Vascular NAD(P)H oxidase activation in diabetes: a double-edged sword in redox signalling

Ling Gao† and Giovanni E. Mann*

Cardiovascular Division, School of Medicine, King’s College London, London, UK

Received 11 November 2008; revised 16 January 2009; accepted 25 January 2009; online publish-ahead-of-print 29 January 2009

Time for primary review: 23 days

Oxidative stress mediated by hyperglycaemia-induced generation of reactive oxygen species (ROS) contributes significantly to the development and progression of diabetes and related vascular complications. NAD(P)H oxidase has been implicated as the major source of ROS generation in the vasculature in response to high glucose and advanced glycation end-products. Sustained activation of NAD(P)H oxidase in diabetes may diminish intracellular levels of NADPH, an essential cofactor for endothelial NO synthase (eNOS) and several antioxidant systems. Recent evidence suggests that basal ROS production via NAD(P)H oxidase may upregulate antioxidant enzyme defenses via redox signalling. Thus, NAD(P)H oxidase may serve as a double-edged sword, with transient activation providing a feedback defense against excessive ROS generation through the activation of receptor tyrosine kinases and the redox-sensitive Nrf2-Keap1 signalling pathway. Overproduction of ROS leads to eNOS uncoupling, mitochondrial dysfunction, and impaired antioxidant defenses owing to depletion of intracellular NADPH. Given the largely negative outcome of antioxidant therapy in the treatment of diabetic complications, targeting the redox-sensitive transcription factor Nfr2 may provide an effective strategy to restore antioxidant defenses in diabetes.

KEYWORDS
Diabetes;
Endothelium;
NAD(P)H oxidase;
Mitochondria;
eNOS;
Redox signalling;
Nrf2-Keap1 signalling;
Antioxidant defense genes

1. Introduction
Diabetes is one of the most serious challenges to healthcare. Diabetic patients have an increased risk of developing microvascular complications and cardiovascular disease (CVD), with progression of the disease leading to blindness, end-stage renal failure, and atherosclerosis.1 Reactive oxygen species (ROS) generated during hyperglycaemia are implicated in the development and progression of diabetic vascular complications and often associated with endothelial dysfunction.2 Vascular NAD(P)H oxidase, mitochondrial dysfunction, and uncoupled endothelial nitric oxide synthase (eNOS) all contribute to oxidative stress in diabetes. However, NAD(P)H oxidase could act as a double-edged sword, with transient activation providing a feedback antioxidant response to ROS via receptor tyrosine kinases and redox-sensitive transcription factors, such as NF-E2-related factor-2 (Nrf2). Prolonged NAD(P)H oxidase activation may lead to depletion of intracellular NADPH and impaired ROS scavenging associated with eNOS uncoupling, mitochondrial dysfunction, and diminished Nrf2-mediated antioxidant gene expression. In this review, we provide a mechanistic overview of the deleterious effects of ROS and their often overlooked physiological role as intracellular mediators of redox homeostasis. Given the largely negative outcome of clinical trials evaluating the benefits of antioxidants in the treatment of diabetes, targeting NAD(P)H oxidase, eNOS, mitochondria, and/or Nrf2 may provide a more effective strategy to restore antioxidant defenses in vascular diseases.

2. NAD(P)H oxidase and modulation of redox signalling
2.1 NAD(P)H oxidase-derived reactive oxygen species and regulation of vascular tone

NAD(P)H oxidases catalyse the transfer of electrons from NADPH to molecular oxygen via their catalytic subunits to generate superoxide (O2•−) and as shown in some studies hydrogen peroxide (H2O2).3 NAD(P)H oxidase in phagocytic cells releases ROS as a defense against pathogens, whereas in endothelial cells (ECs) NAD(P)H oxidase isoforms expressed in the endoplasmic reticulum (ER), perinuclear membranes generating ROS as modulators of redox-sensitive signalling pathways3–5 (see Section 4). Furthermore, anion channels [Cl-channel-3 (ClC-3)] expressed in the plasma...
membrane of ECs may facilitate diffusion of O$_2^-$ between intracellular and/or extracellular compartments.$^{6,7}$

The interaction of O$_2^-$ with nitric oxide (NO) leads to formation of peroxynitrite (ONOO$^-$),$^8$ which in turn can oxidize tetrahydrobiopterin (H$_4$B, a cofactor for eNOS) resulting in eNOS uncoupling and eventually endothelial dysfunction.$^9$ Although H$_2$O$_2$ is generally assumed to be more diffusible across membranes, recent studies suggest that permeation of H$_2$O$_2$ through plasmalemmal aquaporin channels may influence redox signalling.$^{10}$ Numerous studies have reported that H$_2$O$_2$ is an important vasoactive substance capable of modulating vascular tone, although its precise mode(s) of action remains to be elucidated. It is widely recognized that H$_2$O$_2$ can elicit contractile or relaxant responses, depending on the animal species, vascular bed, contractile state, and disease status of vessels.$^{11,12}$ In mice overexpression of the catalase transgene in blood vessels significantly reduces blood pressure compared with wild-type controls.$^{11}$ Moreover, exogenous H$_2$O$_2$-induced contractions of endothelium-denuded femoral arterial rings are attenuated by the iron-chelating agent desferoxamine.$^{12}$ Besides its vasoconstrictor actions, H$_2$O$_2$ is also capable of evoking endothelium-dependent and -independent vasorelaxation by mechanisms dependent on the vessel type and disease status,$^3$ e.g. increased NO production via upregulation of eNOS$^1$ or acutely via PI3-kinase/Akt and ERK1/2-dependent phosphorylation of eNOS;$^{13}$ smooth muscle relaxation via hyperpolarization, which appears to be NO-independent and may account for the compensatory relaxation in hypertensive or atherosclerotic vessels in which eNOS is uncoupled and generates O$_2^-$ rather than NO;$^{15}$ smooth muscle relaxation via a catalase-dependent activation of soluble guanylyl cyclase;$^{14}$ and direct activation of PKGI by thiol oxidants coupling disulphide oxidation with catalytic activity and vascular relaxation.$^{15}$

The precise roles of NAD(P)H oxidase-derived ROS and eNOS-derived NO or O$_2^-$ in regulating vascular tone remain to be investigated. A better understanding of the mechanisms regulating NAD(P)H oxidase and its various subunits in diabetic vascular tissue may provide insights for the design of pharmaceuticals to ‘recouple’ eNOS and restore vascular function. In this review, we examine the mechanisms by which sustained activation of NAD(P)H oxidase in diabetes leads to a cascade of ROS production and impaired antioxidant defenses, and hypothesize that under basal conditions or in early stages of diabetes NAD(P)H oxidase-derived ROS may serve as intracellular mediators to regulate redox-sensitive transcription factors (e.g. Nrf2) involved in adaptive responses to oxidative stress.

2.2 NAD(P)H oxidase, oxygen sensing, and activation of transcription factors

Recent studies have demonstrated that ROS and reactive nitrogen species (RNS) activate a spectrum of transcription factors, including nuclear factor kappa B (NFkB), AP-1, p53, Ets transcriptional factor (Ets-1), hypoxia-inducible factor-1$\alpha$ (HIF-1$\alpha$),$^{16}$ and Nrf2.$^{17}$ Among these, HIF-1$\alpha$ is implicated in oxygen sensing and its stabilization is regulated by NAD(P)H oxidase-derived ROS$^{18}$ and its activity is inversely regulated by oxygen concentration.$^{19}$ Increased expression of the NAD(P)H catalytic isoform Nox4 is associated with elevated levels of HIF-1$\alpha$ and vascular endothelial growth factor (VEGF), and diphenylene iodonium (DPI, a broad inhibitor of flavoproteins including NAD(P)H oxidase) abrogates HIF-1$\alpha$ and VEGF expression and increased ROS production.$^{20}$ Although it remains unclear which Nox isoforms are involved in oxygen sensing, there is consensus that Nox1, Nox4 and their regulator partner p22phox are responsible for the cellular responses to changes in oxygen levels,$^{21}$ and activation of HIF-1$\alpha$ upregulates expression of genes related to angiogenesis, energy metabolism, and cell proliferation.$^{22}$ Other transcription factors are also involved in ROS signalling, e.g. NFXb activation is blocked by overexpression of catalase;$^{23}$ AP-1 activation is associated with ROS generation,$^{14}$ and H$_2$O$_2$ activates Est-1 via an antioxidant response element (ARE)$^{25}$

3. Regulation of the redox-sensitive Nrf2/ARE defense pathway

ROS are generated continuously as natural byproducts of the normal metabolism of oxygen and play important roles in redox signalling.$^{5,26-27}$ Under physiological conditions, ROS are eliminated efficiently by antioxidant defense systems, and oxidative stress occurs when ROS production exceeds the capacity of antioxidant defenses. Nrf2 is a redox-sensitive basic leucine zipper transcription factor, which binds to the ARE in the promoter region of Phase II detoxifying and antioxidant enzymes, leading to an upregulation of antioxidant gene expression in vascular cells (Figure 1)$^{28}$ Keap1 (Kelch-like ECH-associated protein 1) negatively regulates Nrf2 by targeting it for ubiquitination and proteasomal degradation.$^{29}$ Cysteine residues with the BTB domain of Keap1 are involved in the loss of Nrf2 repression and oxidative or electrophilic stress-induced alterations in the Nrf2-Keap1 complex prevent proteasomal degradation, enabling newly synthesized Nrf2 to accumulate in the nucleus and activate ARE-mediated gene expression.$^{30}$

Activation of the Nrf2/ARE pathway appears to reverse endothelial dysfunction induced by prolonged hyperglycaemia.$^{31}$ and we have established that short-term (24-48 h) treatment of human ECs with elevated glucose or advanced glycation end-products (AGE) activates Nrf2-mediated haem oxygenase-1 (HO-1) expression (data not shown). HO-1 protects cells against oxidative stress by degrading the pro-oxidant haem to biliverdin, which is subsequently converted to the radical scavenger bilirubin.$^{32}$ There are reports that bilirubin may directly inhibit NAD(P)H oxidase by interrupting the assembly and activation of enzyme in ECs and in vivo.$^{33,34}$ As hyperglycaemia-induced ROS production and injury to cardiomyocytes and renal tissue is exacerbated in Nrf2 knockout mice,$^{35,36}$ this further implicates the Nrf2/ARE pathway in the defense against oxidative stress in diabetic complications. We propose that basal activity of NAD(P)H oxidases may provide a source of ROS to activate Nrf2/ARE-mediated antioxidant gene expression to maintain redox homeostasis (Figure 1)$^{28}$ In support of this hypothesis, evidence in murine fibroblasts and HepG2 cells suggests that NAD(P)H oxidase plays a key role in regulating Nrf2-mediated induction of glutamate cysteine ligase, the rate-limiting enzyme for synthesis of glutathione.$^{37}$
4. NAD(P)H oxidases in the vasculature

In diabetes the main sources of ROS generation in the vasculature include glucose auto-oxidation, the polyol pathway, AGE, mitochondrial electron transport chain (ETC), uncoupled eNOS, and NAD(P)H oxidases, with the latter enzymes arguably a major source of ROS generation in hypertension, atherosclerosis, and diabetes.5

Vascular NAD(P)H oxidase consists of multiple subunits including p22phox, p40phox, p47phox, p67phox, NoxO1 (Nox Organizer 1), NoxA1 (Nox Activator 1), Rac1, and unique Nox isoforms (based on gp91phox).38 The Nox isoforms are the catalytic subunits for ROS generation that are differentially expressed and regulated in various cell types and pathological conditions but remain to be fully characterized. To date, seven homologues of the Nox family have been identified: Nox1-5, DUOX1 and DUOX2.38 All vascular NAD(P)H oxidases generate O2, and the functions of the different oxidases are related to their localization in specific cells and subcellular compartments as well as their mode of activation.38,39 A recent review3 highlights that in ECs Nox2 (and Nox4) may be predominantly (approximately 90%) associated with perinuclear and/or ER membranes, providing an important link between ER stress and insulin resistance in diabetes.40

Identification of various Nox isoforms, their tissue distribution, and associated regulatory proteins are summarized in Table 1. Nox1, Nox2, Nox4, and Nox5, including recently discovered cytosolic partners (NoxO1, NoxA1), are expressed in vascular cells.5 ROS generated from these Nox isoforms have been implicated in endothelial dysfunction, inflammation, apoptosis, and vascular remodelling.5 Among them, Nox4 appears to control basal levels of ROS production in both endothelial41 and smooth muscle cells (SMCs),42 which may 'pre-condition' cellular defenses against oxidative stress by upregulating endogenous antioxidant enzymes. However, there are conflicting reports concerning the roles of Nox2 and Nox4 in basal ROS production.43

5. Evidence for activation of NAD(P)H oxidase in diabetes

Diabetes is characterized by hyperglycaemia resulting from defects in insulin sensitivity and/or secretion,44 with chronic hyperglycaemia associated with endothelial dysfunction.45,46 Type 1 or juvenile-onset diabetes ( autoimmune destruction of pancreatic β-cells and insulin deficiency) accounts for approximately 5–10% of individuals with diabetes, whereas type 2 or adult-onset diabetes (insulin resistance and relative insulin deficiency) accounts for 90–95% of those with diabetes.44 As the deleterious effects of hyperglycaemia in type 2 diabetes are often amplified by coexisting conditions associated with insulin resistance, such as hypertension, hyperlipidaemia, hyperuricaemia, and advanced atherosclerosis, it is more difficult to resolve the mechanisms underlying vascular pathology. Notably, in a large clinical study observing 74 309 person-years of young men without any signs of diabetic disease, high fasting glucose (<100 mg/dL) resulted in an increased incidence in the onset of type 2 diabetes when a moderately
increased body mass index and a triglyceride level > 150 mg/dL was present.47 Activation of NAD(P)H oxidase is implicated in oxidative stress associated with hyperglycaemia. Table 2 summarizes the different NAD(P)H oxidase isoforms expressed in vascular ECs and SMCs and their activation through different hyperglycaemic conditions. Treatment of human umbilical vein endothelial cells with high glucose increases NAD(P)H oxidase expression, apoptosis, and levels of oxidative stress markers.48 Moreover, ROS production and expression of p22phox are increased in mouse microvascular ECs treated with high glucose.49 As enhanced O2 production in vascular SMCs is unaffected by N(G)-monomethyl-L-arginine methyl ester (eNOS inhibitor), rotenone, or oxypurinol,50 this suggests that eNOS, mitochondrial complex I, or xanthine oxidase, respectively, may not be directly involved in glucose-induced O2 generation. Moreover, in this same study high glucose significantly increased p47phox expression, while Nox1, Nox4, and p22phox were not affected.50 Transfection with p47phox siRNA decreases glucose-stimulated O2 production, implicating involvement of p47phox upregulation and/or phosphorylation in the ROS generation by SMCs in response to hyperglycaemia.

6. Insulin resistance and activation of NADPH oxidase in the vascular wall

Insulin resistance plays a central role in the development of type 2 diabetes and associated cardiovascular complications.46,47,51,52 In a mouse model of obesity, induced by
feeding a high-fat diet for 14 weeks, inflammation and diminished NO production are detected in the vasculature prior similar changes in muscle, liver, or adipose tissue. This observation suggests that the vasculature and eNOS-derived NO production may be a more sensitive target of insulin resistance in type 2 diabetes.

NAD(P)H oxidase overproduction of ROS has been postulated to contribute to the loss of insulin responsiveness. Elevated ROS production from NAD(P)H oxidase may lead to inhibition of signalling at the level of insulin receptor substrate phosphorylation and insulin resistance. Insulin normally exerts vasodilatory, anti-inflammatory, and pro-survival actions. Genetic evidence suggests that a C242T mutation in the p22phox gene is associated with insulin resistance in non-diabetic subjects. In an animal model of insulin resistance (ESMIRO mice), endothelial dysfunction is evident based on attenuated vasorelaxant responses to acetylcholine or calcium ionophore. In these ESMIRO mice, insulin-stimulated phosphorylation of eNOS is blunted and accompanied by increased O2 generation and mRNA expression of Nox2 and Nox4 isoforms in the endothelium. The loss of insulin sensitivity will lead to decreased insulin-induced vasodilation and endothelial dysfunction, probably as a result of an increased ROS production by vascular NAD(P)H oxidase and eNOS uncoupling. Recent studies have shown that transient resistance to insulin and insulin-like growth factor 1 caused by chronic oxidative stress in hepatocytes is associated with activation of the redox-sensitive Nrf2-ARE defense pathway, potentially providing protection for the liver and other tissues against oxidant stress-induced insulin resistance.

The association of obesity with insulin resistance has been recognized for decades. It is well known that adipocytes are a key target for insulin, with loss of insulin sensitivity resulting in glucose/energy storage disorders such as diabetes. Recent evidence indicates that adipose tissue may not just be a storage site for triacylglycerol and a source of free fatty acid (FFA), but also functions as an endocrine and paracrine organ-secreting mediators that influence the actions of insulin. Thus, leptin, adiponectin, resistin, tumour necrosis factor-α (TNF-α), interleukin-6 (IL-6), and FFA have been shown to regulate insulin action. Leptin deficiency in obese ob/ob mice appears to contribute to insulin resistance in this animal model of type 2 diabetes. Moreover, elevated TNF-α levels in Zucker obese fatty rats, used as a model of prediabetic metabolic syndrome, are associated with endothelial dysfunction and increased expression of NAD(P)H oxidase subunits and enzyme activity, potentially providing a molecular link between obesity and insulin resistance. As summarized in Table 3, accumulating evidence suggests that adipocyte-derived mediators in obesity contribute to the development of insulin resistance in the vasculature involving modulation of NAD(P)H oxidase expression or activity.

### 7. Mechanisms underlying activation of NAD(P)H oxidase in diabetes

#### 7.1 Advanced glycation end-products formation and NAD(P)H oxidase in diabetes

NAD(P)H oxidase plays an important role in vascular events triggered by AGE. Incubation of human ECs with AGE (carboxymethyl lysine-modified adducts) promotes intracellular generation of ROS, which is suppressed by DPI and an AGE inhibitor but not by L-NAME. A soluble form of AGE for advanced glycation end-products (sRAGE) partially restores dilation in diabetic mice without affecting vascular reactivity in control mice, and sRAGE significantly inhibits expression of NAD(P)H oxidase in diabetic mice. These results indicate that NAD(P)H oxidase-mediated activation of AGE/RAGE signalling plays a pivotal role in regulating oxidative stress and endothelial dysfunction in type 2 diabetes (Figure 1). Thus, signalling pathways activated by AGE in diabetes, particularly NAD(P)H oxidase, may be a potential target in the treatment of diabetic cardiovascular complications.

#### 7.2 Protein kinase C-dependent NAD(P)H oxidase activation in diabetes

Increased diacylglycerol (DAG) levels and PKC activity, especially α,β1/2, and δ isoforms, in the retina, aorta, heart, renal glomeruli, and circulating macrophages have been reported in diabetes. Activation of NAD(P)H oxidase is abolished in diabetic PKCβ/−/− mice, suggesting that NAD(P)H oxidase is activated via a PKC-dependent pathway. Lack of PKCδ can protect against diabetes-induced renal dysfunction, fibrosis, and Nox-derived ROS production. Other PKC isoforms have also been implicated in NAD(P)H oxidase activation in diabetes, e.g. PKCζ is downstream of AGE–RAGE and mediates ROS generation by NAD(P)H oxidase in the kidney of diabetic rat. PKCζ is responsible for high glucose-induced intracellular ROS production by NAD(P)H oxidase in the adipocytes of diabetic mice, while PKCδ is required for ROS generation from NAD(P)H oxidase in mesangial cells treated with high glucose.

### Table 3 Effects of adipocyte-derived mediators on NADPH oxidase expression/activity and the development of insulin resistance in the vascular wall

<table>
<thead>
<tr>
<th>Mediators</th>
<th>NAD(P)H oxidase</th>
<th>Cell/animal</th>
<th>Insulin resistance</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Leptin</td>
<td>Suppression</td>
<td>HUVEC</td>
<td>Improved</td>
<td>Hwang et al.</td>
</tr>
<tr>
<td>Adiponectin</td>
<td>Suppression of gp91phox</td>
<td>Rat</td>
<td>Improved</td>
<td>Li et al.</td>
</tr>
<tr>
<td>TNF-α</td>
<td>Activation of p22phox and p40phox</td>
<td>Zucker obese fatty rat</td>
<td>Induced</td>
<td>Picchi et al.</td>
</tr>
<tr>
<td>Fatty acids</td>
<td>Activation of NADPH oxidase</td>
<td>BAEC and BASMC</td>
<td>Induced</td>
<td>Inoguchi et al.</td>
</tr>
</tbody>
</table>

**Table 3**

**Mediators of adipocyte-derived mediators on NADPH oxidase expression/activity and the development of insulin resistance in the vascular wall:**

- **Leptin:** Suppression
- **Adiponectin:** Suppression of gp91phox
- **TNF-α:** Activation of p22phox and p40phox
- **Fatty acids:** Activation of NADPH oxidase

**Cell/animal:**
- HUVEC
- Rat
- Zucker obese fatty rat
- BAEC and BASMC

**Insulin resistance:**
- Improved
- Induced

**Reference:**
- Hwang et al. (2012)
- Li et al. (2014)
- Picchi et al. (2016)
- Inoguchi et al. (2018)

**TNF-α:** tumour necrosis factor-α; HUVEC, human umbilical vein endothelial cells; BAEC, bovine aortic endothelial cells; BASMC, bovine aortic smooth muscle cells. Data obtained from cited references.
7.3 Angiotensin II-mediated activation of NAD(P)H oxidase in diabetes

Apart from its role as a vasoconstrictor, angiotensin II is a potent stimulator of NAD(P)H oxidase O$_2^-$ production in the vasculature. Several mechanisms may account for the deleterious effects of angiotensin II, including endothelial dysfunction, activation of NAD(P)H oxidase, mitochondrial dysfunction, and uncoupling of eNOS following activation of NAD(P)H oxidase. Moreover, angiotensin II, acting through angiotensin type 1 receptor, inhibits the actions of insulin in the vasculature. Thus, increased angiotensin type 1 receptor/NAD(P)H oxidase activation appears to contribute to vascular insulin resistance, endothelial dysfunction, apoptosis, and inflammation. Inhibitors of angiotensin II signalling slow the progression of diabetic complications such as nephropathy, retinopathy, and atherosclerosis, independent of their ability to lower blood pressure in both type 1 and type 2 diabetes. Perhaps, the most convincing evidence comes from the HOPE study, which showed that Ramipril significantly reduced cardiovascular events and prevented overt nephropathy in diabetic patients. Although the underlying mechanism(s) require further investigation, insulin resistance appears to be improved.

7.4 NAD(P)H oxidase activation and mitochondrial dysfunction in diabetes

Brownlee and colleagues have implicated generation of ROS by the mitochondrial ETC as a cause of oxidative stress in diabetes. They further postulated that inhibition of eNOS by the mitochondrial ETC as a cause of oxidative stress in insulin resistance appears to be improved.

8. NAD(P)H oxidase activation in diabetes—a double-edged sword

Numerous studies have reported an increase in the expression of Nox isoforms in diabetic vessels (Table 4) and an upregulation of eNOS expression. However, Hink et al. showed that even though eNOS expression was increased in the aorta of diabetic rats, NO production was diminished. It seems likely that NAD(P)H oxidase-derived ROS may increase eNOS expression under basal conditions or in early stages of diabetes, as long as cellular antioxidant defenses are not impaired. Indeed, short-term exposure of human ECs to 25 mmol/L glucose moderately increases eNOS activity and eNOS mRNA and protein expression. In contrast, chronic exposure to elevated glucose

<table>
<thead>
<tr>
<th>Complications</th>
<th>Animal model</th>
<th>Nox isoforms</th>
</tr>
</thead>
<tbody>
<tr>
<td>Retina</td>
<td>STZ-induced diabetic mice</td>
<td>Nox2$^{22}$, Nox2$^{4b}$, Nox4$^{46}$, Nox2$^{93}$, Nox4$^{95}$, p22$^{p22}$, p47$^{p47}$, p67$^{p67}$</td>
</tr>
<tr>
<td>Kidney</td>
<td>STZ-induced diabetic mice</td>
<td>Nox2$^{33}$, Nox4$^{127}$, Rac1$^{108}$, p47$^{p47}$, p67$^{p67}$</td>
</tr>
<tr>
<td>Aorta</td>
<td>STZ-induced diabetic rats</td>
<td>Nox2$^{127}$, Nox2$^{99}$, Nox4$^{127}$, p22$^{p22}$, p40$^{p40}$</td>
</tr>
<tr>
<td></td>
<td>Diabetic db/db mice</td>
<td></td>
</tr>
<tr>
<td></td>
<td>STZ-induced diabetic mice</td>
<td></td>
</tr>
<tr>
<td></td>
<td>STZ-induced diabetic rats</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Diabetic db/db mice</td>
<td></td>
</tr>
</tbody>
</table>

STZ-induced diabetic mice/rats used as type 1 diabetic model; db/db mice used as type 2 diabetic model. Data obtained from cited references.
oxidase leads to eNOS uncoupling, mitochondrial dysfunction, response to ROS/RNS, while sustained activation of NAD(P)H of eNOS, and antioxidant genes may be upregulated in derived ROS may act as a double-edged sword. Under basal diabetes.28,86 Further studies are required to determine whether ROS released from NAD(P)H oxidase initiates activation of this defense mechanism in and influence HIF-1

There is no doubt that antioxidant defenses are impaired in diabetes, but the mechanisms by which diabetes impairs antioxidant gene expression/activity remain to be elucidated. Ahmed et al. reported that expression of antioxidant enzymes such as superoxide dismutase, catalase, and glutathione peroxidase are increased in diabetic animals and patients, implicating upstream activators of antioxidant gene transcription, although other studies suggest that the expression/activity of antioxidant enzymes is downregulated. The Nrf2/ARE pathway may be activated by ROS/RNS as a compensatory mechanism, although it remains unclear whether ROS released from NAD(P)H oxidase initiates activation of this defense mechanism in diabetes.28,86 Further studies are required to determine whether activation of endogenous antioxidant defenses via the Nrf2/ARE can restore vascular function in animal models of diabetes and diabetic patients.

Although lower levels of ROS are involved in oxygen-sensing and influence HIF-1α stability, sustained activation of HIF-1α by excessive ROS generation is deleterious.2,5,27 Prolonged activation of HIF-1α in the diabetic vasculature may cause further damage via the induction of inflammatory responses. Inhibition of the HIF-1α pathway with pigment epithelium-derived factor ameliorates diabetic nephropathy as a consequence of suppressed proinflammatory responses. However, further studies are required to confirm an integral role for NAD(P)H oxidase in modulating HIF-1α expression. Figure 1 summarizes our hypothesis that NAD(P)H oxidase-derived ROS may act as a double-edged sword. Under basal conditions and/or in the early stages of diabetes, expression of eNOS, and antioxidant genes may be upregulated in response to ROS/RNS, while sustained activation of NAD(P)H oxidase leads to eNOS uncoupling, mitochondrial dysfunction, and impaired antioxidant gene expression potentially as a consequence of diminished intracellular NADPH levels.

9. NAD(P)H oxidase activation in diabetic vascular complications

9.1 Diabetic retinopathy

Although it is arguable whether endothelial injury is because of direct actions of high glucose or glucose-induced cytokine release, oxidative stress is present in diabetic retinal cells. Treatment of bovine retinal ECs with high glucose for 7 days increased ROS production by approximately 1.6-fold. Moreover, NAD(P)H oxidase is increased in the retina of diabetic rats and exposure of cultured retinal pericytes to a diabetic environment increases Rac expression and apoptosis. The activity of caspase-3, a marker of apoptosis, was reduced upon treatment of retinal pericytes with dominant negative (DN) p67phox, while overexpression of constitutively active Rac led to an increase in caspase-3.

Overexpression of DNp47phox substantially reduced palmitate-induced ROS production. In STZ-diabetic mice, administration of apocynin (widely used as NAD(P)H oxidase inhibitor, although recently suggested to act as an antioxidant) led to a reduction in retinal ROS. Notably, mice lacking Nox2 are protected against diabetes, indicating that Nox2 activity may play an important role in ROS production and retinal vascular inflammation.

9.2 Diabetic nephropathy

It is increasingly recognized that activation of NAD(P)H oxidase is central to hyperglycaemia-induced oxidative stress in diabetic nephropathy. Nox4 appears to be the predominant isoform expressed in renal cells. Increases in Nox4 and p22phox mRNA were detected in the kidney of diabetic rats 4–8 weeks after the onset of diabetes with similar increases detected in human mesangial cells exposed to high glucose for 2 days. Intensive insulin treatment appears to restore expression levels of Nox4 and p22phox. These findings suggest that ROS generated in response to hyperglycaemia may be derived from Nox4. In agreement, Gorin et al. found an increase in NAD(P)H-dependent ROS generation in kidney cortex and isolated glomeruli of diabetic rats. Subsequent administration of Nox4 siRNA significantly decreased Nox4 expression and O2 production, but had no impact on Nox2 expression. Administration of Nox4 siRNA also significantly reduced renal hypertrophy by modulating fibronectin expression in diabetic animals. These studies highlight a direct effect of O2 on renal hypertrophy, possibly via Nox4-mediated fibronectin expression. However, upregulation of p22phox and p47phox and increased translocation of Rac1, p47phox and p67phox have also been implicated in STZ-induced diabetic nephropathy (Table 4).

9.3 NAD(P)H oxidase and endothelial dysfunction in diabetes and atherosclerosis

Endothelial dysfunction has been postulated as an initial trigger of the progression of atherosclerosis in diabetic patients, with eNOS uncoupling leading to endothelial dysfunction. High concentrations of glucose and insulin resistance are associated with endothelial dysfunction in vitro and in vivo. To date, accumulating evidence suggests that sustained NAD(P)H oxidase ROS generation contributes to endothelial dysfunction in diabetes. Administration of apocynin to OLETF diabetic rats improves dilator responses to acetylcholine by approximately 20%. Adenoviral vectors expressing DN Rac-1 reduce O2 production, and significantly improve vascular relaxation. Although studies in OLETF rats found that vascular p22phox mRNA and O2 production increase concomitantly, p22phox was only implicated indirectly as a potential mediator of vascular dysfunction. In a similar study in basilar arteries...
from OLETF rats, Matsumoto et al.\textsuperscript{103} reported that impaired endothelium-dependent relaxation is associated with increased expression of gp91phox, but not p22phox, and largely abrogated by treatment with apocynin. Thus, different NAD(P)H subunits may modulate endothelial function in conduit and resistance vessels. It is possible that downregulation of specific NAD(P)H oxidase subunits could incrementally improve the redox status in different vascular beds by directly reducing $O_2^-$ production and/or improving bioavailability of NO via ‘recoupling’ of eNOS owing to a reduction of oxidative stress.

**10. Future research and therapeutic implications**

Given the importance of oxidative stress in hyperglycaemia-induced diabetic complications, the treatment choice remains to be administration of blood glucose-lowering agents.\textsuperscript{104} As NAD(P)H oxidase activation may have dual actions in diabetes (Figure 2), selective targeting of the deleterious effects of sustained NAD(P)H oxidase activation, such as eNOS uncoupling, mitochondrial dysfunction, and impaired antioxidant gene expression, may prove beneficial in the treatment of diabetes. Angiotensin II-signalling attenuators and AGE antagonists are effective in ameliorating diabetic vascular complications, possibly as a result of recoupling of eNOS via inhibition of NAD(P)H oxidase.

Statins have emerged as a promising treatment for diabetic cardiovascular complications, even in patients with normal LDL levels because of their beneficial pleiotropic effects.\textsuperscript{105,106} Of note, pitavastatin may restore vascular dysfunction by inhibiting NAD(P)H oxidase activity via inhibition of PKC and downregulation of angiotensin II receptor 1 expression.\textsuperscript{57,107} Moreover, statins recouple eNOS by upregulation of GTPCH-1 in cultured ECs and diabetic mice.\textsuperscript{108} Recoupling of eNOS improves endothelial function, which in turn could feed-forward to reduce NAD(P)H oxidase activity, and thereby ameliorate diabetic vascular complications. As uncoupled protein 2 (UCP-2) has been reported to function as a physiological regulator of mitochondrial ROS generation and may contribute to the prevention of atherosclerosis,\textsuperscript{109} further studies examining the role of UCP-2 in diabetic patients and animal models are warranted. Treatment with anti-inflammatory agents may also reduce the detrimental effects of ROS-induced HIF-1 activation without necessarily compromising the beneficial activation of redox signalling pathways.

While drugs to treat hyperglycaemia, insulin resistance, and hyperlipidaemia have been used to promote favourable outcomes in diabetic patients, non-pharmaceutical approaches involving regular exercise also have a beneficial role in the prevention and treatment of CVD in diabetes.\textsuperscript{110} The underlying mechanisms are poorly understood but may involve improved blood lipid clearance, reduced insulin resistance, reduced levels of inflammatory cytokines, restored mitochondrial function,\textsuperscript{111} restored endothelial function owing to increased eNOS activity and protection afforded by activation of endogenous antioxidant defense enzymes. Exercise training has been reported to recouple myocardial eNOS in diabetic Goto-Kakizaki (GK) rats,\textsuperscript{112} and decrease oxidative stress, inflammation, proteinuria, and increase antioxidant defenses in the kidney via the Nrf2/ARE pathway.\textsuperscript{113} Recent studies have shown that

![Figure 2](https://example.com/figure2.png)

**Figure 2** Role of NAD(P)H oxidase in cellular redox balance in diabetes. Hyperglycaemia-induced activation of NAD(P)H oxidase is potentially a result (or mediator) of diabetic complications involving activation of protein kinase C and AGE–RAGE interactions.\textsuperscript{46} Uncoupling of endothelial nitric oxide synthase, increased mitochondrial ROS production and impaired antioxidant responses are caused by sustained NAD(P)H oxidase activation and inhibition of the pentose phosphate shunt. Diminished NADPH levels and sustained oxidative stress in turn impairs Nrf2-mediated transcriptional activation of Phase II defense (e.g. glutathione-S-transferase, NADPH-quinone oxidoreductase) and antioxidant (e.g. heme oxygenase-1, peroxiredoxin-1) enzymes. The dotted lines represent potential treatments to modulate intracellular redox signalling. AGE, advanced glycation end-products; RAGE; receptor for advanced glycation end-products; ROS/RNS, reactive oxygen species/reactive nitrogen species; G6PD; glucose-6-phosphate dehydrogenase; PPS, pentose phosphate shunt; UCP-2, uncoupling protein 2; GAPDH, glyceraldehyde 3-phosphate dehydrogenase.
exercise training leads to a reduction in the expression of NAD(P)H oxidase subunit expression in human arteries, potentially accounting for decreased ROS production and increased NO bioavailability. Although inhibition of NAD(P)H oxidase activity may prove beneficial for the treatment of diabetes, it is worth noting that exercise-induced ROS production can upregulate antioxidant gene expression. Notably, short-term exhaustive exercise may not be sufficient to upregulate expression of antioxidant stress proteins (e.g. heme oxygenase-1), whereas a half-marathon significantly increases HO-1 expression in human blood cells. Exercise may thus also serve as a double-edged sword in the regulation of NAD(P)H oxidase, with moderate exercise upregulating antioxidant defenses via Nrf2 and other transcriptional pathways and exhaustive exercise leading potentially to an imbalance in NAD(P)H oxidase-mediated ROS production.

The tissue-specific distribution of various NAD(P)H oxidase subunits and distinctive functions of Nox isoforms provides a basis for the design of selective NAD(P)H oxidase inhibitors for the treatment of diabetic vascular complications. Further studies are warranted to discriminate the role(s) of different Nox isoforms and their subunits in redox signalling and the deleterious actions of overproduction of ROS in diabetes and other vascular diseases. The question remains whether basal NAD(P)H oxidase-derived ROS can upregulate Nrf2-dependent gene expression in vascular endothelial and SMCs, providing an antioxidant defense against sustained oxidative stress in diabetes. Evidence implicating NAD(P)H oxidase-derived ROS in the activation of Nrf2/ARE gene transcription is limited, and further studies using NAD(P)H oxidase knockout mice may provide insights for therapies to restore physiological redox signalling and vascular function in diabetes.

We have highlighted that activation of NAD(P)H oxidase affects both cellular redox signalling and oxidative stress in diabetes. NAD(P)H oxidase could act as a double-edged sword, inducing antioxidant defenses via ROS-mediated activation of intracellular redox signalling pathways, whereas overproduction of ROS leads to eNOS uncoupling, mitochondrial dysfunction, and an impaired redox balance owing to depletion of NADPH and impaired Nrf2/ARE-mediated gene expression. As recently reviewed, the feedback inhibition of NAD(P)H oxidase by HO-1-derived bilirubin provides a novel mechanism to regulate vascular redox homeostasis. Thus, the thiol status of vascular cells and the localization of Nox isoforms will markedly influence ROS-mediated signalling under both physiological and pathophysiological conditions. With respect to therapeutic options, lowering blood glucose remains the gold standard, however, strategies to restore basal NAD(P)H oxidase activity, NO production, and more importantly antioxidant defenses offer potential scope for treatment. Recent advances in the identification of vascular NAD(P)H oxidase subunits, their subcellular localization/ regulation, and feedback inhibition of NAD(P)H oxidase via the Nrf2/ARE pathway provides a novel therapeutic target to combat oxidative stress in diabetes.

Conflicts of interest: The authors declare no conflict of interest.

Funding

References

Acknowledgements
We acknowledge our collaborators in the cited references and thank Dr Doan Ngo, Dr Richard C.M. Slow, and Dr Paul A. Fraser for helpful discussions.


30. Xue M, Qian Q, Antonysunil A, Rabbani N, Babaei-Jadidi R, Thornalley PJ.


42. Beyer TA, Werner S. The cytoprotective Nrf2 transcription factor controls insulin receptor signaling in the regenerating liver. Cell Cycle 2008;7:874–878.


NAD(P)H oxidase activation in diabetes


121. Li L, Renier G. Activation of nicotinamide adenine dinucleotide phosphate (reduced form) oxidase by advanced glycation end products links oxidative stress to altered retinal vascular endothelial growth factor expression. Metabolism 2006;55:1516–1523.


