improve cardiovascular
647 outcome in clinical studies, despite successfully achieving glycaemic control (123). These
648 findings support the notion of vascular insulin resistance independently of systemic parameters,
649 which may detrimentally alter vascular insulin signalling, and may be the reason why insulin-
650 regulated glycaemic control fails to translate to improved vascular outcome.

651 Glucagon-like peptide 1 (GLP1) is an incretin with insulin-sensitizing properties, which
652 stimulates insulin secretion in the pancreas and also improves peripheral insulin signalling (12).
653 Although clinically used predominantly for their ability to stimulate pancreatic insulin
654 secretion, GLP1 and its synthetic analogues have also demonstrated anti-inflammatory, anti-
655 oxidant and anti-atherogenic effects in vascular cell culture and animal models (12,88), which

may also be relevant in humans. On the other hand, mechanistic studies have proposed a protective role for GLP1 and its analogues in vascular biology that may be independent of its glucose-lowering abilities (205). Indeed, GLP1 and analogues have been shown to improve local insulin sensitivity, redox state, NO bioavailability and endothelial function, effects partially mediated my activation of AMP-activated kinase alpha (AMPKα) (22,88,205). In line with these pre-clinical findings, the LEADER clinical trial has recently revealed that treatment of diabetic patients with liraglutide, a GLP1 analogue with both glucose-lowering and weight-reducing effects, significantly improved cardiovascular outcome (127). On the contrary, the ELIXA clinical trial studying the cardiovascular effects of lisexenatide, another GLP1 analogue, failed to demonstrate a beneficial cardiovascular effect for this drug, although its main goal was to evaluate the safety of lisexenatide and confirm non-inferiority versus placebo (157). Therefore, there appears to be a drug-dependent variability within the class of GLP-1 analogues, which may reflect pharmacokinetic and pharmacodynamics parameters and the differential ability of each analogue to activate non-canonical, non-GLP1-receptor dependent pathways.

Dipeptidyl peptidase-4 (DPP4) is a protease that cleaves the N-terminal ends of a variety of targets including brain natriuretic peptide (BNP), cytokines such as interleukin 10 and GLP1 (43). DPP4 inhibitors of the –gliptin class have revealed glucose-lowering properties mainly attributed to their ability to increase the bioavailability of GLP1 (36). In addition, a variety of DPP4 inhibitors have displayed direct anti-oxidant, vasodilatory, anti-inflammatory effects that are only partially attributed to the enhancement of GLP1 activity. Indeed, DPP4 inhibition has been associated with reduced inflammation and oxidative stress and improved endothelial function, events likely to involve PKC, AMPKα and uncoupling protein 2 signalling in experimental models (117,173,183). On the other hand, administration of DPP4 inhibitors in diabetic patients has failed to improve cardiovascular outcome in large clinical trials, although

681 those were tested to primarily assess safety (63,186). As such, DPP4 inhibitors appear to have
682 a consistently favourable vascular effect *ex vivo*, but pharmacokinetic or other interactions may
683 compromise their *in vivo* effect. Accordingly, such agents may reveal clinically significant
684 benefit for particular sub-groups of patients or in combination with other novel treatments.

685 Inhibitors of the sodium-glucose co-transporter 2 (SGLT2) have emerged as novel glucose-
686 reducing agents due to their ability to enhance urinary glucose excretion (1). Interestingly, it
687 has been hypothesised that SGLT2 inhibitors may have favourable effects for cardiovascular
688 homeostasis by modulating fluid balance, cardiac metabolism and blood pressure (178).
689 Studies in humans have revealed that empagliflozin, a potent SGLT2 inhibitor, reduces blood
690 pressure while improving parameters of arterial stiffness and resistance in diabetic patients *in*
691 *vivo* (25). Furthermore, a recent study has shown that SGLT2 inhibition reduces systemic and
692 vascular oxidative stress in a mouse model of streptozotocin-induced diabetes (145). The
693 antioxidant effects of SGLT2 inhibition have been confirmed in a variety of tissues in diabetic
694 animal models, including the liver and kidneys (147,196), buts its direct effects on vascular
695 redox state remain elusive. On the other hand, SGLT2 inhibition in mice resulted in
696 vasorelaxant properties in the pulmonary circulation but not the coronaries (72). Accordingly,
697 recent clinical trials have revealed that SGLT2 inhibitors significantly reduce cardiovascular
698 morbidity and mortality in diabetic patients (1), making this class of drugs a promising tool for
699 the efficient treatment of diabetic patients with cardiovascular disease.

700 Sulfonylureas are a commonly used class of anti-diabetic drugs that may also influence
701 vascular biology. In detail, the ability of sulfonylureas to block ATP-regulated potassium
702 (K_{ATP}) channels may be relevant in the vasculature, resulting in increased vasoconstriction (33)
703 and increased infract size in a mouse model of ischaemia-reperfusion (192). On the other hand,
704 some sulfonylureas such as glimepiride have demonstrated the ability to increase vascular NO
705 bioavailability in human aortic endothelial cells (204). However, large clinical trials have

suggested that sulfonylureas may increase the risk for cardiovascular complications in diabetic patients, although these drugs have displayed non-significant effect on cardiovascular mortality (165).

4.3. Paracrine regulation of vascular redox state in obesity and type 2 diabetes mellitus

As described so far, obesity and T2DM result in direct detrimental effects on the vasculature, ranging from stimulation of local inflammation and vascular oxidative stress. Of note, obesity and T2DM are also accompanied by profound alterations on AT biology, particularly inflammatory infiltration and local IR (91,174). Recently it has been revealed that direct paracrine interactions of the vasculature with its neighbouring perivascular AT (PVAT) dynamically regulate vascular biology via mutual communication with the vascular wall, which is achieved with locally secreted signalling molecules (3). Indeed, PVAT is known to secrete factors that are able to diffuse to the vascular wall and regulate vascular tone, ROS generation and inflammation (61,144). Notably, PVAT secretome and lipid content are influenced by signals of vascular origin in an inside-to-outside way (9,124), allowing PVAT to potentially behave as a surrogate marker of vascular disease progression. Therefore, functional changes in PVAT in the context of obesity and T2DM may regulate vascular disease progression (Fig. 8).

PVAT is characterised by adipocyte hypertrophy and inflammatory infiltration in obesity (66,201). In fact, mouse models suggest that PVAT is more sensitive than other AT depots to stimuli such as high-fat diet, rapidly assuming a proinflammatory phenotype as a result (23). In addition, evidence from both animal and human models suggests that obesity is associated with increased oxidative stress within PVAT (4). Crucially, mediators of inflammation and oxidative stress originating from PVAT are able to diffuse towards the vascular wall, detrimentally affecting vascular biology especially in the context of obesity and T2DM (4). Accordingly, obesity and T2DM have been associated with alteration in the secretion of a

variety of adipokines produced in PVAT (4). PVAT of diabetic patients, for example, secretes low levels of adiponectin, resulting in the loss of the antioxidant effects of this hormone on the vasculature (66). Conversely, T2DM is associated with increased production of resistin, a pro-oxidant molecule, by PVAT (153).

Interestingly, PVAT has been directly linked with systemic insulin sensitivity. Indeed, whole body insulin sensitivity is inversely associated with the amount of AT around the branchial artery as well as with intramuscular AT mass (132). Consistently, PVAT around small arterioles may be able to secrete molecules such as TNFα in the lumen of its underlying vessel, thus propagating inflammatory signals within certain vascular beds, a process termed "vasocrine signalling" (215). This is of particular importance in muscle, as this tissue acts as an important buffer for blood glucose in response to insulin (215). Importantly, PVAT is important for preserving the responsiveness of the muscle vasculature to insulin, which is lost in diabetic mice (130,131). These findings identify pathological intramuscular PVAT as a mediator of systemic insulin resistance.

Recently it has been shown that the extent of coronary inflammation can be predicted by evaluating the radiodensity, and thus the lipid content, of peri-coronary AT on coronary tomography images (9). This novel finding demonstrates that characterising the phenotype of PVAT is not only useful in order to understand vascular disease pathophysiology, but it also is of immense translational interest with potential predictive role in the context of obesity and diabetes.

5. Conclusion

Obesity and T2DM comprise overlapping entities reflecting a systemically dysregulated metabolic disease status characterised by systemic inflammation, systemic IR and

hyperglycaemia. Furthermore, these conditions have been strongly associated with increased risk for vascular disease, leading to severe vascular complications. Such complications are mediate by local vascular mechanisms including VSMC proliferation, inflammation and oxidative stress, and are regulated by systemic factors like hyperglycaemia, FFA and AGE.

Obesity and T2DM are associated with increased ROS in the vasculature, which influences vascular biology by inducing oxidative DNA damage, oxidation of NO and activation of pro-inflammatory pathways. This increase in ROS results from increased glucose and FFA influx in the vasculature, as well as from extensive protein glycation and signalling via AGE, all of which lead to increased NADPH-oxidases activity, mitochondrial ROS production and eNOS uncoupling. Crucially, obesity and T2DM may be associated with vascular insulin resistance as well as systemic hyperinsulinaemia. The combination if these parameters may result in increased and dysregulated insulin signalling in the vasculature, which is able to convey detrimental effects via the MAPK pathway. Interestingly, PVAT has been revealed as a significant local regulator of vascular disease the biology of which is altered in cases of obesity and T2DM. Increased inflammation and oxidative stress in PVAT may directly influence vascular redox biology in the context of obesity and T2DM.

In summary, a great body of knowledge that has been gathered regarding the molecular roles of ROS, the biology of obesity and T2DM and the mechanisms of vascular oxidative stress stimulation by metabolic parameters. However, further research is required to understand the heterogeneity of ROS sub-species and functions. Moreover, exploration of the understudied direct effects of insulin in human vessels and the regulatory role of PVAT are crucial to fully elucidate the effects of obesity and T2DM on vascular redox biology if we are to identify novel targets in metabolic and vascular disease.

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Legends to Figures

Legend to Fig. 1: Sources and effects of reactive oxygen species (ROS)

ROS are generated by endogenous enzymes such as the NADPH-oxidases, xanthine oxidase and mitochondrial oxidases and further stimulated by genetic and environmental factors such as smoking and exogenous pro-oxidants. ROS stimulates various signalling molecules such as activation protein 1 (AP1) and NF-κB (nuclear factor kappa beta) and induces DNA oxidative damage and inflammatory activation of immune cells. In endothelial cells, ROS modulates apoptosis and reduces nitric oxide (NO) bioavailability by oxidising NO to form peroxynitrite ($ONOO^-$). In vascular smooth muscle cells (VSMC), ROS regulates migration and proliferation. Furthermore, ROS stimulates endogenous pro-oxidant enzymes, thus establishing a vicious cycle of oxidative stress.

Legend to Fig. 2: Major endogenous antioxidants

A variety of endogenous antioxidant enzymes regulate redox balance in health and disease. Superoxide dismutase (SOD) converts superoxide ($O_2^{\cdot -}$) to hydrogen peroxide (H_2O_2) and water. H_2O_2 is further converted to water by catalase. Paraoxonases and glutathione peroxidase (GPx) reduce a variety of protein and lipid peroxides, thus participating to the endogenous antioxidant defences.

Legend to Fig. 3: Vascular implications of the metabolic syndrome

The metabolic syndrome is characterised by hypertension, dyslipidaemia, systemic inflammation, visceral adiposity and insulin resistance (IR)/type 2 diabetes mellitus (T2DM). These result in increased free fatty acid (FFA) and glucose levels, advanced glycation end-products (AGE), inflammatory mediators and vascular IR, which detrimentally influencing

vascular biology. By promoting vascular remodelling, oxidative stress and inflammation while reducing nitric oxide (NO) bioavailability, the metabolic syndrome leads to a number of vascular complications.

Legend to Fig. 4: The notion of vascular insulin resistance

Vascular insulin signaling requires dimerization and insulin binding of insulin receptor (IR) and/or insulin-like growth factor 1 receptor (IGF1R) monomers in endothelial cells (ECs) and vascular smooth muscle cells (VSMCs), which subsequently undergo crossed auto-phosphorylation. The intracellular phosphorylated receptor residues then bind and phosphorylate various substrates such as the insulin receptor substrate 1 (IRS1) and Shc; at this stage, the pathway can diverge towards the phosphoinositide-3 kinase (PI3K)/Akt pathway or the mitogen-activate protein kinase (MAPK) pathway, which have largely opposing effects on vascular biology. Akt activates endothelial nitric oxide synthase (eNOS) while also promoting eNOS coupling via the phosphorylation of GTP cyclohydrolase I (GTPCH I) which maintains the bioavailability of tetrahydropterin (BH4), a key co-factor for the proper function of eNOS. The PI3K/Akt pathway thus leads to the atheroprotective molecule NO. On the contrary, MAPK such as extracellular signal regulated kinases (ERK) result in endothelin 1 (ET1) production and NADPH-oxidases (NOXs) activation; the subsequent increase in reactive oxygen species such as superoxide ($O_2^{\cdot -}$) anions results in reduced NO bioavailability, peroxynitrite ($ONOO^-$) production and BH4 oxidation, as well as direct oxidative DNA damage, redox-sensitive inflammatory signaling and overall propagation of atherogenesis. Signaling molecules such as protein kinase C (PKC) and c-Jun N-terminal kinase (JNK) are activated by inflammation and evoke insulin resistance via the inhibitory phosphorylation of IRS1 at serine residues. On the other hand, overproduction $O_2^{\cdot -}$ anions and the subsequent

oxidation of BH4 can lead to uncoupling of eNOS, whereby the enzyme, in the absence of its critical co-factor, produces $O_2^{\cdot -}$ instead of NO, thus establishing a vicious cycle of oxidative stress and NO depletion. It is believed that insulin resistance is rather restricted to the IRS1/PI3K/Akt axis of the pathway while the MAPK pathway remains less affected; conversely, the implication of vascular insulin resistance in humans is not well explored.

Legend to Fig. 5: NADPH-oxidase isoforms

NADPH-oxidases exist in several isoforms, namely NOX1-5 and Duox1/2. NOX1 is comprised by the membrane subunit $p22^{phox}$ and requires the co-localization of the cytosolic components Rac1, NOXA1 and NOXO1 for its activity. NOX2, the prototype NOX isoform, includes two membrane subunits, namely $p22^{phox}$ and $gp91^{phox}$, while its activity is regulated by the membrane translocation of Rac1, $p47^{phox}$, $p67^{phox}$ and $p40^{phox}$. The other NOX isoforms are simpler in structure and regulation, consisting only of the $p22^{phox}$ subunit, while NOX5 may also be calcium-regulated, as are the Duox1/2 subunits.

Legend to Fig. 6: Structure and coupling status of endothelial nitric oxide synthase (eNOS)

eNOS is a homodimer that normally converts L-arginine to L-citrulline and nitric oxide (NO) when coupled (panel A). Its activity is regulated via phosphorylation at either stimulatory (e.g., Ser1177 in humans) or inhibitory (e.g., thr495 in humans) sites, and it depends upon the bioavailability of tetrahydropterin (BH4). When BH4 is oxidized, such as in cases of increased oxidative stress, eNOS becomes uncoupled, producing superoxide ($O_2^{\cdot -}$) instead of NO.

Legend to Fig. 7: Protein kinase C and vascular redox signaling in obesity and diabetes mellitus

A variety of factors regulate the endogenous redox enzymatic systems such as NADPH-oxidases, endothelial nitric oxide synthase (eNOS) and mitochondrial oxidases in cases of obesity and diabetes. Protein kinase C (PKC) is a key enzyme comprised of four domains including a substrate-binging subunit (which is regulated by pseuodsubstrate presence), an ATP-binding domain (target of many PKC inhibitors) and two regulatory subunits which display isoform-specific variability and can be subject to regulation by factors such as diacylglycerol (DAG), phorbol esters and calcium. PKC is upregulated in the context of obesity and diabetes by angiotensin II (AngII), tumor necrosis factor alpha (TNFα), and via intracellular DAG increase resulting from metabolites such as free fatty acids (FFA). PKC, in turn, stimulates mitochondrial oxidative stress, regulates eNOS activity and coupling and increased NADPH-oxidases activity via stimulation of endothelin 1 (ET1) and mitogen-activated protein kinase p38 (MAPK p38). Advanced glycation end products (AGE) induce receptor-mediated events upon binding to their receptor (RAGE), which lead to NADPH-oxidases stimulation. Finally, hyperglycaemia modifies Akt thus inhibiting its antioxidant effects.

Legend to Fig. 8: Paracrine regulation of vascular redox state in obesity and diabetes

Perivascular adipose tissue (PVAT) is in mutual cross-talk with the underlying vascular wall. Obesity and diabetes are characterized by qualitative changes in the phenotype of PVAT, promoting local hypoxia, insulin resistance (IR) and inflammation. These eventually lead to a disruption of PVAT's secretome, whereby the secretion of protective adipokines such as adiponectin is reduced while the production of detrimental adipokines such as resistin is

1463 increased. These events promote vascular disease progression by eventually regulating
1464 vascular inflammation, vascular reactive oxygen species (ROS) production and nitric oxide
1465 (NO) bioavailability.