BIOMOLECULES
& THERAPEUTICS

# Function of NADPH Oxidases in Diabetic Nephropathy and Development of Nox Inhibitors

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## Abstract

Several recent studies have reported that reactive oxygen species (ROS), superoxide anion and hydrogen peroxide ($H_2O_2$), play important roles in various cellular signaling networks. NADPH oxidase (Nox) isozymes have been shown to mediate receptor-mediated ROS generation for physiological signaling processes involved in cell growth, differentiation, apoptosis, and fibrosis. Detectable intracellular levels of ROS can be induced by the electron leakage from mitochondrial respiratory chain as well as by activation of cytochrome p450, glucose oxidase and xanthine oxidase, leading to oxidative stress. The up-regulation and the hyper-activation of NADPH oxidases (Nox) also likely contribute to oxidative stress in pathophysiologic stages. Elevation of the renal ROS level through hyperglycemia-mediated Nox activation results in the oxidative stress which induces a damage to kidney tissues, causing to diabetic nephropathy (DN). Nox inhibitors are currently being developed as the therapeutics of DN. In this review, we summarize Nox-mediated ROS generation and development of Nox inhibitors for therapeutics of DN treatment.

**Key Words:** NADPH oxidase, Oxidative stress, Signal transduction, Kidney, Diabetic nephropathy, Nox inhibitor

## INTRODUCTION

Complications of diabetes mellitus (DM) occur in the eyes (retinopathy) (Heng *et al*., 2013; Gorin *et al*., 2015; Khan *et al*., 2017), nerves (neuropathy) (Said, 2007; Vinik *et al*., 2013) and kidneys (nephropathy) (Thomas *et al*., 2006; Caramori *et al*., 2013; Forbes and Cooper, 2013; Thomas *et al*., 2015). Among these complications, diabetic nephropathy (DN) is the leading cause of renal failure in patients with diabetes mellitus which ultimately results in end-stage renal disease (ESRD) (Molitch *et al*., 2004; Thomas *et al*., 2015; Papadopoulou-Marketou *et al*., 2017). In the initial stage of diabetic nephropathy (DN), commonly observed signs include albuminuria mainly resulting from reduction of the glomerular filtration rate (GFR) (Jha *et al*., 2016). Increasing albuminuria, reduction of GFR or mesangial expansion, glomerulosclerosis, podocyte apoptosis, and tubulointerstitial fibrosis are well-established features seen in DN progression (Jha *et al*., 2016). Generally, DN can be divided into multiple stages; (1) increased GFR (hyperfiltration) phase with increased volume of the kidney; (2) normal filtration phase with thickening of the glomerular basement membrane (GBM); (3) microalbuminuria (>30 mg/day); (4) macro-albuminuria (>300 mg/day); (5) uremia with ESRD (Rocco and Berns, 2009; Palatini, 2012; Reidy *et al*., 2014; Lv *et al*., 2015). In addition to functional changes, structural changes are also observed; glomerular basement membrane (GBM) thickening, mesangial expansion and overexpression of ECM proteins, tubulointerstitial fibrosis and glomeruolsclerosis (Mason and Wahab, 2003; Tervaert *et al*., 2010; Caramori *et al*., 2013). Several lines of evidence strongly suggest that oxidative stress serves as an etiological factor in the initiation and progression of DN (Jha *et al*., 2016). In particular, renal oxidative stress is known to be closely associated with the activation or the overexpression of various NADPH oxidase (Nox) isozymes (Jha *et al*., 2016). This review will discuss about the function of Nox isozymes in pathology of DN and recent on-going development of Nox inhibitors for treatment of DN.

## STRUCTURE AND REGULATION OF NOX ISOZYMES

The gp91[phox] (renamed as Nox2) was the first identified isozyme, and it was in phagocytic cells. Subsequently, six additional homologues have been isolated in non-phagocytic cells (Nox1, Nox3, Nox4, Nox5, Duox1 and Duox2) (Bae *et*

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al., 2011). All Nox isozymes are single polypeptide enzymes and share common features. Nox isozymes are structurally divided into two functional domains. The long carboxyl terminal region of Nox contains NADPH- and FAD-binding sites homologous to that of ferredoxin-NADP$^+$ reductase (FNR) (Segal et al., 1992; Sumimoto et al., 1992). The six transmembrane α-helical domains also found in the carboxyl terminal region bearing tandem heme groups homologous to that in ferric reductase (F$_{re}$) (Fig. 1A) (Finegold et al., 1996). The sequential electron transfer from NADPH to FAD and heme groups stimulates the reduction of oxygen molecules leading to generation of reactive oxygen species (ROS) such as superoxide anion and hydrogen peroxide. Among the seven Nox isozymes in human, Nox1, Nox3, and Nox4 have similar structures with Nox2 requiring p22$^{phox}$ integral protein for the activation and formation of Nox complex. Nox5 and Duox1/2 isozymes do not require p22$^{phox}$ integral protein, but contain the EF hand domain responsible for binding of intracellular calcium (Fig. 1B) (Bae et al., 2011).

Nox2 isozyme requires one integral protein, p22$^{phox}$, multiple cytosolic proteins (p47$^{phox}$, p40$^{phox}$, and p67$^{phox}$) and a small GTPase Rac (Bokoch and Zhao, 2006; Bedard and Krause,
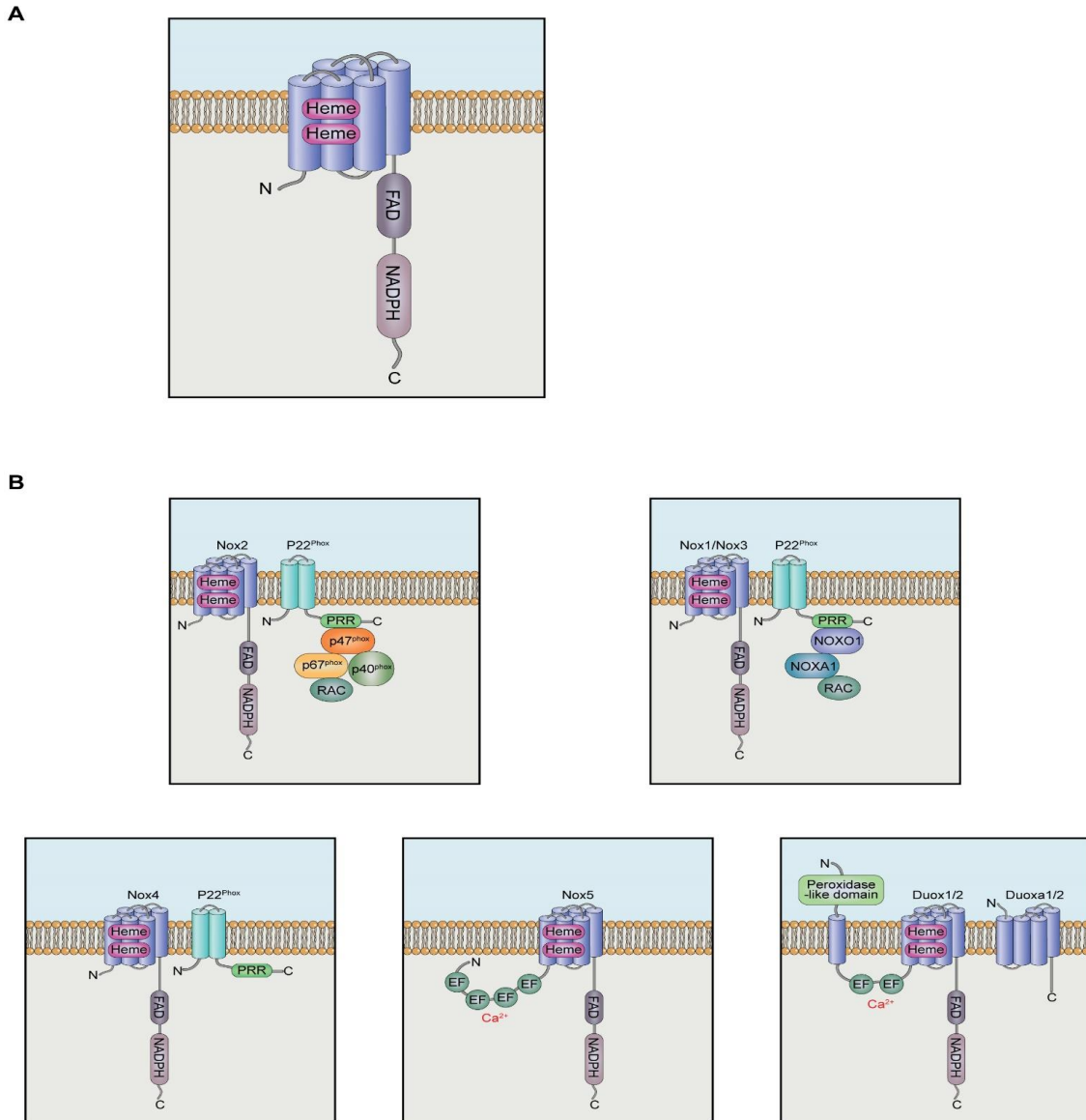


**Fig. 1.** NADPH Oxidase (Nox) structure and Nox isozymes. (A) General structure of Nox isozyme. Nox contains six transmembrane domains with two heme binding sites and long COOH-terminal region for NADPH binding site and FAD-binding site as prosthestic group. (B) Regulation of Nox isozymes. Nox2-p22$^{phox}$ dimer associates p47$^{phox}$ through interaction of SH3 of p47$^{phox}$ with PRR of p22$^{phox}$. The PRR domain of p47$^{phox}$ serves as the binding site for p67$^{phox}$. Subsequently, the p67$^{phox}$ protein recruites p40$^{phox}$ and Rac protein. Nox1 also binds to p22$^{phox}$ and requires Nox organizer 1 (NoxO1), Nox activator1 (NoxA1), and Rac protein. Nox4 requires only p22$^{phox}$ for the activity. Nox5 and Duox 1/2 isozymes contain Ca$^{2+}$-binding EF hand domain at N-terminus region, indicating that these isozymes are regulated by intracellular calcium mobilization.

2007; Bae *et al*., 2011; Jha *et al*., 2016) for its activity. The formation of Nox2 complex is mediated by sophisticated protein-protein interactions (Fig. 1B). Nox2 tightly associates with the integral protein p22phox whose proline-rich region (PRR) functions as the binding site for the Src homology domain 3 (SH3) of p47phox. This interaction is key to subsequent formation of active Nox2 complex. The p47phox also contains phox (PX) domain in the $NH_3$-terminal region, two SH3 domains in the central region, and auto-inhibitory region (AIR) and PRR in the COOH-terminal region. In the resting stage, AIR interacts with tandem SH3 domains to keep intramolecular folding consistent with inactive state. Various serine/threonine kinases including PKC, PKA, MAPK, and Akt phosphorylate the COOH-terminal region of p47phox to interrupt intramolecular SH3-AIR interaction and then to induce the opening of SH3 domain for interaction with PRR of p22phox (Sumimoto *et al*., 1996; Groemping *et al*., 2003; Bedard and Krause, 2007). Subsequently, the PRR domain of p47phox serves as the binding site for p67phox (Bae *et al*., 2011). The p67phox protein contains two SH3 domains in its COOH-terminal region that interacts with the PRR domain of p47phox and four tetratricopeptide repeats (TPR) in the $NH_3$-terminal region that functions as the binding site of small G-protein Rac. The p67phox protein also contains the PB1 domain between the two SH3 domains which mediates molecular interaction with the PB1 domain of p40phox. Receptor-mediated Nox2 complex formation induces electron transfers among NADPH, FAD and heme group, leading to generation of superoxide anions.

Nox1 was identified as a homologue of Nox2 from non-phagocytic colon epithelium (Suh *et al*., 1999; Bae *et al*., 2011). Nox1 also binds to p22phox and requires Nox organizer 1 (NoxO1) and Nox activator1 (NoxA1) as the homologues and functional counterparts of p47phox and p67phox of Nox2 complex respectively as well as the small G protein Rac (Fig. 1B) (Geiszt *et al*., 2003; Kawahara *et al*., 2005; Cheng *et al*., 2006). NoxO1 contains two SH3 domains in the central region for interaction with the PRR domain of p22phox and one PRR domain in the COOH-terminal region for binding to the SH3 domain of NoxA1. It has generally been assumed that the Nox1 complex is constitutively active because a domain homologous to the AIR domain of p47phox is missing in NoxO1 (Sumimoto, 2008; Dutta and Rittinger, 2010). However, it has been reported that Nox1 activity is transiently increased in response to various agonists including EGF (Lassegue *et al*., 2001; Park *et al*., 2006; Schroder *et al*., 2007; Choi *et al*., 2008; Lee *et al*., 2009, 2013), suggesting that an additional mechanism is in operation for the regulation of Nox1 activity. Joo *et al*. (2016) reported a molecular regulatory process in which Casitas B–lineage lymphoma (Cbl) E3 ligase is recruited to Nox1 complex through the interaction between NoxO1 and growth receptor–bound protein 2 (Grb2) leading to the disassembly of Nox1 complex and a subsequent reduction in
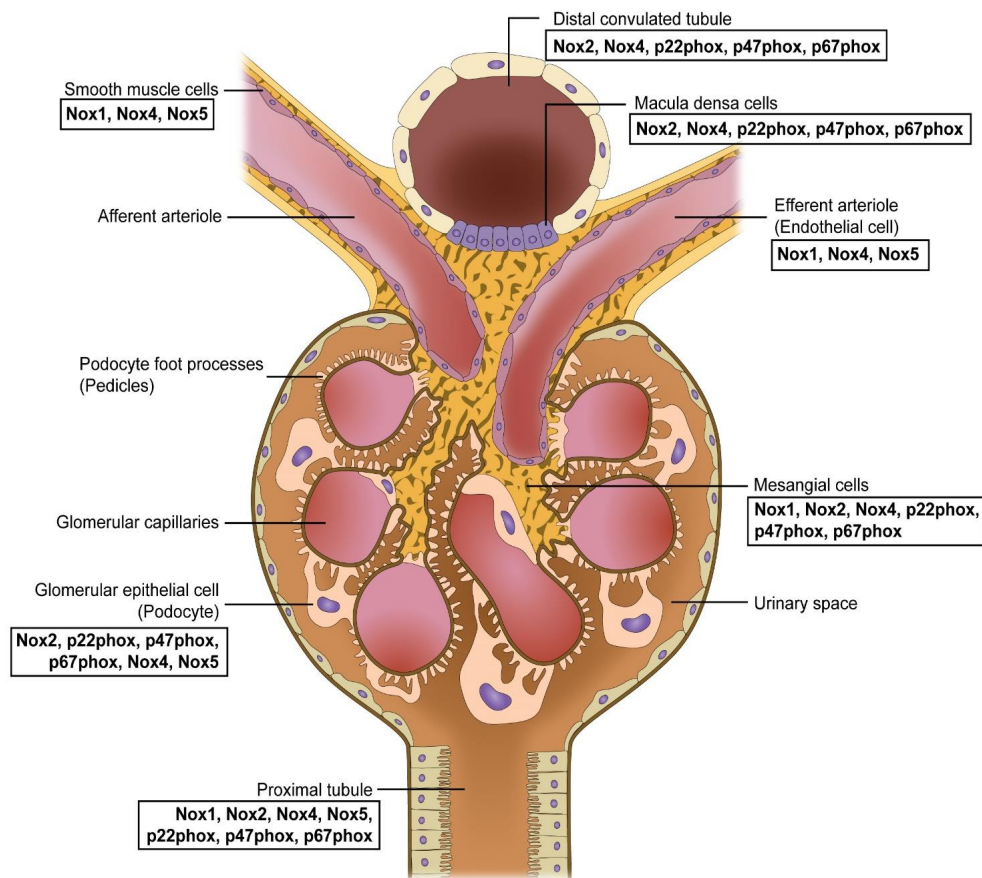


**Fig. 2.** Distribution of Nox isozymes and its components in the kidney.

ROS generation upon epidermal growth factor (EGF) stimulation.

The primary structure of Nox3 shares 56% homology with that of Nox2 (Kikuchi *et al*., 2000; Jha *et al*., 2016). Nox3 also requires p22[phox] and NoxO1 for the activation (Fig. 1B). However, the molecular mechanism for Nox3 complex formation remains to be elucidated (Banfi *et al*., 2004a; Cheng *et al*., 2004; Ueno *et al*., 2005; Ueyama *et al*., 2006; Bedard and Krause, 2007). Nox4 was initially identified from kidney and requires only p22[phox] for the activity (Fig. 1B). However, no cytosolic protein regulating Nox4 activity has been reported. Many reports suggested that Nox4 seems to be constitutively active and Nox4 activity is dependent largely on its expression level in cells. However, Nox4 isozyme activity is regulated by receptor-mediated cell signaling networks, suggesting that unknown protein or proteins regulate Nox4 complex formation and stimulates its activity (Bedard and Krause, 2007; Leto *et al*., 2009; Block and Gorin, 2012; Lassegue *et al*., 2012; Lambeth and Neish, 2014). Poldip2 (polymerase delta-interacting proteins2), a p22[phox] interacting protein, has been identified as a positive regulator for Nox4 activity in SMC migration (Lyle *et al*., 2009). However, molecular mechanisms by which poldip2 associates and interacts with Nox4-p22[phox] complex remain to be determined.

Unlike Nox1-4 isozymes, Nox5 does not require p22[phox] or cytosolic accessory proteins for activation. Given that Nox5 isozyme contains long intracellular $NH_3$-terminus region containing $Ca^{2+}$-binding EF hand domain, Nox5 isozyme is likely regulated by intracellular calcium mobilization (Fig. 1B) (Banfi *et al*., 2001, 2004b). As in Nox5, the EF-hand domain is localized in the $NH_3$-terminus region of Duox 1/2 isozymes and functions as the binding site of intracellular calcium (Fig. 1B) (Ameziane-El-Hassani *et al*., 2005; Bae *et al*., 2011). Recently, we reported that testosterone binds to GPRC6A located in plasma membrane and subsequently induces the activation of Gq protein, $IP_3$ generation, and $[Ca^{2+}]i$ mobilization ultimately leading to Duox1 activation (Ko *et al*., 2014).

## EXPRESSION OF NOX ISOZYMES IN KIDNEY CELLS

It is now clear that multiple Nox isozymes including Nox1, Nox2, Nox4 and Nox5 (in case of human genomes) and their accessory proteins are expressed in kidney tissues (Fig. 2). Together, these isozymes represent one of the major sources of ROS in normal and pathological states of kidney (Jha *et al*., 2014; Holterman *et al*., 2015; Cha *et al*., 2017). The pathological states of kidney in particular includes diabetic nephropathy and fibrosis in which the major function of kidney, elimination of metabolic wastes in blood through glomerular filtration, is severely compromised (Jha *et al*., 2016). The renal vessels are composed of vascular smooth muscle cells (VSMCs) and
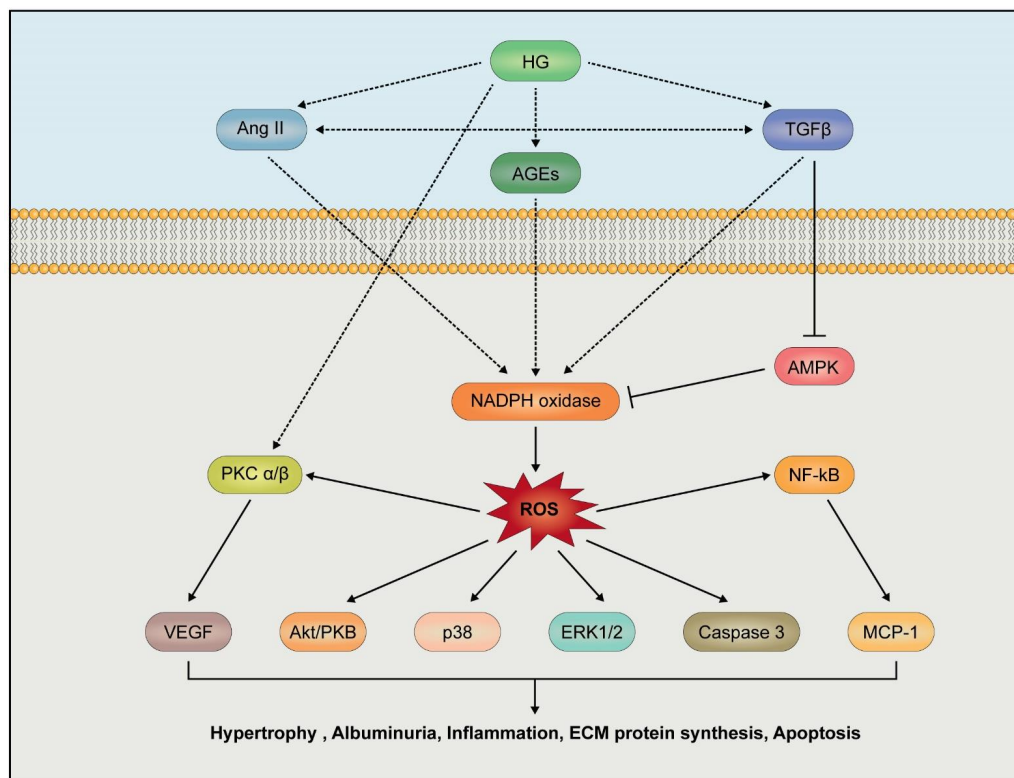


**Fig. 3.** Activation of Nox in diabetic nephropathy. High glucose (HG), transforming growth factor β (TGF β), angiotensin II (Ang II) and advanced glycation end products (AGEs) stimulate Nox activity or its expression, leading to overproduction of ROS. Nox-derived ROS regulate Akt/PKB, p38, and caspase 3 activation leading to podocyte apoptosis. ROS induce inflammation in renal cells by increasing MCP by NF-κB activation. Additionally, the activation of protein kinase C (PKC) and AMP-activated kinase (AMPK) are involved in Nox derived ROS dependent events in the progression of DN.

endothelia cells (ECs) expressing Nox1 and Nox4 which are responsible for ROS generation in response to angiotensin II (Fig. 2) (Wingler *et al*., 2001). Glomerulus plays an important role in maintaining kidney function and is mainly composed of mesangial cells and podocytes. Many reports have shown that upregulation of Nox1, Nox2, Nox4, and Nox5 and the regulatory subunits p22phox, p47phox and p67phox contributes to mesangial cell hypertrophy, tissue expansion, ECM protein accumulation and podocyte apoptosis in glomerulus (Jha *et al*., 2016). Proximal tubule cells express Nox1, Nox2, Nox4 and Nox5 isozymes (Yang *et al*., 2012; Yu *et al*., 2014; Holterman *et al*., 2015), and macula densa expresses Nox2 and Nox4 as well as p47phox, p67phox and p22phox (Fig. 2) (Holterman *et al*., 2015).

## ACTIVATION OF NOX IN DIABETIC NEPHROPATHY

High glucose (HG)-induced kidney damages are associated with several metabolic risk factors (Fig. 3). Activation of renin-angiotensin-aldosterone pathway, activation of PKC, production of advanced glycation end products (AGEs) and TGFβ-induced fibrosis have been reported as high risk factors for the initiation and progression of DN. A high HG level induces increased generation of advanced glycation end products (AGEs) which serve as ligands for cellular receptor RAGE, transducing signals for inflammation (Schmidt *et al*., 1995; Hofmann *et al*., 1999). AGEs from ECM fragments including protein and lipid species thereby lead to structural changes in kidney tissues (Silbiger *et al*., 1993; Forbes *et al*., 2003). Activation of signaling networks by AGE stimulates nuclear factor-kB (NF-kB) activity which induces the generation of proinflammatory cytokines including interleukin-6 (IL-6) and tumor necrosis factor-$\alpha$ (TNF-$\alpha$) (Neumann *et al*., 1999). AGEs are also considered as potent stimulators for VEGF expression and PKC activation, leading to upregulation of TGFβ1 expression, which in turn is causally linked to expression of ECM protein for mesangial expansion (Osicka *et al*., 2000; Lal *et al*., 2002; Singh *et al*., 2014). Activation of TGFβ1 also stimulates Nox4 expression (Fig. 3) (Jiang *et al*., 2014). Importantly, knockdown of Nox4 expression suppressed TGFβ1-dependent fibrotic markers including fibronectin, collagen I, and $\alpha$-smooth muscle actin (Jiang *et al*., 2014). Moreover, Nox inhibitor treatment inhibited TGF-β-induced Smad2/3 activation and then suppressed expression of marker proteins for fibrosis, indicating that Nox-dependent ROS generation may
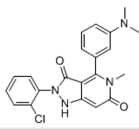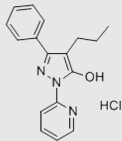
have critical roles in TGF-β-mediated fibrosis in kidney tissues (Jiang *et al*., 2014).

Activation of protein kinase C (PKC) is involved in hyperglycemia-dependent nephropathy. HG-RAGE-phospholipase Cβ (PLC) pathway and angiotensin-angiotensin receptor II-PLCβ pathway can stimulate DAG generation and intracellular calcium mobilization through hydrolysis of phosphatidylinositol 4,5, bisphosphate (PIP2). Elevated levels of intracellular calcium and DAG activate renal PKC affecting vascular functions through inducing changes in endothelial permeability, vasoconstriction, extracellular matrix (ECM) maintenance, cytokine activation and leukocyte adhesion (Fig. 3). Of interest, PKC inhibition ameliorates structural and functional abnormalities in the kidney in db/db mice (Koya *et al*., 2000). Importantly, HG-induced translocation of PKC$\alpha$ to renal membranes stimulates Nox2 activation. In type I diabetic mice, treatment of PKC$\alpha$ inhibitor (Ro-32-0432) attenuates Nox-dependent superoxide production. Renal extracellular matrix accumulation of fibronectin and collagen IV is decreased by Nox inhibitor apocynin (Thallas-Bonke *et al*., 2008). These reports together suggest that Nox2 activation by PKC$\alpha$ is the key downstream event of the AGE-receptor signaling in diabetic nephropathy.

Recently, oxidative stress has come to be recognized as a key player in the pathogenesis of DN. Most ROS produced intracellularly originate from the mitochondrial respiratory chain, and they certainly can bring damages in renal tissues when mitochondria become dysfunctional. Relatively small amounts (1-2%) of total $O_2$ consumed during mitochondrial respiration are normally converted to ROS (Chance *et al*., 1979; Kudin *et al*., 2004). However, the formation of ROS is increased from dysfunctional mitochondria in hyperglycemia stage. ROS accumulation can lead to oxidative stresses in the cell, leading to damages of cellular components including protein and DNA. Ultimately, the oxidative stress leads to renal fibrosis and decline in renal function (Badal and Danesh, 2014). ROS mediate activation of a number of pathways including PKC, NF-kB, hexosamine synthesis and AGE (Nishikawa *et al*., 2000).

As has been described above, ROS can also be produced by activities of Nox isozymes. Many reports have indicated that HG, angiotensin II or TGFβ stimulate Nox4-derived ROS generation, resulting in fibrotic response in mesangial cells, podocytes and tubular cells (Jha *et al*., 2016). The pathogenic process leading to fibrosis has been studied with Nox4-deficient mice (Jha *et al*., 2014; Gorin *et al*., 2015). Interestingly, treatment of Nox inhibitors (GKT137831 or APX-115) resulted

**Table 1.** NADPH oxidase inhibitors

| Compound | Mechanism of action | Chemical name | Chemical structure | Target | References |
|---|---|---|---|---|---|
| GKT137981 | Dual Nox1/4 inhibitor | 2-(2-Chlorophenyl)-4-(3-dimethylamino)phenyl)-5-methyl-1H-pyrazolo[4,3-c]pyridine-3,6(2H,5H)-dione | | Liver fibrosis, kidney fibrosis, lung fibrosis | Laleu *et al*., 2010; Gaggini *et al*., 2011; Aoyama *et al*., 2012; Green *et al*., 2012; Jha *et al*., 2014; Gorin *et al*., 2015; Jha *et al*., 2016 |
| APX-115 | Pan-Nox inhibitor | 3-Phenyl-4-propyl-1-(pyridin-2-yl)-1H-pyrazol-5-olhydrochloride | | Diabetic Nephropathy | Cha *et al*., 2017; Kwon *et al*., 2017; Dorotea *et al*., 2018 |

in resistance to DN progression as evidenced by reduction in ECM accumulation and albuminuria. In addition, inflammation events in Nox4 deficient renal cells were accompanied by reduced levels of NF-kB activity and subsequently of chemokines and MCP-1 and in renal tubular cell or podocyte in response to HG stimulation (Xu *et al*., 2010; Jha *et al*., 2014).

## DEVELOPMENT OF NOX INHIBITOR FOR THE TREATMENT OF DN

Oxidative stress plays an important role in DN, and clinical trials for therapeutic intervention for reduction of such oxidative stress are ongoing. That Nox isozymes are sources of oxidative stress for kidney is now well-established. Therefore, it is tempting to speculate that Nox isozymes will be viable therapeutic targets for DN treatment. Nox isozymes are expressed in various renal cells; Nox4 in EC, Nox1 in SMC, Nox1, Nox2, Nox4, and Nox5 in glomeruli, Nox4 and Nox1 in tubule cells. Of importance, studies with diabetic nephropathy animal models with Nox gene mutation suggest that pan-Nox inhibitors are more effective rather than isozyme-specific inhibitors.

### GKT137831 (Setanaxib)

Pyrazolopyridine compound, a dual inhibitor of Nox1 and Nox4 was developed by Genkyotex. GKT136901 was the lead compound identified from a high-throughput screening (Table 1) (Laleu *et al*., 2010). GKT137831 compound as a Nox1/4 inhibitor was subsequently obtained from a structure-activity relationship study of GKT136901. GKT137831 has been applied to various disease models. These preclinical studies showed that GKT137831 have therapeutic potential in fibrotic and inflammatory diseases of the liver (Aoyama *et al*., 2012), lung (Green *et al*., 2012) and other organs as well as kidney. According to the preclinical data, GKT137831 attenuated various parameters of glomerular injury, such as ECM accumulation, glomerular structural changes and albuminuria in streptozotocin-induced diabetic ApoE–/– mice (Jha *et al*., 2014). In another study, application of GKT137831 significantly reduced mesangial matrix expansion, urinary albumin excretion, and podocyte loss in OVE mice as a type I diabetes model (Gorin *et al*., 2015). GKT137831 is not only a dual inhibitor for the Nox1 and Nox4 isozymes but also a partial inhibitor of Nox5 (Gaggini *et al*., 2011; Jha *et al*., 2016). GKT137831 is now enrolled in a phase 2 clinical trial in primary biliary cholangitis (PBC, a fibrotic orphan disease) and type I diabetes and kidney disease (DKD).

### APX-115

APX-115 is first-in-class pan Nox inhibitor developed by Aptabio (Table 1). The Apx-115 compound protects against diabetic nephropathy in type II diabetic model *db/db* mice. Specifically, treatment of APX-115 significantly improved insulin resistance in diabetic mice. Oxidative stress as measured by plasma 8-isoprostane level was decreased by the APX-115 treatment. APX-115 also suppressed urinary albumin excretion and preserved creatinine clearance in *db/db* mice. In diabetic kidneys, APX-115 significantly suppressed mesangial expansion. Comparative study showed that APX-115 is more effective than dual Nox1/4 inhibitor GKT137831 (Cha *et al*., 2017). Another study compared APX-115 and Losartan (renin-angiotensin system inhibitor) in Streptozotocin (STZ)-induced

type I diabetic mouse. APX-115 inhibited renal oxidative stress and prevented albuminuria, glomerular hypertrophy, tubular injury, podocyte injury, fibrosis and inflammation in diabetic kidneys as effectively as losartan alone which is currently used as the standard treatment against kidney injuries in diabetic patients (Kwon *et al*., 2017). Moreover, treatment with APX-115 in STZ-induced diabetic rat was also as effective as losartan (Dorotea *et al*., 2018). APX-115 is more effective than GKT137831 based on inflammation or fibrosis. This suggests that pan-Nox inhibitors are more effective therapeutics for DN treatment than Nox isozyme-specific inhibitors. This is not surprising given that multiple Nox isozymes are expressed in overlapping manners in multiple cell types of kidney, which all appear to be affected by ROS one way or another. Of note, APX-115 treatment decreased expression of all Nox isozyme expression at the protein level in the kidney. Pan-Nox inhibition might therefore be a promising therapy for diabetic nephropathy.

## CONCLUSION

Currently, no effective therapy exists for treatment of DN. Based on the damage ROS brings to kidney during pathogenesis of DN, Nox isozymes have emerged as promising targets. It should be noted that chronic hyperglycemia in diabetes stimulates the expression and activation of Nox isozymes in renal tissues leading to a sustained oxidative stress. There are various pathways that may contribute to stimulation of Nox-dependent ROS generation. Therefore, Nox isozymes in renal tissues should be effective therapeutic targets for DN treatment. Now, two Nox inhibitors (GKT137831 and APX-115) are enrolled in clinical phase II for DN patients. These Nox inhibitors may represent best hope to date to many DN patients with no other recourse.

## ACKNOWLEDGMENTS

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