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Oxidative Stress, Nox Isoforms and Complications of Diabetes—Potential Targets for Novel Therapies

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Abstract Most diabetes-related complications and causes of death arise from cardiovascular disease and end-stage renal disease. Amongst the major complications of diabetes mellitus are retinopathy, neuropathy, nephropathy and accelerated atherosclerosis. Increased bioavailability of reactive oxygen species (ROS) (termed oxidative stress), derived in large part from the NADPH oxidase (Nox) family of free radical producing enzymes, has been demonstrated in experimental and clinical diabetes and has been implicated in the cardiovascular and renal complications of diabetes. The present review focuses on the role of Noxs and oxidative stress in some major complications of diabetes, including nephropathy, retinopathy and atherosclerosis. We also discuss Nox isoforms as potential targets for therapy.

Keywords Diabetic nephropathy · Cardiovascular disease · NADPH oxidase · Diabetes mellitus · Nox isoforms

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Introduction

Type 2 diabetes is the leading cause of blindness, nontraumatic lower-limb amputation, chronic kidney disease and a major cause of cardiovascular disease, leading to early death [1,2]. With the prediction that the number of individuals with type 2 diabetes will more than triple by 2050 in the USA, with similar trends globally [1–3], there is an urgent need to better understand the processes underlying diabetes-associated complications so that new strategies can be developed to prevent and better manage these conditions. Of the many factors implicated in cardiovascular and renal complications of diabetes, increased ROS bioavailability (oxidative stress) may be particularly important. As such, approaches to reduce ROS generation may have therapeutic potential in complications associated with diabetes.

Many experimental models of both type 1 and type 2 diabetes mellitus exhibit increased cardiovascular and renal ROS generation, triggered in large part by hyperglycaemia [4,5] (Fig. 1). Clinically, patients with diabetes have increased plasma levels of ROS biomarkers such as TBARS, 8α isoprostanes and oxidised LDL, and reduced levels of antioxidant agents and enzymes, such as bilirubin, superoxide

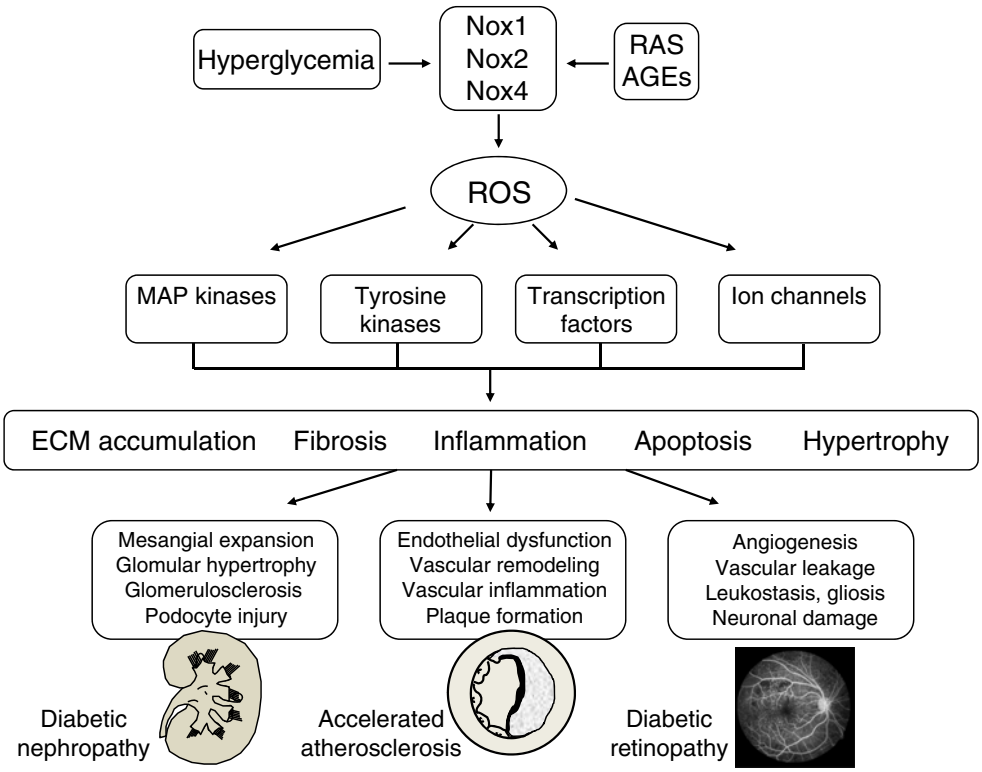

Fig. 1 Schematic of mechanisms whereby Nox-derived reactive oxygen species (ROS) influence development of diabetes-associated complications. The hyperglycaemic milieu, including activation of the renin–angiotensin system (RAS) and production of advanced glycation end products (AGEs), results in an increased activity of Nox isoforms generating increased ROS production, which in turn stimulates signalling pathways associated with cell growth, inflammation, apoptosis and fibrosis. These events contribute to processes underlying nephropathy, atherosclerosis and retinopathy

dismutase and antioxidant vitamins, thus promoting oxidative stress [6–8]. Diabetes-associated factors that increase oxidative stress include hyperglycaemia, formation of advanced glycation end-products (AGEs), activation of the polyol pathway, elevated free fatty acids and increased leptin levels [9,10]. In addition, activation of the renin–angiotensin system (RAS) in diabetes stimulates ROS generation [11] (Fig. 1).

NADPH Oxidase Isoforms, Reactive Oxygen Species and Diabetes

Of the many ROS-generating enzymatic systems, including xanthine oxidase, mitochondrial oxidases and uncoupled NOS, activation of NADPH oxidase is particularly important in diabetes [12,13]. NADPH oxidase catalyses the production of O_2^- by the one-electron reduction of O_2 using NADH or NADPH as the electron donor. Phagocytic NADPH oxidase, the prototype oxidase, is involved in host-defence processes, and comprises membrane-bound gp91phox (Nox2) and p22phox and three cytosolic regulators (p47phox, p67phox, p40phox) [14]. In activated cells, p47phox is phosphorylated, leading to translocation to the membrane and complex formation and association with cytochrome b558 to assemble the active enzyme. Recently, seven novel Nox2 homologues have been identified, including Nox1–7 [15–17], of which Nox1, 2 and 4 levels and/or activity are altered by diabetes, or associated with diabetic complications. All Noxs contain a six-transmembrane domain, and conserved motifs for binding NADPH and FAD and two hemes. The exact (patho)physiological functions of Noxs still remain unclear. However, Nox hyperactivation leads to excessive ROS generation that disrupts redox networks, normally regulated by antioxidant systems, resulting in oxidative stress, which triggers molecular processes contributing to tissue injury.

Nox1 abundance is normally low, but is induced by stimuli such as PDGF, Ang II and glucose and is increased in pathological conditions [18,19]. Nox1 localises with p22phox and requires NADPH oxidase subunits for its activation. Studies from Nox1 knockout mice suggest a possible role for Nox1 in acute, but not chronic, Ang II-mediated hypertension [20,21] and in atherosclerosis, hepatic fibrosis and cancer [22–24]. Nox1 has also been implicated in pancreatic islet beta cell dysfunction and is upregulated in diabetes [25]. Nox2, the phagocytic gp91phox-containing NADPH oxidase, is expressed in non-phagocytic cells including vascular, renal, cardiac and neural cells. Non-phagocytic vascular and renal Nox2 are activated by Ang II and stretch, through c-Src, PKC and EGFR, and by high glucose [26,27]. Phagocytic-like NADPH oxidase activation and increased ROS generation have been demonstrated in Zucker diabetic fatty rat and islets from patients with diabetes [28]. Nox3 is found in foetal tissue and the adult inner ear and is involved in vestibular function [29]. Although there is very little information about Nox3 beyond its function in the ear, a genome wide scan in West Africans with type 2 diabetes showed that

Nox1 (10q22) and Nox3 (6q25.1–q26) are candidate genes associated with diabetic nephropathy [30]. Increased Nox3 gene expression has also been demonstrated in brain nuclei in experimental hypertension [31].

Nox4 is most abundantly expressed in the kidney, hence originally termed renal oxidase (Renox) [32]. Nox4 is distributed in the renal vasculature, mesangial cells, podocytes and tubular cells, and appears to be the major enzymatic source of renal ROS, and as such may play a role in oxidative stress associated with kidney disease in diabetes [33,34]. Similar to Nox1, Nox4 requires cytosolic subunits for it regulation, although exact subunits and mode of regulation are still unclear. p22phox seems to be important and the p47phox and p67phox homologues, NOX organiser 1 [NOXO1($p41^{nox}$)] and NOX activator 1 [NOXA1 ($p51^{nox}$)] may be involved. Nox4 is also regulated by Polydip2, which links Nox4 to the cytoskeleton in vascular cells. In VSMCs, Nox4 co-localises with p22phox and vinculin in focal adhesions and has been implicated in cell migration, proliferation, angiogenesis and cell differentiation [35,36]. Unlike Nox1 and Nox2, Nox4 is constitutively active, producing mainly H_2O_2 rather than $\bullet O_2^-$ [37]. The difference in the type of ROS generated may underlie Nox-specific actions in signalling. Nox4 contributes to basal ROS production through its constitutive activity and to increased ROS generation when stimulated. The pathological role of Nox4 is unclear. It has been suggested to be involved in both vascular injury and protection, and in BP elevation and lowering [38–41]. Growing evidence indicates a role for Nox4 in diabetes-associated complications, including nephropathy, cardiac injury, stroke and atherosclerosis as well as in insulin resistance [33,42–45].

Nox5 (five splice variants—α, β, δ, γ, ε) is the most recently identified Nox and is unique: it is Ca^{2+} sensitive, it possesses a calmodulin-like domain with binding sites for Ca^{2+}, it does not require any NADPH oxidase subunits for its activity and is found in testes, spleen and vascular cells with Nox5α and Nox5β being the ROS generating vascular isoforms [46,47]. Vascular Nox5 is activated by PDGF, Ang II and ET-1 [48,49]. The exact function of vascular Nox5 is unknown, but may regulate growth and migration and has been implicated in atherosclerosis [50]. Unlike Nox1, Nox2, Nox3, Nox4 and Duox1/2 genes, which are present in rodents, Nox5 gene is absent, making it challenging to study the biology of Nox5 in experimental rodent models.

Duox1 and -2, also known as Nox6 and Nox7, are involved in production of thyroid hormone, which requires H_2O_2 for its synthesis [51]. Mutations of duox are associated with congenital hypothyroidism [51]. To our knowledge, there is no evidence that the duoxs are involved in cardiovascular disease or diabetes.

Diabetic Nephropathy, Noxs and Oxidative Stress

Diabetic nephropathy (DN) is a major microvascular complication of diabetes and is the leading cause of end-stage renal disease (ESRD) requiring dialysis and/or transplantation. Diabetic nephropathy is characterised by a progressive increase in albuminuria and proteinuria and a decline in glomerular filtration rate (GFR), which often occur in association with an increase in blood pressure, ultimately leading to ESRD [52]. These renal functional changes are associated with structural abnormalities, including glomerular basement membrane thickening, mesangial expansion with extracellular matrix (ECM) accumulation and glomerulosclerosis [53]. Furthermore, changes in glomerular epithelial cells are associated with a decreased number and/or density of podocytes and podocyte foot process broadening and effacement. Tubulointerstitial accumulation of ECM proteins leads to tubulointerstitial fibrosis which closely correlates with the development of ESRD. Diabetic nephropathy is accompanied by increased generation of ROS in the kidney, due in large part to overactivation of renal Nox 1, 2 and 4 [53].

Increased Nox-mediated superoxide production has been reported in experimental models of diabetes and occurs in parallel with upregulation of Noxs, especially Nox1 and Nox4 [45,53]. Nox-mediated generation of superoxide is an important mediator of matrix accumulation, renal fibrosis and podocyte injury in DN [54].

Expression of glomerular Nox4 increases in STZ-induced diabetic rats causing oxidative damage to the glomeruli, an effect attenuated by insulin treatment [55]. During early stages of diabetes, Nox4-derived ROS mediates renal hypertrophy and increases fibronectin expression. Treatment with antisense oligonucleotides to Nox4 reduced NADPH oxidase-dependent ROS generation in the renal cortex, decreased fibronectin expression with less ECM accumulation and attenuated renal hypertrophy in diabetic rats [53].

Regulation of Noxs in Diabetic Nephropathy

NADPH oxidase in the kidney can be activated by membrane translocation of p47phox via PKC mediated phosphorylation that is upregulated by renal Ang II or by high glucose [56,57]. The molecular link between PKC-β and Noxs is further suggested in PKC-β-deficient mice. Diabetic PKC-β-deficient mice have less proteinuria and are protected from renal hypertrophy, glomerular enlargement and hyperfiltration observed in diabetic wild-type mice. These changes are associated with decreased NADPH oxidase activity accompanied by decreased mRNA expression of p47phox, Nox2 and Nox4 in the renal cortex, suggesting that reduced activity of PKC-β may protect against increased expression of NADPH oxidase subunits in diabetes

[58]. Involvement of AGEs in the activation of a PKC-α-NADPH oxidase pathway has also been reported [59].

ROS derived from Noxs under high glucose can act as second messengers for several transcription factors and signal transduction cascades that are implicated in renal disease and hypertension [53], such as nuclear factor-κB (NF-κB), activator protein-1 (AP-1), serine–threonine kinase Akt/PKB, p38 mitogen-activated protein kinase (p38MAPK) and extracellular-signal-related kinases 1 and 2 (ERK1/2) [60,61]. Oxidative stress increases NADPH oxidase expression and inflammation via activation of NF-κB in renal interstitial cells [62]. NF-κB regulates expression of monocyte chemoattractant protein-1 (MCP-1) in mesangial cells [63], while AP-1 mediates a high glucose-induced increase in TGF-β1 gene promoter activity [64].

Noxs and Proteinuria

Podocyte apoptosis mediated by oxidative stress is an early event in DN that contributes to progressive podocyte depletion and albuminuria [65]. In the presence of high glucose, overproduction of podocyte ROS induces cellular dysfunction and increases albumin excretion. NADPH oxidase is involved in apoptosis of podocytes through proapoptotic p38MAPK and caspase 3 in type 1 and 2 diabetic mice [66] and through generation of ROS via upregulation of CYP4A, Nox1 and Nox4 [54]. High glucose has also been shown to stimulate hypertrophy of podocytes through ROS-dependent activation of ERK1/2 and Akt/PKB pathways [65]. The systemic administration of apocynin, a non-specific NADPH oxidase inhibitor, prevented podocyte apoptosis and ameliorated urinary albumin excretion in diabetic mice [65].

Diabetic Retinopathy, Noxs and Oxidative Stress

Diabetic retinopathy (DR) is the major cause of vision loss and blindness in people of working age, and develops over approximately 10 to 25 years. A recent meta-analysis examining the global prevalence and major risk factors for DR and vision-threatening diabetic retinopathy (VTDR) among 22,896 people with diabetes reported that the overall prevalence was 34.6% for any DR and 10.2% for VTDR [67]. All DR prevalence end points increased with diabetes duration, haemoglobin A(1c) and blood pressure levels, and were higher in people with type 1 compared with type 2 diabetes. DR is largely a disease of the retinal microvasculature, although damage to neurons and glia also occurs [68]. Diabetic retinopathy progresses from the mild non-proliferative stage which features increased vascular permeability, to the moderate and severe non-proliferative stage characterised by blood vessel closure and tissue ischaemia, to the proliferative stage characterised by pathological angiogenesis in the retina often extruding into the vitreous [68]. Vision loss principally occurs from macular oedema, tractional retinal detachment and inner retinal and vitreous haemorrhage. Oxidative stress may be a key contributor to the development of diabetic complications of the eye [68]. The retina is particularly susceptible to oxidative stress because of its high consumption of oxygen and glucose oxidation, high levels of polyunsaturated fatty acids and exposure to light [69]. A major source of retinal ROS is likely to be Nox, although this is yet to be fully explored. Evidence to date indicates that Nox isoforms are widely distributed throughout the retina and upregulated by hyperglycaemia and hypoxia. Nox2 immunolabelling has been identified in retinal endothelial cells [70], Nox 1, 2 and 4 mRNA are present in cultured retinal endothelial cells [71] and ganglion cells [72] and Nox4, Duox1 and Duox2 have been immunolocalised to photoreceptors [73]. Nox2 inhibition has neuroprotective effects in ischaemic injury in the retina [74].

Diabetic retinopathy is a low-grade inflammatory condition [75]. Leukocytes may contribute to microvascular damage by releasing cytokines, inflammatory factors and ROS. Additionally, leukocytes by binding to the vascular endothelium contribute to leukostasis and areas of retinal non-perfusion and subsequent ischaemia. Of interest is that Nox inhibition reduced retinal leukostasis in experimental DR and activated leukocytes stimulated angiogenesis by their adherence to endothelial cells via E-selectin, ICAM-1 and hydrogen peroxide [76]. The inflammatory enzyme, core 2 β-1, 6-N-acetylglucosaminyltransferase (C2GNT) may contribute to DR by eliciting intracellular cross-talk between tumour necrosis factor-α, PKC β1/2 and Nox in human leukocytes to result in leukocyte adhesion to endothelial cells [76].

Nox Isoforms and Diabetic Retinopathy

To date, most studies have focused on the role of Nox2 and Nox4 in retinal inflammation and vascular leakage. Using immunohistochemical techniques, Nox2 has been localised to retinal blood vessels and Nox2 and p47phox were reported increased in retina in experimental DR in association with increased oxidative stress [70,77–79]. In Nox2 knockout mice, retinal ICAM-1 expression and leukostasis are reduced in DR [80]. Moreover, apocynin and Nox2 deletion were equally effective in preventing diabetes-induced increase in retinal ICAM-1 and leukostasis [81]. Nox activity may be required for increased chemokine ligand 2 (CCL2) production in DR and may involve Akt and NF-κB signalling pathways [82]. Finally, there is evidence that PPARγ is involved in ROS derived from Nox2 effects in experimental DR since apocynin or deletion of Nox2 prevented the reduction in retinal PPARγ levels [83].

In terms of Nox4, to our knowledge, only one study to date has evaluated its role in experimental DR. In db/db mice, a model of type 2 diabetes, expression of Nox4 and VEGF was significantly increased in retina and reduced with the hypolipidemic agent, lovastatin, and Nox inhibition [84]. Intravitreal delivery of Nox4 siRNA decreased retinal Nox4 and VEGF expression, Nox activity and retinal vascular permeability [84]. Studies in bovine retinal endothelial cells exposed to high glucose elicited similar results suggesting that Nox4 may contribute to the vascular dysfunction of early DR [84].

Noxs, Ang II and AGEs in Retinopathy

The renin–angiotensin system (RAS) and AGEs have been implicated in the development of DR [85]. Clinical studies have shown that RAS blockade attenuates retinal pathology [86]. Ang II is a known stimulator of Nox and therefore it would be reasonable to expect that damaging effects of Ang II in the retina may be due, in part, to Nox-derived ROS. Although the relationship between Ang II and specific Nox isoforms in the retina has not been comprehensively studied, there is evidence that intravitreal administration of Ang II increases retinal leukostasis, superoxide formation and Nox expression [87], and that these changes are decreased with apocynin. In spontaneously diabetic Torii rats, a type 2 diabetic model, VEGF and p22phox were elevated in retina and reduced with the RAS blocker, candesartan. Furthermore, Ang II infusion increases levels of VEGF and p22phox in retina [87].

Oxidative stress is closely linked to the formation of AGEs and AGE-induced oxidative stress may be partly involved in the detrimental effects of AGEs in the retina. Whether there is interplay between ROS derived from Nox and AGE-related damage in DR is not completely understood. However, AGEs increased ROS generation and VEGF expression in cultured bovine retinal endothelial cells through PKC-dependent activation of Nox [88]. Pigment epithelium-derived factor (PEDF) which has anti-angiogenic, anti-oxidative and anti-inflammatory properties in the retina has also been linked to Nox and AGEs. In endothelial cells, PEDF decreased ROS generation in AGE-exposed retinal endothelial cells by suppressing Nox activity via downregulation of mRNA levels of p22phox and gp91phox. These events were associated with blockade of AGE-elicited actions and NF-κB-dependent VEGF gene induction [89]. Moreover AGE, ROS and Ang II may be interlinked since Ang II modulates expression of retinal glyoxalase I, a key enzyme in the detoxification of AGEs [90].

Diabetes-Associated Atherosclerosis, Noxs and Oxidative Stress

Patients with diabetes have accelerated development of atherosclerosis, predisposing them to a greater risk of stroke, myocardial infarction and peripheral vascular disease. This acceleration in vascular disease is mediated via haemodynamic and metabolic pathways associated with the hyperglycaemic milieu. Direct mechanisms leading to this acceleration in atherogenesis are not clearly identified. However, enhanced production of ROS and reduced antioxidant defences appear to play pivotal roles. The primary source of ROS production in the vasculature is through Noxs, which show enhanced activity in high glucose conditions. Furthermore, diminished activity in the antioxidant defence system has been demonstrated in high glucose conditions.

Nox Isoforms and Diabetes-Associated Atherosclerosis—Lessons from Mouse Models

Studies using mice in which NADPH oxidase subunit/Nox isoform genes were deleted demonstrated a role for Noxs in atherosclerosis. Deletion of the p47phox subunit of the Nox1 and Nox2 complex followed by crossing with the ApoE$^{-/-}$ mouse model of atherosclerosis resulted in a reduction in the development of atherosclerosis irrespective of the diet and without changes in serum lipid concentrations [91]. Disruption of the p47phox gene reduced superoxide production in the vessel wall and inhibited proliferation of vascular smooth muscle cells [91]. These data highlight an important role for p47phox in the development of atherosclerosis. However, they failed to differentiate the specific Nox isoform involved in the development of atherosclerosis.

Nox2 deletion in ApoE$^{-/-}$ mice on high fat diet reduced plaque area in the total aorta, with decreased aortic ROS production [92], suggesting a Nox2-mediated response in the development of atherosclerosis. Regarding Nox1, a causal association has been demonstrated between Nox1 and vascular remodelling in Ang II-infused mice [93] and in neointima formation [93]. Mice deficient in both ApoE and Nox1 on an atherogenic diet for 18 weeks demonstrated reduced atherosclerosis, decreased macrophage infiltration and smaller lesion size at the level of the aortic valve compared with apoE$^{-/-}$ with intact Nox1 [94]. These data suggest that Nox1-derived ROS may modify lesion composition and contribute to atherogenesis, a process which may be accelerated in diabetes [94].

Nox4 may also be important in atherosclerosis and dyslipidemia associated with diabetes. A detailed electron microscopy study investigating the ultrastructural properties of smooth muscle cells and Nox4 in healthy and atherosclerotic human aorta showed a heterogeneous population of smooth muscle cells (SMC) in the atheromatous lesions with associated alteration in Nox4 distribution. In the disease samples, Nox4 expression is preserved in the SMCs that maintain the contractile phenotype, but disappears in the de-differentiated cells that display a myofibroblastic

appearance [95]. Examination of insulin targets in the liver revealed that in experimental models of type 2 diabetes, there is a specific defect in the ability of the Nox4 to inactivate protein tyrosine phosphatase gene family members after stimulation with insulin, which may impact on atherogenesis and vascular injury in diabetes [96].

Endothelial dysfunction, vascular remodelling and inflammation are pivotal in the promotion of atherosclerosis as this leads to infiltration of monocytes and secretion of chemokines such as PDGF and Ang II, which mediate further production of ROS through Nox1 and Nox4 [97,98]. While an increased production of H_2O_2 may be seen as being deleterious, recent studies have suggested a potential protective role for Nox4-derived H_2O_2 on vascular injury during ischaemia, hypertension or inflammatory stress [38–41]. These contrasting effects warrant further clarification.

Nox Inhibitors and Diabetes-Associated Complications

Based on experimental evidence, NADPH oxidase subunits and Nox isoforms are potential therapeutic targets for cardiovascular disease and hypertension. The first pharmacological agents used as NADPH oxidase inhibitors were apocynin and diphenyliodonium (DPI) [99,100]. However, these compounds have been shown to have NADPH oxidase-independent effects and are not Nox-specific isoforms [101,102]. As such, DPI and apocynin are now regarded as oxidative stress suppressers rather than specific inhibitors of NADPH oxidase. More recently, there has been interest in the development of agents that inhibit Noxs in an isoform-specific manner [103,104]. Different strategies have been employed, including small molecule inhibitors, peptide NADPH oxidase inhibitors and siRNA. Several compounds have been registered as NADPH oxidase inhibitors [103,104]. However, none have yet undergone clinical trials, and some have not even completed preclinical studies. However, GKT has recently been tested in a phase 1 clinical trial [105]. The outcomes of this study should reveal interesting information about safety data and pharmacokinetic profiles.

To date, two different classes of compounds have been claimed as potent and orally active bioavailable NADPH oxidase inhibitors: pyrazolopyridines, such as GKT136901 and GKT137831 [106,107], and triazolopyrimidine derivatives, such as VAS2870 and VAS3947 [108,109]. Pyrazolo pyridine derivative compounds such as GKT136901 specifically inhibit Nox1/Nox4-mediated ROS production [106,107]. Although the mechanisms whereby GKT compounds inhibit Nox1/4 have not yet been clarified, they may act as competitive substrate inhibitors since structurally they resemble NADPH. VAS3947 inhibits Nox activity in vascular smooth muscle cells and human umbilical vein endothelial cells [108,109]. Furthermore, it has been shown to inhibit the H_2O_2 burst at the margins of wounds without obvious toxicity in zebra fish, suggesting that it may inhibit Nox4 activity [110]. VAS3947 inhibits H_2O_2 production in addition to inhibiting the production of superoxide and peroxynitrate [102]. These inhibitors seem to target multiple isoforms of the Nox family and may not be highly Nox-specific.

Nox Inhibitors as Potential Therapeutic Agents in Clinical Medicine

Although several compounds have been registered as NADPH oxidase inhibitors [103,104], none have yet undergone clinical trials in a disease-targeted manner, and some have not even completed preclinical studies. However, GKT137831 has recently been tested in a phase 1 trial [105]. The outcomes of this study should reveal interesting information about safety data and pharmacokinetic profiles and should shed light on the potential use of Nox inhibitors in human disease.

Based on the recent findings in experimental models that some Nox isoforms, e.g. Nox4, may have protective functions [38–41] and that inhibiting such Noxs may cause injury, there is much speculation as to the clinical utility of such approaches. However, it should be kept in mind that these studies were conducted in transgenic mice where Nox4 was overexpressed or in mice in which Nox4 gene was knocked out. These are highly artificial models and although they may be interesting to dissect out functions of Nox4, they do not represent human conditions. As such, there is still interest in developing Nox inhibitors in an isoform-specific manner for potential clinical use. Moreover, it may be more appropriate to consider the pharmacological inhibitors as Nox modulators rather than inhibitors because Nox activity seems to be only partially inhibited in vivo.

Pathological conditions in which Nox4 inhibitors have been suggested to have potential clinical benefit include pulmonary hypertension, chronic kidney disease, hepatic disease, diabetic nephropathy and malignant melanoma [111]. Nox1 inhibition may be useful in the treatment of cancer, inflammatory conditions and hepatic disease [112]. However, much research is still needed before such approaches can be considered clinically.

In addition to direct blockade of Nox activation, agents that indirectly reduce oxidative stress may be useful approaches for managing cardiovascular complications of diabetes. Such approaches include PKC inhibitors, pharmacological agents that block the AGE–RAGE axis and drugs that interrupt the RAS, such as angiotensin-converting

enzyme inhibitors and angiotensin receptor blockers. Statins, which inhibit activity of Nox4, may also be a useful strategy to reduce Nox-induced ROS generation.

Conclusions

While there is growing evidence that Nox activation and oxidative stress are important in the development of diabetes-associated complications, including nephropathy, retinopathy and accelerated atherosclerosis, there is still a paucity of information on exactly which Nox isoform generate ROS and what mechanisms regulate Noxs in different tissues in diabetes. However, what is clear is that the hyperglycaemic milieu in diabetes is a potent stimulus for Nox activation and oxidative stress in cardiovascular and renal tissue. The advent of inhibitors of specific Nox isoforms is likely to provide greater insight into the functions of Noxs in diabetes. Isoform-specific Nox inhibitors may be potentially useful agents in managing complications of diabetes. However, much research is still needed before such strategies can be instituted.

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References

1. Emerging Risk Factors Collaboration, Sarwar, N., Gao, P., Seshasai, S. R., Gobin, R., Kaptoge, S., et al. (2010). Diabetes mellitus, fasting blood glucose concentration, and risk of vascular disease: a collaborative meta-analysis of 102 prospective studies. *Lancet, 375*, 2215–2222.
2. Centers for Disease Control and Prevention. National diabetes fact sheet: national estimates and general information on diabetes and prediabetes in the United States, (2011). Atlanta: Department of Health and Human Services, Centers for Disease Control and Prevention.
3. Ismail-Beigi, F. (2012). Clinical practice. Glycemic management of type 2 diabetes mellitus. *The New England Journal of Medicine, 366*(14), 1319–1327.
4. Tikellis, C., Pickering, R.J., Tsorotes, D., Du, X.J., Kiriazis, H., Nguyen-Huu, T.P., Head, G.A., Cooper, M.E., Thomas, M.C. (2012) The interaction of diabetes and ACE2 in the pathogenesis of cardiovascular disease in experimental diabetes. Clinical Science (London) [Epub ahead of print].
5. Huang, A., Yang, Y. M., Feher, A., Bagi, Z., Kaley, G., & Sun, D. (2012). Exacerbation of endothelial dysfunction during the progression of diabetes: role of oxidative stress. *American Journal of Physiology—Regulatory, Integrative and Comparative Physiology, 302*(6), R674–R681.
6. Chang, C.M., Hsieh, C.J., Huang, J.C., Huang, I.C. (2012). Acute and chronic fluctuations in blood glucose levels can increase oxidative stress in type 2 diabetes mellitus. Acta Diabetologia. 2012 May 1. [Epub ahead of print].
7. Tang, W. H., Martin, K. A., & Hwa, J. (2012). Aldose reductase, oxidative stress, and diabetic mellitus. *Frontiers in Pharmacology, 3*, 87–90.
8. Whaley-Connell, A., Sowers, J.R. (2012). Oxidative stress in the cardiorenal metabolic syndrome. Current Hypertension Reports. May 13. [Epub ahead of print].
9. Coughlan, M. T., Patel, S. K., Jerums, G., Penfold, S. A., Nguyen, T. V., Sourris, K. C., et al. (2011). Advanced glycation urinary protein-bound biomarkers and severity of diabetic nephropathy in man. *American Journal of Nephrology, 34*(4), 347–355.
10. Jay, D., Hitomi, H., & Griendling, K. K. (2006). Oxidative stress and diabetic cardiovascular complications. *Free Radical Biology & Medicine, 40*(2), 183–192.
11. Luther, J. M., & Brown, N. (2011). The renin–angiotensin–aldosterone system and glucose homeostasis. *Trends in Pharmacological Sciences, 32*(12), 734–739.
12. Shen, G. X. (2010). Oxidative stress and diabetic cardiovascular disorders: roles of mitochondria and NADPH oxidase. *Canadian Journal of Physiology and Pharmacology, 88*(3), 241–248.
13. Thum, T., Fraccarollo, D., Schultheiss, M., Froese, S., Galuppo, P., Widder, J. D., et al. (2007). Endothelial nitric oxide synthase uncoupling impairs endothelial progenitor cell mobilization and function in diabetes. *Diabetes, 56*(3), 666–674.
14. Babior, B. M. (2004). NADPH oxidase. *Current Opinion in Immunology, 16*(1), 42–47.
15. Al Ghouleh, I., Khoo, N. K., Knaus, U. G., Griendling, K. K., Touyz, R. M., Thannickal, V. J., et al. (2011). Oxidases and peroxidases in cardiovascular and lung disease: new concepts in reactive oxygen species signaling. *Free Radical Biology & Medicine, 1*, 1271–1288.
16. Dworakowski, R., Alom-Ruiz, S. P., & Shah, A. M. (2008). NADPH oxidase-derived reactive oxygen species in the regulation of endothelial phenotype. *Pharmacological Reports, 60*(1), 21–28.
17. Sirker, A., Zhang, M., & Shah, A. M. (2011). NADPH oxidases in cardiovascular disease: insights from in vivo models and clinical studies. *Basic Research in Cardiology, 106*(5), 735–4.
18. Redmond, E. M., & Cahill, P. A. (2012). The NOX–ROS connection: targeting Nox1 control of N-cadherin shedding in vascular smooth muscle cells. *Cardiovascular Research, 93*(3), 386–390.
19. Dikalova, A. E., Góngora, M. C., Harrison, D. G., Lambeth, J. D., Dikalov, S., & Griendling, K. K. (2010). Upregulation of Nox1 in vascular smooth muscle leads to impaired endothelium-dependent relaxation via eNOS uncoupling. *American Journal of Physiology—Heart and Circulatory Physiology, 299*(3), H673–H679.
20. Bayat, H., Schröder, K., Pimentel, D. R., Brandes, R. P., Verbeuren, T. J., Cohen, R. A., et al. (2012). Activation of thromboxane receptor modulates interleukin-1β-induced monocyte adhesion—a novel role of Nox1. *Free Radical Biology & Medicine, 52*(9), 1760–1766.
21. Yogi, A., Mercure, C., Touyz, J., Callera, G. E., Montezano, A. C., Aranha, A. B., et al. (2008). Renal redox-sensitive signaling, but not blood pressure, is attenuated by Nox1 knockout in angiotensin II-dependent chronic hypertension. *Hypertension, 51*(2), 500–506.
22. Paik, Y. H., Iwaisako, K., Seki, E., Inokuchi, S., Schnabl, B., Osterreicher, C. H., et al. (2011). The nicotinamide adenine dinucleotide phosphate oxidase (NOX) homologues NOX1 and NOX2/gp91(phox) mediate hepatic fibrosis in mice. *Hepatology, 53*(5), 1730–1741.

23. Yasuda, M., Kato, S., Yamanaka, N., Iimori, M., Utsumi, D., Kitahara, Y., et al. (2012). Potential role of the NADPH oxidase NOX1 in the pathogenesis of 5-fluorouracil-induced intestinal mucositis in mice. *American Journal of Physiology—Gastrointestinal and Liver Physiology, 302*(10), G1133–G1142.
24. Sheehan, A. L., Carrell, S., Johnson, B., Stanic, B., Banfi, B., & Miller, F. J., Jr. (2011). Role for Nox1 NADPH oxidase in atherosclerosis. *Atherosclerosis, 216*(2), 321–326.
25. Youn, J.Y., Gao, L., Cai, H. (2012). The p47(phox)- and NADPH oxidase organiser 1 (NOXO1)-dependent activation of NADPH oxidase 1 (NOX1) mediates endothelial nitric oxide synthase (eNOS) uncoupling and endothelial dysfunction in a streptozotocin-induced murine model of diabetes. Diabetologia 55, 2069–2079.
26. Liu, J., Ormsby, A., Oja-Tebbe, N., & Pagano, P. J. (2004). Gene transfer of NAD(P)H oxidase inhibitor to the vascular adventitia attenuates medial smooth muscle hypertrophy. *Circulation Research, 95*(6), 587–594.
27. Touyz, R. M., Mercure, C., He, Y., Javeshghani, D., Yao, G., & Reudelhuber, T. (2005). Angiotensin II-dependent chronic hypertension and cardiac hypertrophy in mice do not require gp91phox-containing NADPH oxidase. *Hypertension, 45*, 530–537.
28. Syed, I., Kyathanahalli, C. N., Jayaram, B., Govind, S., Rhodes, C. J., Kowluru, R. A., et al. (2011). Increased phagocyte-like NADPH oxidase and ROS generation in type 2 diabetic ZDF rat and human islets: role of Rac1-JNK1/2 signaling pathway in mitochondrial dysregulation in the diabetic islet. *Diabetes, 60* (11), 2843–2852.
29. Mukherjea, D., Jajoo, S., Sheehan, K., Kaur, T., Sheth, S., Bunch, J., et al. (2011). NOX3 NADPH oxidase couples transient receptor potential vanilloid 1 to signal transducer and activator of transcription 1-mediated inflammation and hearing loss. *Antioxidants & Redox Signaling, 14*(6), 999–1010.
30. Chen, G., Adeyemo, A. A., Zhou, J., Chen, Y., Doumatey, A., Lashley, K., et al. (2007). A genome-wide search for linkage to renal function phenotypes in West Africans with type 2 diabetes. *American Journal of Kidney Diseases, 49*(3), 394–400.
31. Ye, S., Zhong, H., Yanamadala, S., & Campese, V. M. (2006). Oxidative stress mediates the stimulation of sympathetic nerve activity in the phenol renal injury model of hypertension. *Hypertension, 48*(2), 309–132.
32. Streeter, J., Thiel, W., Brieger, K., Miller, Jr F. J. (2012). Opportunity Nox: the future of NADPH oxidases as therapeutic targets in cardiovascular disease. Cardiovascular Therapeutics. doi: 10.1111.
33. Xi, G., Shen, X., Maile, L. A., Wai, C., Gollahon, K., & Clemmons, D. R. (2012). Hyperglycemia enhances IGF-I-stimulated Src activation via increasing Nox4-derived reactive oxygen species in a PKCζ-dependent manner in vascular smooth muscle cells. *Diabetes, 61*(1), 104–113.
34. Fulton, D. J. (2009). Nox5 and the regulation of cellular function. *Antioxidants & Redox Signaling, 11*(10), 2443–2452.
35. Lyle, A. N., Deshpande, N. N., Taniyama, Y., Seidel-Rogol, B., Pounkova, L., Du, P., et al. (2009). Poldip2, a novel regulator of Nox4 and cytoskeletal integrity in vascular smooth muscle cells. *Circulation Research, 105*(3), 249–259.
36. Martin-Garrido, A., Brown, D. I., Lyle, A. N., Dikalova, A., Seidel-Rogol, B., Lassègue, B., et al. (2011). NADPH oxidase 4 mediates TGF-β-induced smooth muscle α-actin via p38MAPK and serum response factor. *Free Radical Biology & Medicine, 50* (2), 354–356.
37. Takac, I., Schröder, K., Zhang, L., Lardy, B., Anilkumar, N., Lambeth, J. D., et al. (2011). The E-loop is involved in hydrogen peroxide formation by the NADPH oxidase Nox4. *Journal of Biological Chemistry, 286*(15), 13304–13313.
38. Schröder, K., Zhang, M., Benkhoff, S., Mieth, A., Pliquett, R., Kosowski, J., et al. (2012). Nox4 is a protective reactive oxygen species generating vascular NADPH oxidase. *Circulation Research, 110*, 1217–1225.
39. Zhang, M., Brewer, A. C., Schröder, K., Santos, C. X., Grieve, D. J., Wang, M., et al. (2010). NADPH oxidase-4 mediates protection against chronic load-induced stress in mouse hearts by enhancing angiogenesis. *Proceedings of the National Academy of Sciences of the United States of America, 107*(42), 18121–18126.
40. Kuroda, J., Ago, T., Matsushima, S., Zhai, P., Schneider, M. D., & Sadoshima, J. (2010). NADPH oxidase 4 (Nox4) is a major source of oxidative stress in the failing heart. *Proceedings of the National Academy of Sciences of the United States of America, 107*(35), 15565–15570.
41. Tong, X., Hou, X., Jourd'heuil, D., Weisbrod, R. M., & Cohen, R. A. (2010). Upregulation of Nox4 by TGF{beta}1 oxidizes SERCA and inhibits NO in arterial smooth muscle of the prediabetic Zucker rat. *Circulation Research, 107*, 975–983.
42. Wu, X., & Williams, K. J. (2012). NOX4 pathway as a source of selective insulin resistance and responsiveness. *Arteriosclerosis, Thrombosis, and Vascular Biology, 32*(5), 1236–1245.
43. Maalouf, R. M., Eid, A. A., Gorin, Y. C., Block, K., Escobar, G. P., Bailey, S., et al. (2012). Nox4-derived reactive oxygen species mediate cardiomyocyte injury in early type 1 diabetes. *American Journal of Physiology. Cell Physiology, 302*(3), C597–C604.
44. Piwkowska, A., Rogacka, D., Audzeyenka, I., Jankowski, M., & Angielski, S. (2011). High glucose concentration affects the oxidant–antioxidant balance in cultured mouse podocytes. *Journal of Cellular Biochemistry, 112*(6), 1661–1672.
45. Sedeek, M., Callera, G., Montezano, A., Gutsol, A., Heitz, F., Szyndralewiez, C., et al. (2010). Critical role of Nox4-based NADPH oxidase in glucose-induced oxidative stress in the kidney: implications in type 2 diabetic nephropathy. *American Journal of Physiology. Renal Physiology, 299*(6), F1348–F1358.
46. Montezano, A. C., Buger, D., Ceravolo, G. S., Yusuf, H., Montero, M., & Touyz, R. M. (2011). Novel Noxes homologues in the vasculature: focusing on Nox4 and Nox5. *Clinical Science, 120* (4), 131–141.
47. Pandey, D., & Fulton, D. J. (2011). Molecular regulation of NADPH oxidase 5 via the MAPK pathway. *American Journal of Physiology—Heart and Circulatory Physiology, 300*(4), H1336–4.
48. Manea, A., Manea, S. A., Florea, I. C., Luca, C. M., & Raicu, M. (2012). Positive regulation of NADPH oxidase 5 by proinflammatory-related mechanisms in human aortic smooth muscle cells. *Free Radical Biology & Medicine, 52*(9), 1497–1507.
49. Hahn, N. E., Meischl, C., Kawahara, T., Musters, R. J., Verhoef, V. M., van der Velden, J., et al. (2012). NOX5 expression is increased in intramyocardial blood vessels and cardiomyocytes after acute myocardial infarction in humans. *American Journal of Pathology, 180*(6), 2222–2229.
50. Pandey, D., Patel, A., Patel, V., Chen, F., Qian, J., Wang, Y., Barman, S.A., Venema, R.C., Stepp, D.W., Rudic, R.D., Fulton, D.J. (2012). Expression and functional significance of NADPH oxidase 5 (Nox5) and its splice variants in human blood vessels. *American Journal of Physiology—Heart and CirculatoryPhysiology, 302*(10), H1919–28.
51. Bhavani, N. (2011). Transient congenital hypothyroidism. *Indian Journal of Endocrinology and Metabolism, 15*(Suppl 2), S117–S120.
52. Cooper, M. E. (1998). Pathogenesis, prevention, and treatment of diabetic nephropathy. *Lancet, 352*(9123), 213–219.
53. Forbes, J. M., Coughlan, M. T., & Cooper, M. E. (2008). Oxidative stress as a major culprit in kidney disease in diabetes. *Diabetes, 57*(6), 1446–1454.

54. Eid, A. A., Gorin, Y., Faff, B. M., Maalouf, R., Barnes, J. L., Block, K., et al. (2009). Mechanisms of podocyte injury in diabetes: role of cytochrome P450 and NADPH oxidases. *Diabetes, 58*(5), 1201–1211.
55. Etoh, T., Inoguchi, T., Kakimoto, M., Sonoda, N., Kobayashi, K., Kuroda, J., et al. (2003). Increased expression of NAD(P)H oxidase subunits, NOX4 and p22phox, in the kidney of streptozotocin-induced diabetic rats and its reversibility by interventive insulin treatment. *Diabetologia, 46*(10), 1428–1437.
56. Asaba, K., Tojo, A., Onozato, M. L., Goto, A., Quinn, M. T., Fujita, T., et al. (2005). Effects of NADPH oxidase inhibitor in diabetic nephropathy. *Kidney International, 67*(5), 1890–1898.
57. Kitada, M., Koya, D., Sugimoto, T., Isono, M., Araki, S., Kashiwagi, A., et al. (2003). Translocation of glomerular p47phox and p67phox by protein kinase C-beta activation is required for oxidative stress in diabetic nephropathy. *Diabetes, 52*(10), 2603–2614.
58. Ohshiro, Y., Ma, R. C., Yasuda, Y., Hiraoka-Yamamoto, J., Clermont, A. C., Isshiki, K., et al. (2006). Reduction of diabetes-induced oxidative stress, fibrotic cytokine expression, and renal dysfunction in protein kinase Cbeta-null mice. *Diabetes, 55*(11), 3112–3120.
59. Cai, W., Torreggiani, M., Zhu, L., Chen, X., He, J. C., Striker, G. E., et al. (2010). AGER1 regulates endothelial cell NADPH oxidase-dependent oxidant stress via PKC-delta: implications for vascular disease. *American Journal of Physiology. Cell Physiology, 298*(3), C624–C634.
60. Lee, H. B., Yu, M. R., Yang, Y., Jiang, Z., & Ha, H. (2003). Reactive oxygen species-regulated signaling pathways in diabetic nephropathy. *Journal of the American Society of Nephrology, 14* (8 Suppl 3), S241–S245.
61. Gorin, Y., Ricono, J. M., Kim, N. H., Bhandari, B., Choudhury, G. G., & Abboud, H. E. (2003). Nox4 mediates angiotensin II-induced activation of Akt/protein kinase B in mesangial cells. *American Journal of Physiology. Renal Physiology, 285*(2), F219–F229.
62. Nava, M., Quiroz, Y., Vaziri, N., & Rodriguez-Iturbe, B. (2003). Melatonin reduces renal interstitial inflammation and improves hypertension in spontaneously hypertensive rats. *American Journal of Physiology. Renal Physiology, 284*(3), F447–F454.
63. Ha, H., Yu, M. R., Choi, Y. J., Kitamura, M., & Lee, H. B. (2002). Role of high glucose-induced nuclear factor-kappaB activation in monocyte chemoattractant protein-1 expression by mesangial cells. *Journal of the American Society of Nephrology, 13*(4), 894–902.
64. Weigert, C., Sauer, U., Brodbeck, K., Pfeiffer, A., Häring, H. U., & Schleicher, E. D. (2000). AP-1 proteins mediate hyperglycemia-induced activation of the human TGF-beta1 promoter in mesangial cells. *Journal of the American Society of Nephrology, 11*(11), 2007–2016.
65. Kim, N. H., Rincon-Choles, H., Bhandari, B., Choudhury, G. G., Abboud, H. E., & Gorin, Y. (2006). Redox dependence of glomerular epithelial cell hypertrophy in response to glucose. *American Journal of Physiology. Renal Physiology, 290*(3), F741–F751.
66. Susztak, K., Raff, A. C., Schiffer, M., & Böttinger, E. P. (2006). Glucose-induced reactive oxygen species cause apoptosis of podocytes and podocyte depletion at the onset of diabetic nephropathy. *Diabetes, 55*(1), 225–233.
67. Yau, J. W., Rogers, S. L., Kawasaki, R., Lamoureux, E. L., Kowalski, J. W., Bek, T., Wong, T. Y., & Meta-Analysis for Eye Disease (META-EYE) Study Group. (2012). Global prevalence and major risk factors of diabetic retinopathy. *Diabetes Care, 35*(3), 556–564.
68. Wilkinson, C. P., Ferris, F. L., 3rd, Klein, R. E., Lee, P. P., Agardh, C. D., Davis, M., et al. (2003). Proposed international clinical diabetic retinopathy and diabetic macular edema disease severity scales. *Ophthalmology, 110*, 1677–1682.
69. Arden, G. B., & Sivaprasad, S. (2011). Hypoxia and oxidative stress in the causation of diabetic retinopathy. *Current Diabetes Reviews, 7*, 291–304.
70. Al-Shabrawey, M., Bartoli, M., El-Remessy, A. B., Ma, G., Matragoon, S., Lemtalsi, T., et al. (2008). Role of NADPH oxidase and Stat3 in statin-mediated protection against diabetic retinopathy. *Investigative Ophthalmology and Visual Science, 49*, 3231–3238.
71. Li, J., Wang, J. J., Yu, Q., Chen, K., Mahadev, K., & Zhang, S. X. (2010). Inhibition of reactive oxygen species by Lovastatin downregulates vascular endothelial growth factor expression and ameliorates blood–retinal barrier breakdown in db/db mice: role of NADPH oxidase 4. *Diabetes, 59*, 1528–1538.
72. Dvoriantchikova et al. (2012) Neuronal NAD(P)H oxidases contribute to ROS production and mediate RGC death after ischemia. Investigative Ophthalmology & Visual Science 53, 2823–2830.
73. Bhatt, L., Groeger, G., McDermott, K., & Cotter, T. G. (2010). Rod and cone photoreceptor cells produce ROS in response to stress in a live retinal explant system. *Molecular Vision, 16*, 283–293.
74. Yokota, H., Narayanan, S. P., Zhang, W., Liu, H., Rojas, M., Xu, Z., et al. (2011). Neuroprotection from retinal ischemia/reperfusion injury by NOX2 NADPH oxidase deletion. *Investigative Ophthalmology and Visual Science, 52*(11), 8123–8131.
75. Tang, J., & Kern, T. S. (2011). Inflammation in diabetic retinopathy. *Progress in Retinal and Eye Research, 30*, 343–358.
76. Tarr, J. M., Ding, N., Kaul, K., Antonell, A., Perez-Jurado, L. A., & Chibber, R. (2012). Cellular crosstalk between TNF-alpha, NADPH oxidase, PKCbeta2, and C2GNT in human leukocytes. *Cellular Signalling, 24*, 873–878.
77. Chen, P., Guo, A. M., Edwards, P. A., Trick, G., & Scicli, A. G. (2007). Role of NADPH oxidase and ANG II in diabetes-induced retinal leukostasis. *American Journal of Physiology—Regulatory, Integrative and Comparative Physiology, 293*, R1619–R1629.
78. Garrido-Urbani, S., Jemelin, S., Deffert, C., Carnesecchi, S., Basset, O., Szyndralewiez, C., et al. (2011). Targeting vascular NADPH oxidase 1 blocks tumor angiogenesis through a PPAR-alpha mediated mechanism. *PLoS One, 6*, e14665.
79. Arbiser, J. L., Petros, J., Klafter, R., Govindajaran, B., McLaughlin, E. R., Brown, L. F., et al. (2002). Reactive oxygen generated by Nox1 triggers the angiogenic switch. *Proceedings of the National Academy of Sciences of the United States of America, 99*, 715–720.
80. Saito, Y., Geisen, P., Uppal, A., & Hartnett, M. E. (2007). Inhibition of NAD(P)H oxidase reduces apoptosis and avascular retina in an animal model of retinopathy of prematurity. *Molecular Vision, 13*, 840–853.
81. Al-Shabrawey, M., Rojas, M., Sanders, T., Behzadian, A., El-Remessy, A., Bartoli, M., et al. (2008). Role of NADPH oxidase in retinal vascular inflammation. *Investigative Ophthalmology and Visual Science, 49*, 3239–3244.
82. Zhang, W., Rojas, M., Lilly, B., Tsai, N. T., Lemtalsi, T., Liou, G. I., et al. (2009). NAD(P)H oxidase-dependent regulation of CCL2 production during retinal inflammation. *Investigative Ophthalmology and Visual Science, 50*, 3033–3040.
83. Tawfik, A., Sanders, T., Kahook, K., AkeeL, S., Elmarakby, A., & Al-Shabrawey, M. (2009). Suppression of retinal peroxisome proliferator-activated receptor gamma in experimental diabetes and oxygen-induced retinopathy: role of NADPH oxidase. *Investigative Ophthalmology and Visual Science, 50*, 878–884.
84. Wilkinson-Berka, J. L., Heine, R., Tan, G., Cooper, M. E., Hatzopoulos, K. M., Fletcher, E. L., et al. (2010). RILLKKMPSV influences the vasculature, neurons and glia, and (pro)renin receptor expression in the retina. *Hypertension, 55*, 1454–1460.

85. Sarlos, S., & Wilkinson-Berka, J. L. (2005). The renin–angiotensin system and the developing retinal vasculature. *Investigative Ophthalmology and Visual Science, 46*, 1069–1077.
86. Wilkinson-Berka, J. L. (2006). Angiotensin and diabetic retinopathy. *The International Journal of Biochemistry & Cell Biology, 38*, 752–765.
87. Fukumoto, M., Takai, S., Ishizaki, E., Sugiyama, T., Oku, H., Jin, D., et al. (2008). Involvement of angiotensin II-dependent vascular endothelial growth factor gene expression via NADPH oxidase in the retina in a type 2 diabetic rat model. *Current Eye Research, 33*, 885–891.
88. Li, L., & Renier, G. (2006). 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, 55*, 1516–1523.
89. Yamagishi, S., Nakamura, K., Matsui, T., Inagaki, Y., Takenaka, K., Jinnouchi, Y., et al. (2006). Pigment epithelium-derived factor inhibits advanced glycation end product-induced retinal vascular hyperpermeability by blocking reactive oxygen species-mediated vascular endothelial growth factor expression. *Journal of Biological Chemistry, 281*(29), 20213–20220.
90. Miller, A. G., Tan, G., Binger, K. J., Pickering, R. J., Thomas, M. C., Nagaraj, R. H., et al. (2010). Candesartan attenuates diabetic retinal vascular pathology by restoring glyoxalase-I function. *Diabetes, 59*, 3208–3215.
91. Barry-Lane, P. A., Patterson, C., Van der Merwe, M., Hu, Z., Holland, S. M., Yeh, E. T. H., et al. (2001). p47phox is required for atherosclerotic lesion progression in ApoE$^{-/-}$ mice. *Journal of Clinical Investigation, 108*, 1513–1522.
92. Judkins, C. P., Diep, H., Broughton, B. R. S., Mast, A. E., Hooker, E. U., Miller, A. A., et al. (2010). Direct evidence of a role for Nox2 in superoxide production, reduced nitric oxide bioavailability, and early atherosclerotic plaque formation in ApoE$^{-/-}$ mice. *American Journal of Physiology—Heart and Circulatory Physiology, 298*, H24–H32.
93. Dikalova, A., Clempus, R., Lassegue, B., Cheng, G., Mccoy, J., Dikalov, S., et al. (2005). Nox1 overexpression potentiates angiotensin II-induced hypertension and vascular smooth muscle hypertrophy in transgenic mice. *Circulation, 112*, 2668–2676.
94. Shimizu, H., Nakagawa, Y., Murakami, C., Aoki, N., Kim-Mitsuyama, S., & Miyazaki, H. (2010). Protein tyrosine phosphatase PTPepsilonM negatively regulates PDGF beta-receptor signaling induced by high glucose and PDGF in vascular smooth muscle cells. *American Journal of Physiology. Cell Physiology, 299*(5), C1144–C1145.
95. Perrotta, I., Sciangula, A., Perrotta, E., Donato, G., & Cassese, M. (2011). Ultrastructural analysis and electron microscopic localization of Nox4 in healthy and atherosclerotic human aorta. *Ultrastructural Pathology, 35*(1), 1–6.
96. Fenyo, I. M., Florea, I. C., Raicu, M., & Manea, A. (2011). Tyrphostin AG490 reduces NAPDH oxidase activity and expression in the aorta of hypercholesterolemic apolipoprotein E-deficient mice. *Vascular Pharmacology, 54*(3-6), 100–106.
97. Lassègue, B., & Griendling, K. K. (2010). NADPH oxidases: functions and pathologies in the vasculature. *Arteriosclerosis, Thrombosis, and Vascular Biology, 30*, 653–661.
98. Matsuno, K., Yamada, H., Iwata, K., Jin, D., Katsuyama, M., Matsuki, M., et al. (2005). Nox1 is involved in angiotensin II-mediated hypertension: a study in Nox1-deficient mice. *Circulation, 112*, 2677–2685.
99. Hart, B. A., Elferink, J. G., & Nibbering, P. H. (1992). Effect of apocynin on the induction of ulcerative lesions in rat skin injected with tubercle bacteria. *International Journal of Immunopharmacology, 14*(6), 953–961.
100. Gatley, S. J., & Sherratt, H. A. S. (1976). The effects of diphenyleneiodonium on mitochondrial reactions. Relation of binding of diphenylene[^{125}I]iodonium to mitochondria to the extent of inhibition of oxygen uptake. *Biochemical Journal, 158*, 307–315.
101. Aldieri, E., Riganti, C., Polimeni, M., Gazzano, E., Lussiana, C., Campia, I., et al. (2008). Classical inhibitors of NOX NAD(P)H oxidases are not specific. *Current Drug Metabolism, 9*, 686–696.
102. Wind, S., Beuerlein, D., Eucker, T., Müller, H., Scheurer, P., Armitage, M. E., et al. (2010). Comparative pharmacology of chemically distinct NADPH oxidase inhibitors. *British Journal of Pharmacology, 161*, 885–898.
103. Drummond, G. R., Selemidis, S., Griendling, K. K., & Sobey, C. G. (2011). Combating oxidative stress in vascular disease: NADPH oxidases as therapeutic targets. *Nature Reviews. Drug Discovery, 10*(6), 453–457.
104. Kim, J. A., Neupane, G. P., Lee, E. S., Jeong, B. S., Park, B. C., & Thapa, P. (2011). NADPH oxidase inhibitors: a patent review. *Expert Opinion on Therapeutic Patents, 21*(8), 1147–1158.
105. http://www.genkyotex.com/cdpdf/1320395578_GenKyoTex_Phase_I_Release_31-10-11.pdf.
106. Laleu, B., Gaggini, F., Orchard, M., Fioraso-Cartier, I., Cagnon, I., Houngninou-Molango, S., et al. (2010). First in class, potent, and orally bioavailable NADPH oxidase isoform 4 (Nox4) inhibitors for the treatment of idiopathic pulmonary fibrosis. *Journal of Medicinal Chemistry, 53*, 7715–7730.
107. Page, P., Orchard, M., Fioraso-Cartier, l., Mottironi, B. (2008). Pyrazolo pyridine derivatives as NADPH oxidase inhibitors, Patent WO 2008/113856 A1. Switzerland patent application.
108. Stielow, C., Catar, R. A., Muller, G., Wingler, K., Scheurer, P., Schmidt, H. H. H. W., et al. (2006). Novel Nox inhibitor of oxLDL-induced reactive oxygen species formation in human endothelial cells. *Biochemical and Biophysical Research Communications, 344*, 200–205.
109. Ten Freyhaus, H., Huntgeburth, M., Wingler, K., Schnitker, J., Bäumer, A. T., Vantler, M., et al. (2006). Novel Nox inhibitor VAS2870 attenuates PDGF-dependent smooth muscle cell chemotaxis, but not proliferation. *Cardiovascular Research, 71*, 331–341.
110. Niethammer, P., Grabher, C., Look, A. T., & Mitchison, T. J. (2009). A tissue-scale gradient of hydrogen peroxide mediates rapid wound detection in zebrafish. *Nature, 459*, 996–999.
111. Spychalowicz, A., Wilk, G., Sliwa, T., Ludew, D., Guzik, T.J. (2012). Novel therapeutic approaches in limiting oxidative stress and inflammation. Current Pharmaceutical Biotechnology. [Epub ahead of print].
112. Bonner, M.Y., Arbiser, J.L., Targeting, N.A.D.P.H. (2012 May 13). oxidases for the treatment of cancer and inflammation. Cellular and Molecular Life Sciences. [Epub ahead of print].