Nox as a target for diabetic complications

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Abstract
Oxidative stress has been linked to the pathogenesis of the major complications of diabetes in the kidney, the heart, the eye or the vasculature. NADPH oxidases of the Nox family are a major source of ROS (reactive oxygen species) and are critical mediators of redox signalling in cells from different organs afflicted by the diabetic milieu. In the present review, we provide an overview of the current knowledge related to the understanding of the role of Nox in the processes that control cell injury induced by hyperglycaemia and other predominant factors enhanced in diabetes, including the renin–angiotensin system, TGF-β (transforming growth factor-β) and AGEs (advanced glycation end-products). These observations support a critical role for Nox homologues in diabetic complications and indicate that NADPH oxidases are an important therapeutic target. Therefore the design and development of small-molecule inhibitors that selectively block Nox oxidases appears to be a reasonable approach to prevent or retard the complications of diabetes in target organs. The bioefficacy of these agents in experimental animal models is also discussed in the present review.

Key words: diabetes, diabetic complications, hyperglycaemia, oxidative stress, Nox, reactive oxygen species, small-molecule inhibitor

INTRODUCTION

Diabetes is associated with numerous microvascular and macrovascular complications [1,2]. The major microvascular complications are DN (diabetic nephropathy), DR (diabetic retinopathy) and diabetic neuropathy [1,3–7]. Diabetic macrovasculopathy consists of accelerated atherosclerosis in large arteries (e.g. carotid arteries, aorta, and coronary and femoral arteries) and is associated with increased risk of cardiovascular diseases such as stroke, coronary artery disease and peripheral vascular disease [1,2]. Diabetes also affects cardiac structure and function in the absence of coronary artery disease, a complication referred to as DCM (diabetic cardiomyopathy) [8–11].

Oxidative stress has emerged as a critical pathogenic factor in the initiation and development of diabetic complications [12–22]. Diabetes is accompanied by increased generation of ROS (reactive oxygen species) in the kidney, retina, heart and the macro- or micro-vasculature [8–27]. The deleterious role of ROS in the pathogenesis of diabetic complications is suggested by the findings that antioxidants or overexpression of SOD (superoxide dismutase) or catalase are relatively effective in preventing tissue and cellular alterations in experimental animal models of diabetes [6,8–13,22,23,26,28–44]. Although chronic hyperglycaemia alone may be sufficient to trigger diabetes-mediated pathologies, data from animal models and cultured cells indicate that a combination of growth factors, hormones and cytokines act on cells to generate ROS that induce and maintain tissue or cell injury [1,2,8–13,18,20,24,25,45–47]. Multiple pathways may lead to ROS generation, i.e. mitochondrial oxidative phosphorylation, xanthine oxidase or uncoupled NOS (nitric oxide synthase). However, recent studies indicate that the phagocyte-like NADPH oxidases of the Nox family are a major source of ROS in many non-phagocytic cells, including cells from the cardiovascular and cardioenrenal systems [13,23,48–70].

The present review will focus on the role of the enzymes of the NADPH oxidase pathway in the pathogenesis of the macro- and micro-vascular complications of diabetes (Figure 1). To date, the Nox family comprises seven members: Nox1–Nox5, Duox (dual oxidase) 1 and Duox2 [56,60,71,72]. Nox1, Nox2 (also known as gp91phox) and Nox4 are the NADPH oxidase homologues that are predominantly expressed in the cardiovascular and cardioenrenal systems, including most of the vascular

Abbreviations: ACE, angiotensin-converting enzyme; ACEi, ACE inhibitor; AGE, advanced glycation end-product; AMPK, AMP-activated protein kinase; AngII, angiotensin II; AOPP, advanced oxidation protein product; ApoE, apolipoprotein E; AT1 receptor, AngII type 1 receptor; ARB, AT1 receptor blocker; CYP4A, cytochrome P450 4A family; DCM, diabetic cardiomyopathy; DN, diabetic nephropathy; DR, diabetic retinopathy; Duox, dual oxidase; EMT, epithelial–mesenchymal transition; HDL, high-density lipoprotein; IGF, insulin-like growth factor; LDL, low-density lipoprotein; mTOR, mammalian target of rapamycin; mTORC1, mTOR complex 1; NOx, nitric oxide synthase; eNOS, endothelial NOx; Nox01, Nox organizer 1; O2•−, superoxide; PDGF, platelet-derived growth factor; PEDF, pigment epithelium-derived factor; PKC, protein kinase C; PPARγ, peroxisome proliferator-activated receptor γ; RAGE, receptor for AGEs; raptor, regulatory associated protein of mTOR; RAS, renin–angiotensin system; ROS, reactive oxygen species; Sp, specificity protein; TGF-β, transforming growth factor β; TNFR, thioredoxin-interacting protein; UUO, unilateral ureteral obstruction; VEGF, vascular endothelial growth factor.

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Figure 1 Role of Nox oxidases in the development of diabetic complications

In diabetic milieu, hyperglycaemia, together with local RAS, AGEs, AOPPs, TGF-β, oxidized or glycated LDL, VEGF and PDGF, induce an increase in Nox subunit expression and Nox activity that result in enhanced ROS generation. Nox-derived ROS contribute to the cell and tissue injury characteristic of diabetic complications in the kidney, retina, heart, vessels and vasculature.

pericytes and endothelial cells. This encompasses most of the cells from the heart, the kidney, the retina and the circulatory vessels [13,56,58,62,63,69,73–76]. Nox1, Nox2 and Nox4 consist of six membrane-spanning regions with binding sites for NADPH, FAD and haem. The latter comprise electron transfer centres that pass electrons from NADPH to oxygen-forming O2− (superoxide anion) and H2O2 [56,63,69–72,77,78]. The calcium-dependent homologue Nox5 is also found in the human vasculature, but, as this enzyme is not present in mice and rats, the investigation of its role in vascular pathologies such as diabetic complications has been hampered [63,74]. No data related to Nox3 or Duox1/Duox2 expression in the organs involved in diabetic complications are available yet. Mechanistically, Nox2, Nox1 and Nox4 require p22phox as an activating, stabilizing and/or regulatory subunit. Nox2 and Nox1 activation involves complex formation with regulatory subunits. Nox4 does not require the regulatory subunits essential to other Nox isoforms and is a constitutively active enzyme regulated primarily at the level of its expression in response to various stimuli [56,62,63,70,72].

In the present review, we describe the numerous reports suggesting that these enzymes are a primary source of ROS in the diabetic environment and are key mediators of diabetic complications. The findings demonstrate that impairment of NADPH oxidase function ameliorates diabetes-induced end-organ damage. Note that the present review will highlight the role of the Nox catalytic subunits without covering the function of the regulatory subunits. Although the review addresses diabetic complications and not its aetiology, it is important to mention that there is evidence that Nox oxidases such as Nox2 are implicated in pancreatic β-cell dysfunction in diabetes [79]. Similarly, the importance of Nox oxidases in adipocytes and their role in diabetes-induced cardiovascular damage [80,81] will not be detailed here. Because Nox-derived ROS mediate diabetic complications, the NADPH oxidases of the Nox family are attractive therapeutic targets. This is particularly relevant since clinical trials based on treatment with antioxidants, such as vitamins E and D, have been demonstrated failures in the amelioration of complications related to cardiovascular disease or diabetes [82–85]. The lack of efficiency of manipulating the antioxidant enzymes has also been reported [86]. Consequently, aiming to directly inhibit the sources of ROS in the setting of diabetes may be a superior approach compared with non-selective scavengers.

ROLES OF NOX OXIDASES IN DIABETIC KIDNEY DISEASE

DN is a major microvascular complication of Type 1 or Type 2 diabetes, and the most common cause of end-stage renal
disease in adults, affecting approximately 20–40% of diabetic patients [87]. A number of redox-sensitive mechanisms orchestrate key events of DN such as glomerular and tubular hypertrophy, mesangial cell injury, extracellular matrix accumulation, thickening of glomerular or tubular basement membranes, as well as podocyte dysfunction, leading ultimately to proteinuria, glomerulosclerosis and tubulointerstitial fibrosis [18,20,22,24–26,35–37,39,40,44,52,66,88–92]. A growing array of evidence suggests that Nox enzymes contribute to the pathogenesis of DN. This is because of the fact that multiple factors implicated in this pathology, such as hyperglycaemia, the RAS (renin–angiotensin system), including AngII (angiotensin II) and aldosterone, TGF-β (transforming growth factor-β), AGEs (advanced glycation end-products), AOPPs (advanced oxidation protein products), PDGF (platelet-derived growth factor), oxidized LDL (low-density lipoprotein), IGF (insulin-like growth factor)-1, VEGF (vascular endothelial growth factor), and endothelin, have been shown to alter the activity or expression of the Nox proteins and their regulatory subunits, and ultimately the amount of ROS produced [56,58,60,66,68,92–110]. Renal cells from the two major compartments of the kidney, glomeruli and tubulointerstitium, are particularly sensitive to the chronic hyperglycaemia that is an effective activator of Nox oxidases in these cells. Up-regulation of Nox4, Nox2, and Nox1 (mRNA and protein), together with increased O$_2^-$ or H$_2$O$_2$ generation, has been reported in response to high glucose concentrations in renal cells in vitro, as well as in experimental models of diabetes [66,68,92,98,111]. Chronic hyperglycaemia further amplifies Nox-derived ROS generation in renal cells and tissue via an elevation in the concentration of the agonists and mediators mentioned above, especially AngII and TGF-β. For instance, elevation of Nox4 or Nox2 expression, as well as increased oxidative stress, in glomeruli and tubules of animals with diabetes are inhibited by treatment with ACEis [ACE (angiotensin-converting enzyme) inhibitors] or ARBs [AT$\_1$ (AngII type 1) receptor blockers] [111–117]. Similarly, TGF-β redox signalling pathways are triggered by the mediators up-regulated by hyperglycaemia, including AngII and AGEs [24,117–119]. It is important to note that Nox oxidase subunits (e.g. Nox4) can be simultaneous targets and regulators of agonists such as TGF-β in renal cells [119,120]. The essential role of Nox subunits in diabetes-induced renal damage is supported further by the observation that most of the agents improving renal pathologies in DN prevent the increased expression of these Nox components in diabetic glomeruli and tubules [66,97,116,121–124]. In this section, we will consider the role of Nox oxidase subunits in renal cell injury induced by hyperglycaemia and the various stimuli known to be involved in the pathogenesis of DN.

Role of Nox oxidases in diabetes-induced glomerular cell injury

The initial morphological alteration that takes place during the early phase of DN is characterized by an increase in mesangial matrix accumulation accompanied by mesangial cell hypertrophy that contributes to glomerular basement membrane thickening [20,24,125]. As the disease advances, the deposition of matrix protein (e.g. fibronectin, type IV collagen or laminin) is exacerbated further and damage of glomerular epithelial cells or podocytes occurs, as evidenced by the widening of foot processes subsequent to the loss of key proteins contributing to the structural organization of the slit diaphragm [20,24]. Podocyte injury and reduction of podocyte number from apoptotic cell death also occurs during early DN and plays a key role in early proteinuria [20,24]. Nox-derived ROS, especially when produced by Nox4, appear to participate in each of these events.

Role of Nox oxidases in glomerular mesangium/ mesangial cell injury

During the initial stages of diabetic kidney disease, Nox4 protein expression increases in the glomeruli, including the mesangium, and Nox4-derived ROS contribute to oxidative stress [66,97,98,116,121–124]. Importantly, the elevation in Nox4 protein and ROS generation can be reversed by insulin treatment, confirming that hyperglycaemia and hyperglycaemia-induced mediators most likely account for these effects [66,121]. Studies by our group have provided evidence that Nox4-dependent ROS generation mediates glomerular hypertrophy and mesangial matrix accumulation [66]. Antisense oligonucleotides for Nox4 reduced glomerular enlargement, as well as fibronectin accumulation, in glomeruli from streptozotocin-induced Type 1 diabetic rats [66].

In cultured mesangial cells, glucose elicits a rapid up-regulation in Nox4 protein levels, including that found in mitochondria [68,126]. This is associated with an increase in ROS production [68,126]. Moreover, prolonged exposure of mesangial cells to high glucose has also been described to augment Nox4 mRNA and protein expression [121,127,128]. Nox4 is required for the high-glucose-induced (acute or chronic) increase in ROS production and accumulation of fibronectin in these cells [66]. Furthermore, previous findings have shown that Nox4 participates in the generation of mitochondrial ROS after treatment of mesangial cells with high glucose [68]. NADPH-dependent O$_2^-$ and H$_2$O$_2$ production measured in intact Percoll-purified mitochondria from mesangial cells are significantly reduced after Nox4 knockdown [68]. Evidence for an active Nox4 in mitochondria is also reported in cardiac myocytes and a potential mitochondrial localization signal was identified in the N-terminus of Nox4 [56,63,67–70,129–131]. The presence of a functional and glucose-sensitive Nox4 in the mitochondria suggests that a short paracrine loop may exist, by which ROS production by mitochondrial Nox4 alters mitochondrial respiratory chain activity, thereby leading to mitochondrial dysfunction. This contention is supported by the observation that ROS derived from mitochondrial Nox4 are able to oxidize and affect the activity of mitochondrial proteins in cardiac myocytes [67] and that Nox4-derived ROS inactivate mitochondrial respiratory chain complex I [132]. It is important to consider that, in mesangial cells, Nox4 is also found in membranes [68]. Membrane Nox4 is likely to also contribute to high-glucose-induced oxidative stress. This may also include high-glucose-dependent mitochondrial ROS production, since interplays between mitochondria and Nox enzymes localized outside the mitochondria have been described in vascular cells [133].

The molecular mechanisms by which diabetes and high glucose regulate Nox4 remain unclear. PKC (protein kinase C)
The signalling intermediates that have been often implicated in the control of Nox4 expression by glucose *in vitro* and *in vivo*. The fact that statins, known to inhibit Rho and Rac, prevent Nox4 expression and oxidative stress concomitantly to ameliorate mesangial matrix expansion and renal function in diabetic kidney (including glomeruli), indicate that small GTPase pathways may contribute to the modulation of Nox oxidases [123, 136]. miR-25 has been shown to serve as silencer for Nox4 gene expression, and its down-regulation by high glucose results in Nox4-mediated ROS production [127]. TxNIP (thioredoxin-interacting protein) has been recently identified as a mediator of high-glucose-induced ROS generation by Nox4 in mesangial cells [126]. Interestingly, TxNIP controls the expression of mitochondrial Nox4 in cells exposed to glucose [126]. It should be noted that glucose itself could modulate Nox oxidase expression via glycolytic metabolites, fructose 6-phosphate and the hexosamine pathway, that results in O-GlcNAcylation of transcription factor controlling Nox promoters in vascular cells [16]. For instance, nuclear factors known to bind to the Nox4 promoter, such as Sp (specificity protein) 1, Sp3 and NF-κB (nuclear factor-κB) have been shown to undergo post-translational modification by O-GlcNAc in vascular cells exposed to high glucose [16, 137–139]. Another means for glucose to regulate the Nox oxidases may be via the pentose phosphate pathway that provides NADPH for Nox activity [80].

In mesangial cells, Nox4 has also been demonstrated to confer AngII-mediated harmful effects. AngII elicits an acute increase in Nox4 protein expression, as well as a chronic prolonged up-regulation of Nox4 mRNA and protein levels associated with enhanced ROS production in cultured mesangial cells [140]. We have documented that, in *vitro*, Nox4-derived ROS are required for AngII-induced generation, mesangial cell hypertrophy and increased protein synthesis and/or fibronectin expression [55, 93, 141, 142]. The same observations can be made with AGEs or AOPPs, as they have also been documented to mediate mesangial cell injury in *vitro* or in *vivo* through Nox4-dependent mechanisms [119]. The role of Nox4 in TGF-β-mediated ROS generation and matrix protein accumulation has not been directly established in mesangial cells. The role of other Nox catalytic homologues in mesangial cell injury in the diabetic kidney has been less well studied. Some of these studies in streptozotocin-induced Type 1 diabetic rats employed apocynin, an inhibitor that blocks the recruitment of NADPH oxidase cytosolic subunits to the membrane-bound subunits of the Nox complexes, particularly those containing Nox2 and Nox1. Type 1 diabetic rats treated with apocynin demonstrated less glomerular fibronectin and collagen than controls, implicating roles for Nox2 and/or Nox1 [88]. It should be noted that the use of apocynin has serious limitations as it is a non-specific Nox inhibitor due to its ability to scavenge H2O2 and directly acts as an antioxidant [143]. The involvement of Nox2 in the mesangium is unclear because Nox2 is not detected in cultured human or rat mesangial cells. Nox2 is detected in isolated mouse mesangial cells and in diabetic glomeruli [102, 144, 145]. Genetic deletion of p47<sup>phox</sup> in Type 1 diabetic Akita mice attenuates diabetes-induced and high-glucose-induced Nox2 expression in glomeruli and cultured mesangial cells respectively [145]. Therefore p47<sup>phox</sup>-dependent activation of Nox2 may be a determinant for the progression of DN. However, a recent study has shown that glomerular mesangial matrix expansion and albuminuria are not attenuated in streptozotocin-induced Type 1 diabetic Nox2-knockout mice [146]. Interestingly, this may be due to an up-regulation of Nox4 [146]. Taken together, these findings warrant a reassessment of the role of Nox2 in DN. Although Nox1 is thought to promote the deleterious effects of glucose or AngII in the vasculature [147, 148], its function in mesangial cell injury in the diabetic milieu has not been reported.

**Role of Nox oxidases in glomerular epithelial cell/podocyte injury**

Up-regulation of Nox4 and Nox1 protein expression in diabetic glomeruli from OVE26 Type 1 diabetic mice, including podocytes, is accompanied by increased oxidative stress, loss of podocytes and foot process effacement with resultant albuminuria [97, 98, 149]. Cultured podocytes exposed to high glucose for a prolonged time period augments Nox4 protein expression and Nox4-derived ROS, promoting apoptotic cell death [97, 98, 149, 150]. In contrast with what is observed in mesangial cells, glucose does not acutely regulate Nox4 protein expression in cultured podocytes [98]. A sequential regulation of Nox4 oxidases by CYP4A (cytochrome P450 4A family) has been identified in podocytes in which 20-HETE (20-hydroxyeicosatetraenoic acid) generation by CYP4A mediates the stimulatory effect of high glucose on Nox4 and Nox1 expression and the resultant ROS production [98]. In the presence of high glucose concentrations, Nox4 promotes podocyte cell death via activation of p53- and PUMA (p53 up-regulated modulator of apoptosis)-dependent apoptotic pathways [97]. Importantly, inactivation of AMPK (AMP-activated protein kinase) by high glucose accounts for the increase in Nox4 mRNA and protein expression, as well as subsequent ROS production and podocyte apoptosis [97]. AMPK activators significantly reduced Nox4 expression, oxidative stress and podocyte injury in *vitro* or *in vivo* [97, 149, 151]. Note that AMPK activation is also able to counteract the induction of ROS production by high glucose via blockade of NADPH oxidase activity in mesangial cells [152]. A recent report indicates that mTOR (mammalian target of rapamycin) is an upstream regulator of Nox4 in podocytes and that AMPK acts by inhibiting the mTOR pathway via tuberin activation [153].

Although AngII- or TGF-β-induced oxidative stress mediates podocyte injury [46, 75, 154], very little is known regarding the role of the Nox oxidases in the podocyte dysfunction promoted by these agonists or the other major mediators of DN. Similar to what is observed in mesangial cells, the AngII-dependent increase in NADPH oxidase activity is associated with the up-regulation of Nox4 and Nox2 expression in podocytes [75, 155]. To date, the modulation of NADPH oxidase subunits by TGF-β, including Nox4, has not been reported in podocytes.

**Role of Nox oxidases in diabetes-induced tubular and interstitial cell injury**

Hyperglycaemia-induced ROS generation has been associated with AngII- and TGF-β-induced tubular and interstitial cellular dysfunction [20, 24]. This mechanism may lead to
tubulointerstitial fibrosis and promote tubular EMT (epithelial–mesenchymal transition) [20,24]. In addition, diabetes-induced oxidative stress can be pro-apoptotic or hypertrophic for tubular cells [20,24].

Analogous to glomeruli, tubules from streptozotocin-induced Type I diabetic rats demonstrate an increase in Nox4 mRNA and protein expression. When Nox4 is suppressed with antisense oligonucleotides in vivo, diabetes-mediated ROS production and extracellular matrix protein synthesis are both reduced in the renal cortex [66,121,122]. Given that most of the renal cortex comprises tubular epithelium, this observation may reflect an effect on the tubules. Interestingly, Nox4 protein expression is increased in the renal cortex, but is unchanged in the medulla from Type 2 diabetic mice [156]. The presence of Nox4 in mitochondria isolated from the renal cortex suggests that the protein is localized to mitochondria in tubular cells [68]. Although Nox2 levels are not affected in the renal cortex from Type 2 diabetic db/db mice [156], Nox2 is increased in the cortex from Type 1 diabetic rats [66]. However, a role for Nox2 is challenged by a recent report indicating that tubulointerstitial disease is not ameliorated in Type 1 diabetic Nox2-knockout mice [146]. Statins are known to inhibit Nox expression and activation, and attenuate increased Nox4 expression and oxidative stress in the cortex from mice made Type 1 diabetic with streptozocin. This is suggestive of a mechanism whereby Nox4 contributes to tubular injury [123,136,157].

Exposure of cultured renal proximal tubular epithelial cells to high glucose leads to the up-regulation of Nox4 protein expression, but has no effect on Nox2 or Nox1 expression [156]. Additionally, Nox4-dependent ROS production is required for the glucose-induced increase in fibronectin accumulation and TGF-β expression in these cells [156]. The pro-fibrotic action of the oxidase is corroborated by the finding that overexpression of Nox4 in tubular cells causes a robust increase in fibronectin synthesis [103].

AngII has also been shown to employ Nox4 as a mediator of injurious effects in tubular epithelial cells. Chronic AngII treatment up-regulates Nox4 expression and induces EMT in cultured renal epithelial cells through Nox4-dependent ROS production [158–160]. Furthermore, it was reported recently that AngII up-regulates Nox4 expression in the mitochondrial and membrane fractions and is required for AngII-mediated mitochondrial and intracellular ROS production in cultured renal tubular cells [161]. The ROS derived from Nox4 upon AngII stimulation contributes to apoptosis in these cells [161]. Unlike glucose, AngII seems to enhance the expression of the Nox2 subunit along with Nox4 in cultured tubular cells [160,162]. The effects on Nox4 appear to be mediated by lectin-like oxidized LDLR-1 (LDL receptor-1) [160,162].

There is a paucity of information regarding the role of the oxidase or other Nox enzymes in TGF-β signalling in tubular cells. Although it is clear that oxidative stress is implicated in TGF-β-mediated tubular cell injury [163], there is a deficit of causal evidence supporting the role of the oxidase or other Nox enzymes in TGF-β signalling in cultured tubular cells. TGF-β promotes the up-regulation of Nox4 protein, but not that of Nox2 [164]. AMPK activation is able to attenuate the high-glucose-

AngII- and TGF-β-induced increase in Nox4 protein expression in cultured tubular cells, supporting the concept that the kinase acts as a suppressor of oxidative stress [165].

Among the other factors known to mediate tubular damage in DN, IGF-I has been identified as a potent regulator of Nox activity in tubular cells. Hence it has been established that Nox4-derived ROS serve as signal transducers for the fibrotic effects of IGF-I in cultured renal tubular epithelial cells [103]. The role of VEGF even though is ambiguous. In vivo, VEGF is thought to preserve the integrity of glomerular filtration barrier, as well as podocyte function [166,167]. IGF-I is also known to increase the expression of VEGF in the renal cortex [173]. Thus it is conceivable that Nox4-dependent up-regulation of VEGF may participate in renal cell injury in the diabetic kidney. However, the role of other Nox proteins such as Nox2, which is known to be regulated by VEGF [174], should not be excluded.

Information regarding the role of hyperglycaemia-mediated oxidative stress or Nox-derived ROS in interstitial cell injury, particularly in the transformation of renal fibroblast into myofibroblasts, remains very sparse. Although glucose has been shown to elicit extracellular matrix protein synthesis in kidney fibroblasts, there is no evidence that the ROS produced by Nox4 or any other Nox oxidases mediate these effects. Most of the data available concern the effect of TGF-β. Studies with fibroblasts derived from the heart, lung and kidney indicate that Nox4 is central to TGF-β-induced ROS generation and myofibroblast differentiation to a pro-fibrotic phenotype [55]. Importantly, Nox4 is also the predominant Nox homologue implicated in kidney myofibroblast differentiation and expression of fibronectin in response to TGF-β [55,94].

Note that the up-regulation of Nox protein expression by hyperglycaemic conditions may be a sort of putative protective mechanism. For instance, the fibrogenesis taking place in the kidney (as well as in the heart and the vasculature) could be considered as a series of events that attempt to repair tissue damage caused by pathological factors such as hyperglycaemic conditions. Thus the findings that Nox oxidases (e.g. Nox4) are critical for fibrosis suggest that the up-regulation of Nox oxidases induced by glucose could be part of a protective mechanism in response to injury. Sustained exposure to chronic hyperglycaemia may result in excessive Nox-dependent ROS production, leading to aberrant extracellular matrix protein accumulation and organ injury.

ROLES OF NOX OXIDASES IN DIABETIC RETINOPATHY

DR is a widespread complication of diabetes and a major cause of blindness in people of working age [4,6]. The sequence of patho-
logical changes promoted by hyperglycaemia in DR include an increase in vascular permeability, retinal vascular pericyte and endothelial cell death, capillary occlusion, tissue ischaemia and abnormal growth of new vessel in the retina or neovascularization via activation of angiogenesis [4,6,13]. The elevation in vascular permeability promoted by blood–retinal barrier breakdown leads to diabetic macular oedema, resulting in vision loss. Similar to DN, key mediators of retinal injury in synergy with glucose include AngII, AGEs or modified/glycated LDL [4,6,13,175–180]. The potent angiogenic factor VEGF is crucial for the development of retinal angiogenesis and neovascularization, as demonstrated by the observation that blockade of the VEGF system improves vascular pathologies associated with DR [3]. Importantly, there are interplays between these different factors during the course of DR. For instance, a complete RAS has been identified in the retina and its inhibition attenuates hyperglycaemia-mediated microvascular complications in the retina via disruption of VEGF- or AGE-dependent pathways [179,181–184].

It has been proposed that oxidative stress plays a pivotal role in the pathogenesis of DR [13,175]. ROS derived from Nox enzymes are likely to be implicated in this process [13]. Many findings regarding the role of Nox subunits in DR are correlative and suggest that the up-regulation of Nox2 or Nox4 expression is associated with increased ROS generation. The evidence is derived from studies of retinae from diabetic animals and cultured retinal endothelial cells and pericytes in response to high glucose [30,181,185–188]. The strongest causative evidence that Nox oxidases are an important source of ROS in the retina and directly contribute to DR was provided by studies demonstrating that depletion of Nox4 by intravitreal delivery of specific siRNAs significantly decreased retinal NADPH oxidase activity, ROS production, VEGF expression and reduced vascular permeability in Type 2 diabetic db/db mice [188]. This has been confirmed in cultured retinal endothelial cells, where Nox4-dependent ROS generation mediated the increase in VEGF caused by high glucose and hypoxia [188]. The implication of Nox4 in neovascularization witnessed in DR is supported by studies in Nox4-overexpressing or Nox4-deficient mice models, showing that the enzyme promotes myocardial and endothelial angiogenesis in a VEGF-dependent manner [189,190]. A role of Nox4 in angiogenesis is also suggested by the finding that aldosterone can increase its expression in the retina [191]. Moreover, Nox4-derived ROS are essential for VEGF expression and angiogenesis induced by insulin in human microvascular endothelial cells [192]. In Nox4-deficient mice, ischaemic brain injury was lessened [193]. The mechanism lends itself to similar roles in ischaemia in DR. Although Nox4 appears to be critical for diabetic microvascular complications and angiogenesis, the role of Nox2 should not be ignored. Indeed, it is known that Nox2 plays an important role in VEGF-induced angiogenesis in vitro in vascular cells [174].

Nox oxidases may be critical for neovascularization in diabetes. Oxidative stress, vascular permeability and blood–retinal barrier breakdown are significantly reduced in Nox2-deficient mice rendered diabetic with streptozotocin [194]. It should be noted that it is difficult to delineate the role of Nox2 expressed in retinal cells from that present in the leucocytes infiltrating the retinal tissue and vasculature. Indeed, it was documented that vascular inflammation due to leucocyte adhesion to vascular endothelial cells via adhesion molecules or leukostasis occurs in DR, leading to cytokine and growth factor release, capillary plugging, vascular injury and hyperpermeability [4,13]. The fact that leucocyte–endothelial interaction, leukostasis and ICAM-1 (intracellular adhesion molecule-1) are decreased in retina from streptozotocin-induced Type 1 diabetic Nox2-knockout mice or diabetic wild-type mice treated with apocynin indicates that this Nox homologue is critical for retinal inflammation in DR [194]. Nox-derived ROS are involved in the pathogenesis of DR. Treatment with agents that are known to prevent cell injury in experimental animal models, such as statins or PEDF (pigment epithelium-derived factor), blocks diabetes-induced Nox4 and Nox2 expression and oxidative stress, as well as retinal cell injury. Similar findings have been demonstrated in vitro using cultured retinal cells [185,187,188]. Additionally, diabetes-mediated suppression of the protective anti-angiogenic factor PPARγ (peroxisome-proliferator-activated receptor γ) is not observed in streptozotocin-induced Type 1 diabetic mice lacking Nox2, suggesting a pathogenic role of the enzyme in DR [195]. This is also a reminder that Nox2 may be important for the development of angiogenesis in the diabetic retina.

AngII is a widely recognized stimulator of Nox oxidases in the renal and cardiovascular systems [55,63]. It is likely that the deleterious actions of AngII in the retina are mediated by Nox-derived ROS. In streptozotocin-induced Type 1 diabetic rats, AngII mimics the stimulatory effect of diabetes on retinal leukostasis, and Nox inhibition with apocynin attenuates the pro-inflammatory action of AngII or diabetes, suggesting that AngII may be a mediator of diabetes-induced vascular retinal damage [196,197]. The relationship between AngII and Nox oxidases in DR warrants further investigation.

There is less data available related to the connection between Nox enzymes and AGEs in DR as compared with DN. Treatment of cultured retinal endothelial cells with apocynin significantly reduced the stimulatory effects of AGEs on ROS production and VEGF expression, indicating that Nox oxidases are involved in AGEs redox signalling [198]. Interestingly, in vivo injection of PEDF to AGE-treated rats is able to inhibit AGE-induced retinal vascular permeability through blocking Nox2-dependent ROS generation and VEGF expression [199]. In vitro, exposure of cultured retinal endothelial cells to PEDF attenuates the stimulatory actions of AGEs on Nox2 and VEGF gene expression, as well as ROS production [199]. These studies clearly raise the possibility of the existence of functional links between AGEs and Nox oxidase subunits in diabetes-induced retinal pathology.

**ROLES OF NOX OXIDASES IN DIABETIC CARDIOMYOPATHY**

DCM has been defined as ventricular dysfunction that may occur independently of coronary artery disease and hypertension [8–11]. The pathogenesis of DCM is multifactorial and includes autonomic dysfunction, abnormal ion homoeostasis, alteration in structural proteins and interstitial fibrosis [8–11]. The structural and cellular abnormalities of DCM include, among
AT1 receptor up-regulation is linked to that of Nox4 and TGF-β increase in oxidative stress, reduced fibrosis and pro-fibrotic factor the myocardium [217]. These effects are accompanied by a decrease in oxidative stress, reduced fibrosis and pro-fibrotic factor TGF-β expression, as well as an improvement in cardiac function [217]. AT1 receptor up-regulation is linked to that of Nox4 and Nox2 in the heart from streptozotocin-induced Type 1 diabetic mice and Type 2 diabetic KK/Ta mice [112,213]. It is important to point out that numerous examples of the interaction between AngII and Nox enzymes in cardiac injury are reported outside of the DCM setting. ROS derived from mitochondrial Nox4 and a p47phox-dependent Nox oxidase (most likely Nox2) located in the membrane seems to contribute to cultured cardiac myocyte hypertrophic and fibrotic responses to AngII [220]. In vivo studies with Nox2-deficient mice have clearly established a role for Nox2 in AngII-induced interstitial cardiac fibrosis [203,221,222]. In vitro, it has been documented that Nox2 mediates AngII-dependent cardiac myocyte hypertrophy [223,224].

As for DN, the pro-fibrotic factor TGF-β is likely to operate downstream of hyperglycaemia and AngII in the heart [225]. Increased TGF-β expression typically parallels Nox4 and Nox2 up-regulation in the myocardium from streptozotocin-induced Type 1 diabetic rats and mice [112,208,212,215]. Since hyperglycaemia modulates AngII and TGF-β levels in the heart, it is conceivable that the control of Nox4 by AngII or TGF-β may contribute to cardiac fibrosis in DCM. The observation that AngII stimulates Nox4 and Nox2 expression [226], together with the fact that AngII-induced TGF-β expression in cultured cardiac fibroblasts is required for AngII-mediated fibrosis [225], suggests that a regulatory network involving AngII, TGF-β and Nox oxidases exists in these cells. Although the role of AGEs is documented in the pathogenesis of DCM [227–229], no data are available concerning their interaction with Nox oxidases. There are circumstantial observations in the myocardium from streptozotocin-induced Type 1 diabetic rats showing that the increase in AGEs and AGE receptors is associated with the augmentation in Nox2 and ROS production [216].

**ROLES OF NOX OXIDASES IN DIABETIC VASCULAR COMPLICATIONS**

Diabetes conveys a risk for vascular complications, such as peripheral artery disease, coronary artery disease and myocardial infarction [1,2]. Abnormal vascular endothelial dysfunction and accelerated atherosclerosis are prominent features of diabetes [1,2,12,13]. Chronic hyperglycaemia lends itself to atherosclerosis by altering vascular smooth muscle and endothelial cell function, promoting macrophage recruitment [12,13]. Endothelial dysfunction is characterized by reduced bioavailability of the vasodilator and anti-atherogenic factor NO [12,230]. eNOS (endothelial NOS), one of the enzymes that produce NO, may be a source of ROS under certain pathological conditions, including diabetes. This is due to a phenomenon called ‘uncoupling’, which will not be detailed in the present review, and leads to a decrease in NO bioavailability [12,230].

The molecular mechanisms underlying the complications of diabetes in the vasculature and atherosclerosis are not completely understood, but there is evidence supporting the role of oxidative stress in these processes [12,13]. Most of the results available are just suggestive of a role for Nox oxidases in diabetes-induced vessel pathology and atherosclerosis, but do not establish cause and effect. The findings appear to vary with the experimental models used and the vessels considered. Nox1, but not Nox4, levels are augmented in aortic vessels of streptozotocin-induced Type 1 diabetic rats, an event that is associated with increased ROS generation and endothelial dysfunction [231]. Aortic Nox1 is also found increased in streptozotocin-induced Type 1 diabetic
mice [232]. In contrast, another study indicates that Nox4, but not Nox1, is augmented in the aorta from Type 1 diabetic mice, a process that is PKC-dependent [233]. In mesenteric arteries or aortic sinus from ApoE (apolipoprotein E)−/− mice made Type 1 diabetic with streptozotocin, the expression of Nox4 and Nox2 are enhanced concomitantly to ROS production [234,235]. Nox4 is up-regulated in the aorta of streptozotocin-induced Type 1 diabetic rats [236,237], whereas Nox2 or Nox1 levels are not altered [237]. This contrasts with an older report showing an up-regulation of Nox2 in the aorta of streptozotocin-induced Type 1 diabetic rat [238]. Similar results are obtained in experimental models of Type 2 diabetes. When compared with control mice, aortas from db/db Type 2 diabetic mice display enhanced Nox2, Nox1 and Nox4 expression that are linked to an increase in oxidative stress and impaired vasodilation [239–241]. In Type 2 diabetic OLETF (Otsuka Long Evans Tokushima Fatty) rats, increased ROS generation in aortic rings correlate with elevated levels of Nox2, as well as endothelial dysfunction [242–245]. Aorta from a rat model where Type 2 diabetes is induced with low doses of streptozotocin and high-fat diet displays enhanced Nox4 expression [246]. Similarly, Nox4 is increased in arterial smooth muscle of pre-diabetic Zucker rats [247]. The direct demonstration of a critical role for a Nox homologue in vascular pathology is provided by a study performed in Type 1 diabetic Nox1-null mice [231]. It is reported that, in contrast with Nox2-deficient mice or mice injected with siRNA for Nox4, Nox1-null mice are protected from diabetic endothelial dysfunction [233]. In p47phox-deficient mice or in mice for which the Nox1 regulatory subunit NoxO1 (Nox organizer 1) was depleted using RNA interference in vivo, the impairment of endothelial function by diabetes was also markedly attenuated [233]. Mechanistically, that study demonstrates that the molecular event triggering diabetic endothelial cell dysfunction is eNOS uncoupling and the subsequent decline in NO production is initiated by the p47phox- and NoxO1-regulated Nox1 [233]. A recent report using ApoE- and Nox1-double-knockout mice made diabetic with streptozotocin confirms the importance of Nox1 in diabetes-accelerated atherosclerosis [248]. The studies indicate that, in the setting of diabetes, Nox1, but not Nox4, mediates oxidative stress, inflammation and fibrosis and determines atherosclerotic plaque size [248].

Numerous in vitro data in cultured vascular cells are available. Cultured vascular endothelial cells appear to primarily express the homologues Nox2 and Nox4, rather than Nox1 [62,63,70]. Prolonged exposure of cultured endothelial cells isolated from arteries or veins to high glucose elicits an up-regulation of Nox2 and Nox4 expression; effects that are linked to an increase in ROS formation [95,249–256]. siRNA- or antisense-mediated Nox4, Nox1 and Nox2 knockdown are reported to reduce high-glucose-induced ROS generation in cultured endothelial cells [95,248,255,257]. Moreover, Nox4 is critical for high-glucose-induced inflammatory signalling in these cells [255,257]. Nox4-derived ROS have been shown to be required for endothelial cell activation and monocyte binding in response to high glucose [255]. A role of Nox oxidases was also suggested in the apoptotic response of endothelial cells to high glucose, but only circumstantial observations are reported [249,250]. In vascular smooth muscle cells, the expression of the Nox homologues seems to be dependent on the type and the size of the vessel. The homologues present in vascular smooth muscle cells from resistance arteries are Nox2 and Nox4, but not Nox1 [62,63,70,258]. In cultured vascular smooth muscle cells from conduit arteries such as the aorta, Nox1 and Nox4, not Nox2, are expressed [62,63,70,258]. High glucose elicits an increase in Nox4 expression in cultured vascular smooth muscle cells [233,251]. These effects are dependent on PKC activation [233]. The direct implication of Nox4 in the action of glucose in cultured vascular smooth muscle cells is demonstrated by the observation that depletion of Nox4 prevents high-glucose-induced ROS generation, proliferation and migration [233]. It was also reported that glucose up-regulates Nox1 and that high-glucose-stimulated ROS generation by Nox1 accounts for the decreases in the expression of two protective factors of vascular smooth muscle cell function: ACE2 and Ang-(1–7) [angiotensin-(1–7)] [148]. Importantly, Nox1- and Nox4-derived ROS are responsible for the failure of NO to inhibit the migration of smooth muscle cells exposed to high glucose [107]. Another report shows that high glucose had no effect on Nox1 and Nox4 expression in primary aortic vascular smooth muscle cells [259]. The findings also imply that both a Nox4-based oxidase and a Nox1-based oxidase may contribute to glucose-induced vascular smooth muscle cell injury.

The RAS has been implicated as a major contributing factor in the pathogenesis of vascular disease and atherosclerosis based on studies in both human and experimental animal models [12,13,62,63,70,260,261]. Under diabetic conditions, increased AngII levels in the vasculature are associated with enhanced ROS generation, thereby contributing to diabetes-associated endothelial dysfunction and atherosclerosis [12,13,237,262–268]. In vivo, AngII infusion in transgenic mice with smooth-muscle-cell-specific Nox1 overexpression results in oxidative stress and impaired endothelium-dependent relaxation via eNOS uncoupling [269]. In vitro, conflicting data exist regarding Nox4 modulation by AngII. Some studies have found that AngII diminishes Nox4 mRNA levels [147], whereas other have shown that the hormone promotes an increase in Nox4 mRNA and protein expression, an effect associated with enhanced ROS production [109,270]. Importantly, only the impairment of Nox1 function, but not Nox4, seems to be able to abrogate AngII-mediated ROS production in cultured vascular smooth muscle cells [271]. In vivo, Nox1, Nox4 and Nox2 expression is found enhanced in the aorta from AngII-infused rats [272,273]. In vascular smooth muscle cells from resistance arteries, chronic exposure to AngII enhances Src-dependent Nox2 expression [258,274]. Interestingly, a study has indicated that Nox5 is a key mediator of AngII signalling in cultured human vascular smooth muscle cells [275]. In endothelial cells, AngII up-regulates Nox2 and Nox4 expression [276,277] and is able to promote the recruitment of Nox4 to membranes [278]. In vitro studies show that down-regulation of Nox2 and Nox4 attenuate AngII-mediated ROS generation and endothelial cell activation [276,277,279]. Stimulation of Nox2-dependent ROS generation by AngII triggers mitochondrial ROS production that, in turn, further activate Nox2, thereby amplifying oxidative stress and endothelial cell injury [279]. AngII treatment also enhances the association of Nox2 with its regulatory subunit p47phox [276]. Nox2 is critical for AngII-induced endothelial cell...
Atherosclerosis observed in ApoE−/− mice is accompanied by a decrease in Nox2 expression, as well as ROS production [281]. Note that PPARγ agonists may also wield their beneficial effects via suppression of Nox2, Nox1 and Nox4 expression in aortas from Type 2 diabetic db/db mice [239].

AGEs or AOPPs has been linked to vascular lesions in diabetes and atherosclerosis [12,13,282,283]. For instance, AGEs have been demonstrated in atherosclerotic lesions from patients with diabetes and have been shown to contribute to the damage of the vasculature in experimental models of diabetes [12,13,282,283]. There is evidence suggesting a link between AGEs, oxidative stress and endothelial dysfunction, as well as vascular injury in diabetes [12,13]. However, the demonstration of the in vivo relationship between AGEs and Nox oxidases in the vasculature is mostly based on circumstantial evidence. The attenuation of atherosclerosis observed in ApoE−/− mice rendered Type 1 diabetic with streptozotocin and treated with AGE inhibitors or in diabetic mice that are deficient for RAGE (receptor for AGEs) and ApoE−/− is accompanied by a decrease in Nox2 levels, suggesting that a Nox oxidase may mediate the effects of AGEs [284,285]. Endothelial cell dysfunction in resistance arteries from Type 2 diabetic mice is associated with increased Nox2 expression and ROS production, and this is prevented by treatment of the mice with agents that block AGE formation [286]. In vitro, treatment of cultured endothelial cells isolated from resistance arteries with the AGE inhibitor abrogates high-glucose-induced Nox2 expression and ROS production [286]. ROS derived from a Nox1-based Nox oxidase are required for vascular smooth muscle cell activation by AGEs [287]. Methylglyoxal, a precursor of AGEs, increases Nox4 and ROS production in cultured endothelial cells and vascular smooth muscle cells [251]. AOPPs promote vascular endothelial cell injury via RAGE- and Nox-dependent signalling pathways [288]. Specifically, the Nox subunits that seem to be involved in these events are Nox2 and Nox4, with AOPPs stimulating the association of Nox2 to p47phox [288]. AGE-modified LDL causes an increase in Nox1 and Nox4 expression that correlates with enhanced ROS formation and vascular smooth muscle cell injury [289]. Similarly, AGE-modified LDL promotes an increase in oxidative stress and Nox4 expression in endothelial cells, an effect enhanced by high glucose [290]. The induction of vascular endothelial cell injury markers by glycated LDL is mediated by a Nox2-containing oxidase [291]. Importantly, there is evidence for interactions between AGEs, RAS and Nox oxidases in the development of vascular cell injury [292].

PDGF, TGF-β and IGF-1 signalling are modulated by high glucose and are thought to play a key role in vascular pathology and the development of atherosclerosis [293–297]. Nox1-derived ROS are required for growth responses to PDGF in vascular smooth muscle cells exposed to high glucose or not [295]. It is worth noting that the homologue Nox5 was found to be critical for PDGF redox signalling in human vascular smooth muscle cells [298]. Nox4 mediates TGF-β-induced smooth muscle cell activation [299]. Additionally, up-regulation of Nox4 by TGF-β in smooth muscle cells from pre-diabetic rats (Zucker rats) is responsible for the impaired response to NO [247]. Interestingly, the ROS generated by Nox4 mediate high-glucose-induced enhancement of vascular smooth muscle cell responsiveness to IGF-1, including enhanced proliferation and migration [233].

Monocytes and macrophages are also important cells implicated in the development and progression of atherosclerosis in diabetes. Although Nox2 is a primary source of ROS in macrophages, Nox2 deficiency in these cells does not attenuate the atherosclerotic lesions in mice [300]. A critical role for Nox4 in macrophage and monocyte pathobiology has been described. Nox4 is required for the enhanced production of ROS triggered by oxidized LDLs that promote macrophage death, thereby involving monocyctic Nox4 in atherogenesis [301]. Furthermore, chronic exposure of monocytes to diabetic conditions mimicked by exposure of the cells to LDL and high glucose results in Nox4 up-regulation, and silencing of Nox4 prevents monocyte migration and macrophage recruitment [302]. Diabetic milieu also seems to regulate other Nox subunits since exposure of human monocyte-derived macrophages to high glucose stimulates Nox2 expression and cellular ROS production [303]. These effects are attenuated by incubation of the cells with HDL [303].

**NOX OXIDASES AS THERAPEUTIC TARGETS FOR DIABETIC COMPLICATIONS**

It is apparent from the present review that the in vivo and in vitro experimental evidence support a fundamental role for NADPH oxidases of the Nox family in the pathogenesis and pathophysiology of DN, DR, DCM and diabetes-associated macrovascular complications (Figure 1). The corollary of these observations is the consideration of the Nox homologues and their associated subunits as relevant therapeutic targets for the treatment of diabetic complications. There has been recently a considerable effort for the generation and development of agents able to inhibit the Nox enzymes in a homologue-specific manner [84,304–309]. The data obtained in the kidney, the heart, the retina and the macrovasculature described in the present review suggest that the catalytic subunits Nox4, Nox2 and Nox1 may be primary targets for these inhibitors. The search for the identification of inhibitors was...
generally centred on the discovery of agents that act on the catalytic subunits, rather than on the regulatory subunits since it is more difficult to design molecules that effectively alter protein-protein interactions. Among these inhibitors, the orally administrable small-molecule inhibitors from the Pyrazolo pyridine chemical series, referred to as GKT136901 and GKT137831, have drawn considerable attention. In contrast with most of the other compounds that have been described as Nox inhibitors but have not undergone or completed pre-clinical studies, intensive research is currently being conducted to test the bioefficacy of the GKT inhibitors in animal models of disease and they have recently been used in a Phase 1 clinical trial [13,308,310–313]. They are presented as dual Nox4 and Nox1 inhibitors with (to a lesser extent) some inhibitory actions on Nox5 and Nox2 [156,305,308,310]. Although the mechanisms by which these compounds inhibit Nox4 and Nox1 remain unclear, it is possible that they act as competitive inhibitors since their structures resemble NADPH [84]. However, the design of inhibitors that do not target such highly conserved regions and prevent the interaction of critical and unique components of the enzyme is necessary. With the enormous progress in nanotechnologies and delivery systems, there is seemingly great potential to develop inhibitors that may not be conventional small-molecule inhibitors.

Numerous pre-clinical studies performed with GKT136901 or GKT137831 in experimental animal models indicate that the inhibitors effectively attenuate the pathological changes observed in renal complication of diabetes, atherosclerosis, liver fibrosis and idiopathic pulmonary fibrosis [13,308,310–313]. Interestingly, that the efficacy of the dual Nox4/Nox1 inhibitors was primarily demonstrated in disease models for which the role of Nox4 as mediator of the pathologies is established (i.e. lung and liver fibrosis) [311,314,315] suggests that the protective actions of the compound may be predominantly due to the targeting of Nox4 in these conditions. Note that the effectiveness of Nox4 inhibition by GKT136901 is supported by recent in vivo studies in skeletal muscle. Indeed, GKT136901 is able to prevent overload-mediated hypertrophy in a context where Nox4 is the only Nox homologue accounting for the hypertrophic response [316]. Indeed, the data indicate that, in muscle only expressing Nox4 and Nox2, the hypertrophic effect of overload is abolished in Nox4-knockout mice or GKT136901-treated mice, but not in Nox2-deficient mice [316].

However, the perception of the role of Nox4 has become more complex in regards to the divergent data obtained in mouse models with Nox4 deletion or overexpression. Nox4 is reported to have a deleterious role in some experimental models and a protective function in others. Studies using transgenic mice overexpressing cardiac-specific active or inactive forms of Nox4, as well as mice with cardiac-specific deletion of Nox4, show that Nox4 up-regulation by aging or hypertrophic stimuli, including an acute model of pressure overload, leads to cardiac dysfunction [67] and that the oxidase is a major source of oxidative stress in the failing heart [206], thereby corroborating the injurious effects of Nox4. In contrast, another report that also employed mice with a genetic deletion of Nox4 or a cardiomyocyte-targeted overexpression of Nox4 demonstrates that the enzyme mediates protection against chronic load-induced stress in mouse hearts by enhancing angiogenesis [189]. Studies in global Nox4-deficient mice indicate that endogenous Nox4 may protect the vasculature during ischaemic and inflammatory stress [317]. In the kidney, some studies show that Nox4-knockout mice display exacerbated tubulointerstitial damage in UUO (unilateral ureteral obstruction), a model of renal fibrosis [173]. Interestingly, these studies suggest that, in the absence of Nox4, the global antioxidant defence may be decreased [173,317]. Experiments using global and inducible Nox4-knockout mice with murine models of kidney disease, such as streptozotocin-induced Type 1 diabetes, UUO and remnant kidney model, indicate that Nox4 does not promote renal disease, but may have a small protective effect against proteinuria, fibrosis and inflammation [318]. This indicates that the role of Nox4 depends on the model system used and seems to vary between in vivo experimentation and cultured cell models. It is relevant since most of the observations defining the deleterious actions of Nox4 or other Nox oxidases rely on in vitro studies.

In the heart, it is proposed that the divergences observed are due to the fact that the models used are challenging the cardiac function with different time course [304]. Hence Nox4 may be acutely harmful to cardiac myocytes [67] and be chronically beneficial to the heart function via facilitation of angiogenesis that has time to take place [189]. In addition, the magnitude of the pressure overload used in the two studies was different, suggesting that the function of Nox4 may vary with the severity of the pathology. These aspects should be taken into account when considering the role of Nox4 in the pathogenesis of the complications of diabetes. Indeed, it is conceivable that the role of Nox4 varies in the function of the stages and the duration of the disease, i.e. early or advanced diabetes, as well as the severity of the disease at these different phases of diabetic complications.

Additionally, another factor susceptible to critically influence the outcome of the work with Nox4-knockout mice and may be responsible for conflicting data is the type of experimental animal model of diabetes that is used. For instance, the severity of the renal damages seen in DN differs considerably in the different mouse models of Type 1 (OVE26, Akita mice and streptozotocin-induced diabetes models) or Type 2 diabetes (db/db and BTBR ob/ob mice) [319,320]. Importantly, similar variations exist for mice backgrounds with some animals, such as C57BL6 mice the background of most of the Nox4-deficient mice described in the literature, being more resistant to DN than others [319,320]. The fact that few murine models of DN progress to develop advanced lesions [319,320] may also weaken the possibility of understanding the function of Nox4 over the course of the disease and whether Nox4 exerts a particular role at these time points may not be revealed.

It should also be pointed out that the discrepancy between the studies could be due to the type of genetic manipulations performed to generate the global or cell-specific Nox4-deficient mice. For instance, podocyte-specific genetic reduction of mTORC1 (mTOR complex 1)-associated raptor (regulatory associated protein of mTOR) gene by ablation of two alleles in diabetic mice results in worsening of renal function. In contrast, ablation of one allele leads to renal protection, revealing that excessive raptor/mTORC1 inhibition aggravates pathology probably due to the fact that the enzyme is required for podocyte integrity [321].
If Nox4 is essential for maintaining basal functions, similar issues could occur for the mouse models with genetic manipulation of the oxidase and especially the mice where the Nox4 gene is completely deleted. These problems may not happen in experimental animal models where Nox4 is targeted with antisense oligonucleotides or siRNA.

All of these observations are a critical reminder of the highly artificial character of the genetic mouse models in which the Nox4 gene is knocked out or Nox4 is overexpressed and that these conditions are far from representing human conditions. Therefore, although it is certain that the studies reporting that Nox4 play a protective role clearly raise salutary questions about the approaches based on Nox4 inactivation and the clinical relevance of using Nox4 inhibitors, we believe that there is still interest in developing Nox inhibitors as therapeutic targets for the treatment of diabetic complications in humans, especially in regard to the promising outcome of the pre-clinical studies performed so far. Note that the potential caveats linked to the utilization of these inhibitors may be overcome by the fact that studies indicate that treatment of normal mice with these inhibitors does not interfere with physiological processes and also the observation that Nox4-knockout mice have no phenotype under normal conditions [304]. However, it is important to point out the potential limitation of the GKT inhibitors as being non-specific and their potential to inhibit a widely described role of Nox4 in the differentiation of multiple cardiovascular cells [56,63,70]. This would appear to have serious repercussions for tissue homeostasis. There is no ultimate proof that these inhibitors are safe and the information existing related to the role of Nox4 as a pro-differentiation factor in various cell types and thus the potential for neoplastic effects should be taken into account.

Beside the direct inhibition of Nox oxidases, the present review suggests that adjunct therapies targeting the agonists or signalling intermediates that regulate the expression or function of Nox subunits and subsequent ROS production should be considered for the treatment of diabetic complications. In regards to the observation reported above, such strategies could involve the use of PKC inhibitors, agents that disrupt the AGE signalling, inhibitors of the RAS (ACEis and ARBs), statins (that inhibit Rac1 and Rho), Rho/Rho kinase inhibitors, AMPK activators (i.e. metformin) and cholesterol absorption inhibitor (ezetimide), as well as PPARγ activators (pioglitazone or rosiglitazone).

Figure 2 shows the possible therapeutic approaches that could target Nox oxidases expression and activity in the treatment of diabetic complications in the kidney, retina, heart, blood vessels and vasculature.

**CONCLUSIONS**

Although significant progress has been made in the investigation of the role of Nox oxidases in the complications of diabetes that manifest in the kidney, retina and macrovasculature, there
is still a need for more direct and less circumstantial evidence establishing which Nox homologues and subunits are involved in redox-dependent pathologies in various tissues. This is particularly important given the conflicting data obtained from studies with transgenic and knockout mice. It is ironic that most of the discrepancies seen in these models are related to Nox4, the Nox homologue for which more causal evidence supporting the role in DR, DN and DCM is available. Nevertheless, it is clear that diabetes and hyperglycaemia are strong stimulators of Nox-dependent ROS generation in the macrovascular and microvascular complications of diabetes. The understanding of the individual role of Nox homologues in the different molecular mechanisms and signalling cascades altered should be pursued concomitantly with the translation to therapeutic interventions. The arrival of homologue-specific Nox inhibitors will be instrumental in characterizing the function of Nox homologues in the diabetic environment. The wide range of Nox actions in pathological processes imply that the therapeutic potential of these inhibitors for the treatment of diabetic complications could be considerable.

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Nox as a target for diabetic complications


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