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REVIEW

# The NADPH Oxidase Family and its Inhibitors

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**Abstract** The classical nicotinamide adenine dinucleotide phosphate (NADPH) oxidase was originally detected in neutrophils as a multicomponent enzyme that catalyzes the generation of superoxide from oxygen and the reduced form of NADPH. This enzyme is composed of two membrane-bound subunits (p22phox and gp91phox), three cytosolic subunits (p67phox, p47phox, and p40phox) and a small G-protein Rac (Rac1 and Rac2). Recently, it has been demonstrated that there are several isoforms of nonphagocytic NADPH oxidase. Endothelial cells, vascular smooth muscle cells or adventitial fibroblasts possess multiple isoforms of this enzyme. The new homologs, along with gp91phox are now designated the Nox family of NADPH oxidases and are key sources of reactive oxygen species in the vasculature. Reactive oxygen species play a significant role in regulating endothelial function and vascular tone. However, besides the participation in the processes of physiological cell, these enzymes can also be the perpetrator of oxidative stress that causes endothelial dysfunction. This review summarizes the current state of knowledge of the structure and functions of NADPH oxidase and NADPH oxidase inhibitors in the treatment of disorders with endothelial damage.

**Keywords** NADPH oxidase · Nox family · Nox inhibitors · Reactive oxygen species · Cardiovascular disease

**Abbreviations**

| | |
|---|---|
| ROS | Reactive oxygen species |
| PKC | Protein kinase C |
| $H_2O_2$ | Hydrogen peroxide |
| GSH | Reduced glutathione |
| NO | Nitric oxide |
| MPO | Myeloperoxidase |
| DPI | Diphenyleneiodonium chloride |
| AEBSF | 4-(2-aminoethyl)-benzenesulphonyl fluoride; |
| | S17834: 1,4-dimethyl-2,3,5,6-tetraiodobenzene |
| VAS-2870 | 3-benzyl-7-(2-benzoxazolyl)thio-1,2,3- |
| | triazolo(4,5- d) pyrimidine |
| PR-39 | Proline-arginine-rich antimicrobial peptide |
| VEGF | Vascular endothelial growth factor |
| TNF-α | Tumor necrosis factor α |
| ox-LDL | Oxidized low-density lipoprotein |
| AGEs | Advanced glycation end-products |
| NEFAs | Non-esterified fatty acids |
| PDGF | Platelet-derived growth factor |
| VSMCs | Vascular smooth muscles cells |
| FAD | Flavin adenine dinucleotide |

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## Introduction

Nicotinamide adenine dinucleotide phosphate (NADPH) oxidase was first described in phagocytic cells by Rossi and Zatti in 1964, as the enzyme responsible for the respiratory burst. Nine years later, Babior et al. (1973) demonstrated the first evidence that this enzyme is responsible for

reactive oxygen species (ROS) generation. Babior (1999) also presented the structure of the classical NADPH oxidase of phagocytes as consisting of five subunits: p67phox ("phox" stands for phagocyte oxidase), p47phox, p40phox, p22phox and the catalytic subunit gp91phox (Vignais 2002). Nowadays, we know that there is a whole family of nonphagocytic NADPH oxidases, consisting of a few members, which are homologues of the catalytic subunit gp91phox. These isoforms of NADPH oxidase are named Nox1, Nox3, Nox4, Nox5, Duox1 and Duox2 as well as Nox2, known as gp91phox (Dworakowski et al. 2008; Geiszt 2006; Miller et al. 2006). Nox1, Nox2, Nox3, and Nox5 are transmembrane proteins that transport electrons across biological membranes to reduce oxygen to superoxide (Miller et al. 2006). Nox4, Duox1 and Duox2 do not produce superoxide, they produce directly hydrogen peroxide ($H_2O_2$) (Chen et al. 2008). Therefore, the essential function of Nox enzymes is generation of ROS that participates in the induction of host defense genes, phosphorylation of kinases, activation of transcription factors, mobilization of ion transport systems (Touyz and Schiffrin 2004). In the vascular system, ROS plays a significant role in regulating endothelial function and vascular tone. However, besides the participation in the processes of physiological cell, the Nox family of enzymes can also be the perpetrator of oxidative stress that causes endothelial dysfunction, inflammation, apoptosis, migration, hypertrophy, fibrosis, angiogenesis, and rarefaction (Landmesser and Harrison 2001). Due to the participation of NADPH oxidases in cardiovascular diseases, there is a need to develop selective inhibitors of these enzymes (Selemidis et al. 2008).

## Structure

In phagocytic cells, NADPH oxidase is composed of two membranes—bound elements gp91phox and p22phox, three cytosolic proteins: p40phox, p47phox, p67phox and a small G-protein Rac.

Gp91phox and p22phox proteins form a heterodimer linked permanently with the plasma membrane. This complex is called flavocytochrome b558 due to its maximum absorption at 558 nm, although it is catalytically inactive in resting cells, moving toward the cytosolic subunits of the cell membrane and forming an active complex with NADPH oxidase (Babior 1999; Leusen et al. 1996).

Wallach and Segal (1997) first presented the structure of the catalytic subunit gp91 phox also called Nox2. The gp91phox consists of 570 amino acids. Its polypeptide chain is composed of six domains in the membrane and contains at least three glycosylation sites (Asn131, Asn148, and Asn239). Raad et al. (2009) have shown that gp91phox (Nox2) is phosphorylated during activation of human neutrophils. They have provided evidence that protein kinase C (PKC) is involved in this process, and they have proved that phosphorylation potentiates intrinsic diaphorase activity of gp91phox and interaction with Rac, p67phox, and p47phox. These results suggest that phosphorylation of gp91phox by PKC also participates in the regulation of phagocyte NADPH oxidase activity. The C-terminal region is cytosolic and contains a flavoprotein domain, which is homologous to known flavoprotein dehydrogenase flavin adenine dinucleotide (FAD)-binding sequences, as well as a consensus sequence representing a putative NADPH-binding site (Vignais 2002).

Biberstine-Kinkade et al. (2001) have proved that the Gp91phox is a hemoprotein whose two heme centers are located between the third and fifth membrane domain. The iron atom of the first heme group is located closer to the surface of the cytoplasm and is associated with histidine residues His101 and His209. A second heme center is associated with residues His115 and His222. This protein is encoded by the gene CYBB (cytochrome b beta), which is located on the X chromosome at position 21.1 (Bolscher et al. 1991).

The p22phox polypeptide chain consists of 195 amino acids and has four membrane domains. This protein has a proline-rich sequence involved in the binding of p47phox-SH3 domains (DeLeo et al. 1995). Zhu et al. (2006) have demonstrated that this subunit possesses a cytoplasmic N-terminus, two transmembrane α-helices connected by a short extracellular loop and a C-terminus that extends back into the cytoplasm. Dahan et al. (2002) have proved that the p20phox protein has an additional binding site for p47phox and two potential binding sites for p67phox. The p22phox is phosphorylated on threonine residues by a phosphatidic acid activated kinase and PKC. This protein is encoded by the gene CYBA (cytochrome b alpha), which is located on chromosome 16q24 (Dinauer et al. 1990). All Noxes except for Nox5 seem to have an obligatory need for p22phox (Takeya and Sumimoto 2006).

Subunit p67phox consists of 526 amino acids with two src-homology (SH)3 domains, four tetratricopeptide-rich regions and a proline-rich region (Groemping and Rittinger 2005; Lapouge et al. 2002; Koga et al. 1999). Tetratricopeptide repeat motifs are responsible for binding Rac proteins. The C-terminal domain houses the Phox and Bem1p (PB1) domain, whose function is to create heterodimers between proteins containing the PB1 domain. P67phox is phosphorylated on its serine and threonine residues by PKC-dependent and PKC-independent pathways (Dang et al. 2003). This protein is encoded by the gene neutrophil cytosol factor (NCF)2, which is located on chromosome 1q25 (Francke et al. 1990) and acts as a link between p40phox and p47phox in complex.

The p40phox protein consists of 339 amino acids, one SH3 domain and one Px domain. The PC motif is located at the C-terminus of the protein, which is an evolutionarily conserved fragment of the PB1 domain and is necessary for connecting heterodimers at the PB1 domain (Lapouge et al. 2002). The p40phox is phosphorylated at the serine 315 and threonine 154 residues by a PKC mechanism (Bromberg and Pick 1985). This protein is encoded by the gene NCF4, which is located on chromosome 22q13.1 (Zhan et al. 1996). The p40phox protein is not necessary to activate the oxidase complex; its function is not clear—might be to stabilize the complex of p67phox-p47phox in the cytoplasm (Babior 2004).

Subunit p47phox is composed of 390 amino acids. This protein contains two SH3 domains, one phox homology Px domain, a proline-rich region and one auto-inhibitory region (Selemidis et al. 2008). The p47phox protein acts as a connector between the components of the membrane and the cytoplasm. Cytoplasmic p47phox occurs in a conformation in which its activity is inhibited (auto-inhibited conformation), thus preventing the combination of these two components. Both SH3 domains are blocked by intramolecular interaction with a region rich in proline residues in the C-terminus of the protein. Through phosphorylation by PKC, the SH3A domain can bind to the proline-rich region in the p22phox protein. This process leads to complex integration and encourages the flow of electrons and the production of superoxide anions. In the presence of an appropriate stimulus such as phorbol 12-myristate 13-acetate (PMA), serine residues on p47phox become phosphorylated (Fontayne et al. 2002). Dewas et al. (2003) have proved that tumor necrosis factor (TNF)-α induces phosphorylation of p47phox in human neutrophils. PKC is reported to be involved in regulating the phosphorylation of NADPH oxidase components in human neutrophils. However, the regulatory role of specific isoforms of PKC in phosphorylating particular oxidase components has not been determined (Bey et al. 2004).

The novel cytosolic subunits were named NoxO1 (Nox organizer 1 = p47phox homolog) and NoxA1 (Nox activator 1 = p67phox homolog) (Quinn et al. 2006). In transfected cells, Nox1 is also able to use the p47phox and p67phox subunits, suggesting that cytosolic subunits are not specific for a given Nox protein (Banfi et al. 2003). It seems likely that p47phox acts as a subunit of Nox1 in the vascular system. In addition, using the mouse proteins suggests a constitutive activity of the Nox1/NoxO1/NoxA1 system; studies using human proteins have shown only a weak constitutive activity, and full activation depends on activation through the PKC activator PMA (Geiszt et al. 2003). There are significant differences between the mouse and the human proteins, in particular, in the region of the phox homology domain, which is distinct in human NoxO1. It is not clear whether the difference in PKC dependence is really due to a difference between the mouse and human Nox1/NoxO1/NoxA1 system or whether this reflects some experimental details, such as cell lines or the use of transient versus stable expression systems (Bedard and Krause 2007).

In nonphagocytic cells, the gp91phox component may be replaced by homologous Nox and Duox proteins. Nox1, Nox2, Nox4, and Nox5 have been also identified in vascular tissue.

Nox1 protein was the first homologue of gp91phox to be described (Lee et al. 2005; Szanto et al. 2005). It is composed of 564 amino acid residues and although it shows 56 % similarity to gp91phox, the FAD-binding site and heme-binding sites are nearly identical in both proteins (Bataller et al. 2003). The Nox1 protein is encoded by the gene Nox1 located on chromosome X (Xp21) (Suh et al. 1999). This isoform is found mainly in the colon epithelium, but also at lower levels in vascular smooth muscle cells, endothelial cells, uterus, placenta, prostate, osteoclasts, and retinal pericytes (Nisimoto et al. 2008). Nox1 needs p22phox, p47phox and p67phox for its activity. Fernandes et al. (2009) have described that in vascular smooth muscle cells, this process is regulated by the redox chaperone protein disulfide isomerase. Nox1 produces mostly superoxide, which is later converted to $H_2O_2$.

Nox3 was identified by Kikuchi et al. (2000). This protein consists of 568 amino acids and shows 58 % similarity to gp91phox. Nox3 was detected in several fetal tissues, including kidney, liver, lung, and spleen (Banfi et al. 2004).

Nox4 is a 578 amino acid protein that shares 39 % homology with gp91phox. It has been described as an NADPH oxidase homologue in the kidney, as well as endothelial cells, smooth muscle cells, heart, pancreas, and osteoclasts (Kawahara and Lambeth 2007; Petry et al. 2006) and has also been found in the nucleus of vascular cells, mitochondria and endoplasmic reticulum. According to Chen et al. (2008), Nox4 generates mainly $H_2O_2$. It is possible that this isoform does not need p47phox, p67phox, p40phox or Rac for its activation. Clempus et al. (2007) have suggested that Nox R1 may be relevant during the activation of Nox4.

Nox5 is a $Ca^{2+}$ dependent homologue, composed of 737 amino acids, which has been identified in human lymphoid tissues, testis, spleen, and endothelial cells (Jagnandan et al. 2007; Serrander et al. 2007). Si et al. (2007) have demonstrated that mouse and rat genomes do not include the Nox5 gene. This isoform has an amino-terminal calmodulin-like domain with four binding sites for $Ca^{2+}$. These Noxes do not need p22phox or other subunits for their activation. Nox5 is regulated by intracellular $Ca^{2+}$ high levels of which can lead to excessive ROS production

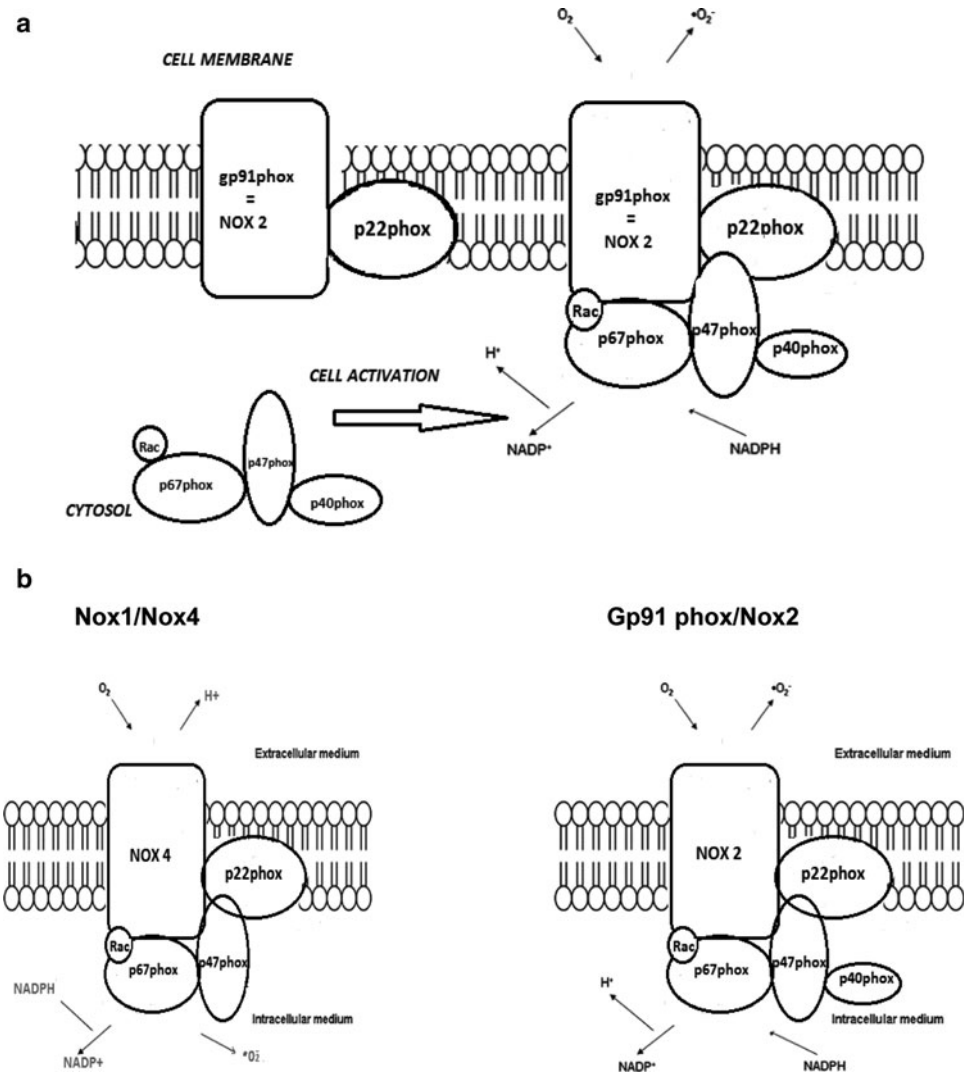


**Fig. 1** **a** The mechanism of NADPH oxidase activation. NADPH oxidases are multisubunit membrane proteins that are activated by translocation of the cytosolic subunits p47phox, p67phox, and Rac to the Nox/p22phox complex. **b** The vascular NADPH oxidase complex may contain Nox1 or Nox4 as substitutes for catalytic gp91 phox subunit (Nox2) of the phagocytic oxidase. Superoxide is produced intracellularly (in opposite to the Nox2—superoxide is produced extracellularly). The Nox subunit is bound to p22 phox in the plasma membrane and they stabilize each other. $O_2^-$: superoxide anion. Based on Dusting et al. 2005

(BelAiba et al. 2007). Schulz and Münzel (2008) have proved that Nox5 has participated in endothelial cell proliferation, migration and angiogenesis, as well as oxidative damage in atherosclerosis. According to recent reports, thrombin, platelet-derived growth factor (PDGF), Ang II and endothelin-1 are all Nox5 activating agents (Montezano et al. 2011).

Summing up, Nox1, Nox2, Nox4, and Nox5 have been detected in vascular tissue. All of these Nox family members play a significant role in the generation of ROS in the vasculature but the main causative factor overproduction of ROS in endothelial cells is Nox2 (Fig. 1).

## Inducers for Activating NADPH Oxidase

NADPH oxidases are activated and regulated by several factors such as agonists of G-protein-coupled receptors (angiotensin II and endothelin 1); growth factors: thrombin and vascular endothelial growth factor (VEGF); cytokines: TNF-α; metabolic factors: increased glucose, insulin, non-esterified fatty acids (NEFAs) or advanced glycation end-products (AGEs), oxidized lipids, oscillatory shear stress, hypoxia/re-oxygenation and nutrient deprivation (Table 1) (Brandes and Schröder 2008; Ibi et al. 2008; Ray and Shah 2005). Essential for p47phox, phosphorylation has been presented for acute activation by angiotensin II, phorbol esters, TNF-α, VEGF and oscillatory shear, using multiple specific molecular approaches such as the use of p47phox-deficient cells and p47phox transfection (Ray and Shah 2005; Li et al. 2003). Hu et al. (2000) have described that primary oxidase activity in endothelial cells is inhibited by a dominant-negative Rac mutant as is oxidase activation by shear stress, depolarization, hypoxia/re-oxygenation, nutrient deprivation or VEGF. Rueckschloss et al. (2001) have shown that atherosclerotic levels of low-density lipoprotein (LDL) stimulate NADPH oxidase-generated superoxide anion, which may contribute to LDL oxidation.

**Table 1** Inducers for activating NADPH oxidase

| Activators of NADPH oxidase | Regulatory subunit-involved p47phox | Regulatory subunit-involved Rac |
|---|---|---|
| G-protein-coupled-receptor agonist | | |
| Angiotensin II | √ | √ |
| Endothelin 1 | | |
| Growth factors | | |
| Thrombin | | √ |
| VEGF | √ | √ |
| Cytokines | | |
| TNF-α | √ | |
| Metabolic | | |
| ox-LDL | | |
| NEFAs | | |
| Glucose | | |
| Insulin | | |
| AGEs | | |
| Mechanical factors | | |
| Shear stress | √ | √ |
| Flow cessation | | |
| Nutrient deprivation | | √ |
| Hypoxia re-oxygenation | | √ |

Adapted from Ray and Shah 2005

*VEGF* vascular endothelial growth factor, *TNF-α* tumor necrosis factor α, *ox-LDL* oxidized low-density lipoprotein, *AGEs* advanced glycation end-products, *NEFAs* non-esterified fatty acids, √ activating effect

This oxidized LDL (ox-LDL) acts as an especially potent stimulus for oxidase activation, both in the vasculature and in macrophages. Li et al. (2005) have demonstrated that TNF-α-induced endothelial oxidase activation and downstream phosphorylation of extracellular signal-regulated kinase 1/2 involves its binding to TNF-receptor-associated factor 4. In the cardiovascular system, angiotensin II activates NADPH oxidase via AT1 receptors through stimulation of signaling pathways involving c-Src p21Ras, phospholipase D, phospholipase A2, and PKC (Touyz and Briones 2011; Block 2008). Li and Shah (2003) have reported that endothelin 1 is an important pathogenic factor responsible for the increased oxidase activity.

Lyle et al. (2009) have proved that ClC-3, Poldip2, and protein disulfide isomerase can also regulate Nox enzymes. In diabetes, NADPH oxidase is activated by several factors such as hypercholesterolemia, hyperglycemia, elevated NEFAs, hyperinsulinemia, increased AGEs and activation of the renin–angiotensin system (Lassegue and Clempus 2003)

Recent studies have proved that NADPH oxidase activation is sensitively regulated by a wide range of pathophysiological and physiological factors.

## Inhibitors of NADPH Oxidases

NADPH oxidases inhibitors may block their electron transferring components, can remove and/or prevent assembly of the oxidase by interacting with their subunits at the molecular level, can also reduce subunit gene and protein expression and modify the oxidases and/or their activators (Selemidis et al. 2008).

There are currently no potent specific Nox inhibitors. Studies include compounds that reduce oxidative stress through inhibiting ROS production by NADPH oxidases: apocynin, diphenyleneiodonium chloride, pefabloc, proline-arginine-rich antimicrobial peptide (PR-39), and new peptide inhibitors that have been developed to specifically target NADPH oxidases include the gp91ds-tat and novel non-peptide VAS-2870. In this review, we will briefly describe some of the most commonly used compounds.

The best known inhibitor of NADPH oxidase is apocynin. This blocker (4′-hydroxy-3′-methooxyacetophenone) also known as acetovanillone, was first isolated from the roots of Apocynum cannabinum by Schmiedeberg in 1883 (Stefanska and Pawliczak 2008). Acetovanillone is a selective blocker of NADPH-oxidase activity. Apocynin inhibits the association of p47phox with the membrane heterodimer in leukocytes, monocytes, and endothelial cells and also acts as a scavenger of $H_2O_2$ (Johnson et al. 2002). This blocker does not inhibit enzyme in cells devoid or deficient of myeloperoxidase (MPO). Recently it has been indicated that the features of apocynin as a blocker of NADPH-oxidase and its subordination on MPO-catalyzed oxidation might be associated with the reaction of the acetovanillone radical and/or its dimer radical, intracellular reduced glutathione (GSH) or with the thiols of the p47phox (Ximenes et al. 2007; Vejrazka et al. 2005). Apocynin in very high concentrations has been found to block Nox4 and Nox5 (Ellmark et al. 2005), making it more effective against Nox2, Nox4 and Nox5-dependent NADPH oxidase activity. However, apocynin inhibition of Nox1 and Nox3 isoforms remains to be determined. Interestingly Riganti et al. (2004) have demonstrated that apocynin may really stimulate ROS generation in non-phagocytic cells.

Heumüller et al. (2008) provide evidence that acetovanillone acts as an antioxidant but not as a blocker of NADPH oxidases in nonphagocytic cells. They have indicated that the inhibitory action of apocynin is narrowed to MPO-expressing leukocytes and that the compound does not block NADPH oxidases in MPO-free vascular cells. It seems that apocynin has relatively few side effects in vivo (Lafeber et al. 1999), future work is needed to determine any further impact on human disease.

The most commonly used Nox inhibitor is diphenyleneiodonium (DPI) chloride (also known as dibenziodolium

chloride). Its activity is associated with the abstraction of electrons from an electron transporter and the creation of a radical which then blocks the appropriate transporter of electrons through a covalent binding step (Selemidis et al. 2008). DPI is not selective towards any concrete isoform of NADPH oxidase; it inhibits almost all of the Nox family. DPI also inhibits NADH dehydrogenase, nitric oxide synthase, glucose phosphate dehydrogenase, xanthine oxidase, mitochondrial complex I and cytochrome P-450 reductase. Hutchinson et al. (2007) have proved that this blocker stimulates glucose uptake in skeletal muscle cells and reduces in oxygen consumption. DPI can be used as a neuroprotective blocker in the treatment of symptoms associated with reperfusion injury including stroke and cerebral hemorrhage (Li et al. 2006). DPI also reduces ROS formation under normal conditions (Stielow et al. 2006) which could be an advantage for therapy targeted against excessive ROS levels. However, due to potentially toxic non-specific effects, DPI is inadequate for clinical use.

One of the inhibitors of NADPH oxidases is also 4-(2-aminoethyl)-benzenesulphonyl fluoride (AEBSF; known as pefabloc). This blocker inhibits the binding of the cytoplasmatic subunit p47phox to the flavocytochrome b558 (Diatchuk et al. 1997). This process is dependent on its reactive sulphonyl fluoride group and positively charged aminoalkyl moiety. AEBSF inhibits NADPH oxidase-dependent superoxide generation in vascular smooth muscle cells. Pefabloc inhibits Nox2 including activity of NADPH oxidase. This effect is associated with acute interruptions to the blood–brain barrier after a stroke (Kahles et al. 2007). However, Martyn et al. (2006) have suggested that AEBSF has no effect on Nox4. So far, nothing is known about the properties and toxicity of this blocker and it has not been proven yet, whether AEBSF really inhibits any homologues of NADPH oxidase (Bedard and Krause 2007).

Another pharmacological inhibitor of NADPH oxidases is gp91ds-tat. This 18-amino acid peptide inhibits Nox2 by mimicking a sequence within the enzyme. By binding to p47phox, Gp91ds-tat not only inhibits the activity of Nox2, but quite probably that of the Nox1-dependent NADPH oxidases in the vascular wall. This inhibitor demonstrates the effectiveness of the blocking the increase in ROS generation caused pro-atherogenic stimuli—Ang II (Yang et al. 2005). Gp91ds-tat was described as strongest in inhibiting superoxide generation in the vascular system, where Nox1 and Nox4 play a key role. However, this blocker would have poor oral bioavailability and a propensity to induce immunogenic responses in patients, making it of limited use in treating cardiovascular disease.

Toxin B produced by clostridium difficile inhibits Rac-dependent NADPH oxidase activation. It seems that Clostridium toxin B affects Nox1, Nox2 and Nox3 but does not demonstrate any significant inhibitory effect on Nox4 and Nox5 (Selemidis et al. 2008). Rho proteins (Rac, Cdc42) inhibited by Clostridium toxin B play a very important function in the regulation of endothelial cell morphology, adhesion and motility. These proteins also affect on transcription factors (nuclear factor $\kappa$B) and the expression of E-selectin, P-selectin, vascular cell adhesion molecule-1 and intercellular adhesion molecule-1 levels. Clostridium toxin B can modulate endothelial cell phenotype, which leads to vascular permeability and inflammation (Cernuda-Morollon and Ridley 2006). Like any toxin, this inhibitor has cytotoxic properties and hence limited clinical application.

PR-39, cathelicidin antimicrobial peptide is a naturally-occurring peptide inhibitor of NADPH oxidase. This blocker binds to p47phox hence inhibiting the subsequent binding of p47phox to p22phox. PR-39 has an inhibitory effect on NADPH oxidase isoforms dependent on p47phox and NoxO1 for activity Nox1 and Nox2 (Kawahara et al. 2005). In vivo, PR-39 stimulates angiogenesis and protects against ischemia. However, because it is a peptide, its use is severely restricted.

Another inhibitor of NADPH oxidase is S17834, a synthetic polyphenol compound. S17834 (1,4-dimethyl-2,3,5,6-tetraiodobenzene) suppresses superoxide generation and NADPH oxidase activity in human umbilical vein endothelial cells in response to TNF-$\alpha$ and NADPH oxidase activity (Kalinowski and Malinski 2004; Selemidis et al. 2008). Therefore it may have an inhibitory effect on Nox2 and Nox4. This blocker reduces pro-atherogenic effect associated with the production of superoxide (Zang et al. 2006). However, the pharmacokinetic properties and toxicity of S17834 are still largely unclear.

VAS-2870, 3-benzyl-7-(2-benzoxazolyl) thio-1,2,3-triazolo(4,5-d) pyrimidine was described as a new non-peptide inhibitor of NADPH oxidase by Stielow et al. (2006). However, its mechanism of action has not been determined yet. VAS-2870 blocks NADPH oxidase activity in Nox2-including HL-60 cell lines and in vascular endothelial cells including Nox2 and Nox4. It is likely that VAS-2870 interacts with the Nox and p22phox subunits and does not act by interfering with the assembly process of the oxidase and with the signaling pathways. This blocker shows no superoxide scavenging features but its pharmacokinetic properties and toxicity have not been assessed yet. However, although this blocker was found to be effective in suppressing PDGF-BB-dependent activation of NADPH oxidase and generation of intracellular ROS. It seems that VAS-2870 does not reduce ROS generation in normal conditions and so isn't suitable for this kind of therapy (Stielow et al. 2006).

Recently, statins have been shown to inhibit NADPH oxidase activity. Mevastatin decreases superoxide

production by inhibiting synthesis of farnesylpyrophosphate and geranylgeranylpyrophosphate which are crucial for membrane attachment of Rac and NADPH oxidase assembly. Mevastatin also decreases p22phox and Nox1 expression. This blocker is able to influence Nox1 and Nox2 activation. However, statins cannot be used as specific NADPH oxidase inhibitors (Bokoch and Prossnitz 1992; Wassmann et al. 2002).

Losartan, drug used mainly to treat high blood pressure, decreases Ang II-dependent activation of NADPH oxidase via AT1 receptors. This AT1 receptor antagonist is unlikely to display Nox selectivity as Ang II stimulates Nox1 and Nox4 oxidation (Rajagopalan et al. 1996).

One of the inhibitors of NADPH oxidases is a $\beta$1 receptor blocker nebivolol. This blocker inhibits membrane association, interaction of p67phox and Rac and decreases oxidase expression. Nebivolol also inhibits Nox1-dependent superoxide production (Mason et al. 2005; Mollnau et al. 2003; Oelze et al. 2006; Troost et al. 2000).

Gliotoxin (GT), a metabolite of pathogenic fungi, such as Candida and Aspergillus inhibits phosphorylation of p47phox by preventing PKC co-localization with p47phox. GT also inhibits electron transport through the flavocytochrome before oxidase activation. This blocker has low potency for blocking Nox4 (Kawahara et al. 2004).

Other blocker, metformin is now believed to be the most widely prescribed antidiabetic drug in the world. It inhibits PMA and Ang II-dependent ROS production from NADPH oxidase. Metformin is a scavenges hydroxyl radicals but not superoxide (Bonnefont-Rousselot et al. 2003).

Shukla et al. (2005) have described sildenafil-citrate as a blocker that inhibits endothelial superoxide production and gp91phox expression in response to the thromboxane mimetic U46619 via increases in cGMP.

Bilirubin can inhibits superoxide production in neutrophil lysates when applied to cytosolic fractions prior to reconstitution with membrane fractions and sodium dodecyl sulphate stimulation. It is unlikely to influence expression of NADPH oxidase subunits Nox2, p22phox and p47phox but may reduce p47phox phosphorylation (Pflueger et al. 2005).

Next blocker, benzylisothiocyanate, can be in concentration-dependently blocks 12-O-Tetradecanoylphorbol-13-acetate-induced superoxide production in a human leukemia cell line without influencing PKC activity and p47phox translocation. This mechanism may involve covalent cysteine modification of the NADPH (Ippoushi et al. 2002; Miyoshi et al. 2004; Xiao et al. 2006).

Resveratrol, an important antioxidant found in grapes and wine decreases superoxide production in intact macrophages and homogenates. This blocker does not scavenge superoxide in cell-free systems. It is an inhibitor of PKC (Deby-Dupont et al. 2005; Leiro et al. 2004; Poolman et al. 2005).

Choi et al. (2005) have proved that tetracycline derivative—minocycline inhibits NADPH oxidase-derived superoxide production in microglia and dopaminergic neurons in response to stimuli such as thrombin by down regulating p67phox expression.

Next blocker of NADPH oxidase—perhexiline inhibits superoxide production in intact neutrophils stimulated by PMA, by an undefined mechanism. Perhexiline does not inhibit enzyme assembly. It is an efficacious anti-anginal agent that inhibits carnitine-palmitoyl-transferase (Ashrafian et al. 2007; Kennedy et al. 2006).

Roxithromycin is a semi-synthetic macrolide antibiotic. It is used to block superoxide generated by intact neutrophils activated by PMA. This antibiotic does not inhibit PKC-dependent phosphorylation. Roxithromycin inhibits translocation of p47phox and/or p67phox to the plasma membrane. It inhibit RNA-dependent protein synthesis. Moreover, it is a capable of inhibiting cytochrome P450 (Abdelghaffar et al. 2005).

Taurine, or 2-aminoethanesulfonic acid, is an organic acid widely distributed in animal tissues. Taurine chloramine is responsible for inhibition of PMA-dependent superoxide anion production in human neutrophils by interfering with the translocation of p47phox and p67phox to the membrane. Barua et al. (2001) have proved that this blocker also inhibits phosphorylation of p47phox.

Next blocker, plumbagin inhibits NADPH-dependent superoxide production in various cell lines that express Nox4. Napthoquinone structure may impart ROS-scavenging effects. However, mechanism of action of this blocker is unknown (Fan et al. 2005).

Pharmacological inhibitors of NADPH oxidase which block directly the activity of this enzyme are also shown in the Table 2 and Fig. 2.

## Role of Nox Inhibitors in Hypertension and Atherosclerosis

### Nox Inhibitors in Hypertension

There are many factors that can contribute to hypertension; genetics, age, obesity and salt sensitivity are only some of them. Many of them lead to or are connected with, increased total peripheral resistance, whose attenuation can be caused by ROS. ROS, as well as reactive nitrogen species, are important signaling molecules in cardiovascular cells. Among ROS are superoxide anions, hydroxyl radicals and $H_2O_2$, which all take part in growth and apoptosis, as well as the expression of proinflammatory compounds and the modulation of endothelial functions

**Table 2** Inhibitors of NADPH oxidases

| Name of inhibitor | Mechanism of action | Farmacological effects | References |
|---|---|---|---|
| Apocynin (extracted from the *Picroria kurroa* plant) | Oxidase assembly inhibitor: inhibits association of p47phox with membrane heterodimer. Scavenger of hydrogen peroxide | Possible remedy for inflammation-mediated diseases, including asthma, arthritis, and cardiovascular diseases | Simons et al. (1989); Stolk et al. (1994 ); t Hart et al. (1990); |
| DPI (non-peptide) | Flavoprotein inhibitor: abstracts electrons from FAD and prevents electron flow through the flavocytochrome conduit | Inhibitor of NADH ubiquinone oxidoreductase, NADH dehydrogenase, xanthine oxidase, cytochrome P450 oxidoreductase, NOS, bacterial nicotine oxidase, decreased ox-LDL | Brandsch and Bichler (1987); Cross and Jones (1986); Cross et al. (1984); Gatley and Sherratt (1976); O'Donnell et al. (1993, 1994); Stuehr et al. (1991) |
| AEBSF (non-peptide) | Oxidase assembly inhibitor: inhibits association of Nox2 with p47phox. Does not scavenge superoxide generated in cell-free systems | Non-selective serine protease inhibitor | Diatchuk et al. (1997) |
| Gp91dstat (peptide) | Oxidase assembly inhibitor: inhibits association of Nox2 with p47phox. Does not scavenge superoxide generated by cell-free systems | No other reported effects. Probably propensity to induce immunogenic responses | DeLeo et al. (1995); Rey et al. (2001) |
| PR-39 (peptide) | Binds to SH3 domains of the p47phox subunit and prevents binding to p22phox | Stimulates angiogenesis and protects against ischemia | Shi et al. (1996) |
| Statins (non-peptide) | Decrease superoxide production by inhibiting synthesis of farnesylpyrophosphate and geranylgeranylpyrophosphate which are crucial for membrane attachment of Rac and NADPH oxidase assembly. May also decrease p22phox and Nox1 expression. Likely to influence Nox1 and Nox 2 activities | HMG-CoA reductase inhibitor. Decreases AT1 receptor expression; increases eNOS expression, most effective for treating cardiovascular disease with questionable benefit in those without previous CVD but with elevated cholesterol levels | Bokoch and Prossnitz (1992); Wassmann et al. (2002) |
| AT1 receptor antagonists (non-peptide) | Decrease Ang II-dependent activation of NADPH oxidase via AT1 receptors. Unlikely to display Nox selectivity as Ang II stimulates Nox1 and Nox4 oxidases | No other reported effects, used for controlling high blood pressure | Rajagopalan et al. (1996) |
| Nebivolol (non-peptide) | Inhibits membrane association and also interaction of p67phox and Rac and decreases oxidase expression. Inhibits Nox1-dependent superoxide production | $\beta$-adrenoceptor blocker, used in treatment of hypertension | Mason et al. (2005); Mollnau et al. (2003); Oelze et al. (2006); Troost et al. (2000) |
| Gliotoxin (non-peptide) | A fungal metabolite, thiol-modifying toxin thought to inhibit phosphorylation of p47phox by preventing PKC co-localization with p47phox. Also, inhibits electron transport through the flavocytochrome before oxidase activation. Low potency for blocking Nox4 | Stimulation of cGMP release. Cytoskeletal re-organisation. Disrupts the mitochondrial membrane potential, possesses immunosuppressive properties, anti-inflammatory activity. | Comera et al. (2007); Kawahara et al. (2004); Kweon et al. (2003); Serrander et al. (2007); Tsunawaki et al. (2004); Yoshida et al. (2000) |
| S17834 (non-peptide) | Flavonoid derivative proposed to directly inhibit NADPH oxidase activity; although the mechanism is still undefined | No other reported effects | Cayatte et al. (2001) |
| Clostridium difficile toxin B (peptide) | Glucosylation of threonine-35 on Rac, which modifies GTPase activity | Likely to inhibit all Rac-dependent protein activity. High toxicity, vascular permeability and inflammation | Sehr et al. (1998) |

**Table 2** continued

| Name of inhibitor | Mechanism of action | Farmacological effects | References |
|---|---|---|---|
| Nordihydroguaia-retic acid (non-peptide) | Blocks $H_2O_2$ production in macrophages in response to phorbol esters and in endothelial cells in response to thrombin | Lipoxygenase inhibitor. Blocks arachidonic acid metabolism | Holland et al. (1998); Ozaki et al. (1986); Tsunawaki and Nathan (1986) |
| SKF525A (non-peptide) | Decreases superoxide and $H_2O_2$ production in endothelial cells | Cytochrome P450 inhibitor | Holland et al. (1997, 1998) |
| Metformin (non-peptide) | Scavenges hydroxyl radicals but not superoxide. Could also inhibit PMA and Ang II-dependent ROS production from NADPH oxidase. However, this is likely to be due to inhibition of PKC activity | Antihyperglycemic agent. PKC inhibitor | onnefont-Rousselot et al. (2003); B Mahrouf et al. (2006); Ouslimani et al. (2005) |
| Sildenafil-citrate (non-peptide) | Inhibits endothelial superoxide production and gp91phox expression in response to the thromboxane mimetic U46619 via increases in cGMP | An inhibitor of phosphodiesterase type 5. Unlikely to be selective for any particular isoform of NADPH oxidase or a direct inhibitor of NADPH oxidase enzyme. Its PDE inhibitory actions are likely to influence many cellular systems. Changes in gp91phox expression could influence immune system | Koupparis et al. (2005); Marco and Michele (2007); Muzaffar et al. (2005); Shukla et al. (2005) |
| Bilirubin (non-peptide) | Can inhibit superoxide production in neutrophil lysates when applied to cytosolic fractions prior to reconstitution with membrane fractions and SDS stimulation. Unlikely to influence expression of NADPH oxidase subunits Nox2, p22phox and p47phox but may reduce p47phox phosphorylation | May also scavenge ROS | Kwak et al. (1991); Lanone et al. (2005); Pflueger et al. (2005) |
| Minocycline (non-peptide) | A tetracycline derivative that inhibits NADPH oxidase-derived superoxide production in microglia and dopaminergic neurons in response to stimuli such as thrombin by downregulating p67phox expression | Antibiotic | Agwuh and MacGowan (2006); Choi et al. (2005) |
| Perhexiline (non-peptide) | Inhibits superoxide production in intact neutrophils stimulated by fMLP or PMA, by an undefined mechanism. Does not inhibit enzyme assembly | Efficacious anti-anginal agent that inhibits carnitine-palmitoyl-transferase | Ashrafian et al. (2007); Kennedy et al. (2006) |
| Roxithromycin (non-peptide) | Blocks superoxide generated by intact neutrophils activated by fMPL or PMA but not by cell lysates. Does not inhibit PKC-dependent phosphorylation. May inhibit translocation of p47phox and/or p67phox to the plasma membrane | Antibiotic belonging to the macrolide class. Inhibit RNA-dependent protein synthesis. Capable of inhibiting cytochrome P450 | Abdelghaffar et al. (2005); Anderson (1989); Perry et al. (1992) |
| Taurine chloramine (non-peptide) | Reversible inhibition of PMA-dependent superoxide anion production in human neutrophils by interfering with the translocation of p47phox and p67phox to the membrane. Also inhibits phosphorylation of p47phox | Inhibits inducible NOS in alveolar macrophages | Barua et al. (2001); Kim et al. (1996) |
| Mastoparan (peptide) | Inhibition of superoxide production by neutrophil lysates most likely via interaction with N-terminal of p67phox. Does not interfere with translocation of p47phox and p67phox to membrane | An amphiphilic cationic tetradecapeptide isolated from wasp venom. Shows affinity towards SH3 domains. May interact with G-proteins | Jones and Howl (2006); Tisch et al. (1995); Tisch-Idelson et al. (2001); Yibin et al. (2005) |

**Table 2** continued

| Name of inhibitor | Mechanism of action | Farmacological effects | References |
|---|---|---|---|
| Ghrelin (peptide) | Inhibits superoxide production by thoracic aorta most probably via release of NO. Does not scavenge superoxide | The peptide is capable of releasing growth hormone releasing peptide. Stimulates gastric acid secretion | Kawczynska-Drozdz et al. (2006); Schubert (2007); Sun et al. (2007) |
| Alpha tocopherol (non-peptide) | Decreases stimulated superoxide production, inhibits p67phox-p47phox translocation and p47phox phosphorylation in monocytes, neutrophils and microglial cells. This effect is likely to be due to PKC inhibition | Quenches ROS | Cachia et al. (1998); Egger et al. (2001); Kanno et al. (1996); Venugopal et al. (2002) |
| Benzylisothiocyanate (non-peptide) | Concentration-dependently blocks TPA-induced superoxide production in a human leukemia cell line without influencing PKC activity and p47phox translocation. Mechanism may involve covalent cysteine modification of the NADPH oxidase | May inhibit NO, PGE2 and TNF-$\alpha$ production. Can cause apoptosis via induction of Bak and Bax proteins | Ippoushi et al. (2002); Miyoshi et al. (2004); Xiao et al. (2006) |
| Probucol (non-peptide) | Decreases superoxide production in rabbit aorta, which may be attributed to down-regulation of p22phox | Free radical scavenger | Inoue et al. (1998); Itoh et al. (1997, 2002); Keaney et al. (1995); tocker and Keaney (2004) |
| Resveratrol (non-peptide) | Decreases superoxide production in intact macrophages and homogenates. Does not scavenge superoxide in cell-free systems | Inhibitor of PKC | Deby-Dupont et al. (2005); Leiro et al. (2004); Poolman et al. (2005) |
| Curcurmin (non-peptide) | Decreases superoxide production in intact macrophages and homogenates. Does not scavenge superoxide in cell-free system | Is a potent irreversible inhibitor of thioredoxin reductase via alkylation of cysteine residues | Deby-Dupont et al. (2005); Fang et al. (2005) |
| Nitrolinoleate (non-peptide) | A nitrated lipid which inhibits PMA- and FMLP-dependent superoxide production and degranulation in human neutrophils by increasing cAMP but not cGMP levels | Other effects associated with raising cAMP. Vasorelaxation | Coles et al. (2002); Lima et al. (2005) |
| Mycophenolate acid (non-peptide) | A fungal derivative that inhibits endothelial and neutrophil-derived superoxide by reducing Rac levels and activation in the membrane. Does not alter mRNA levels of Nox2, Nox4 and p47phox | A potent inhibitor of inosine monophosphate dehydrogenase involved in purine synthesis in B and T lymphocytes | Allison and Eugui (2000); Krőtz et al. (2007) |
| Plumbagin (non-peptide) | Inhibits NAD(P)H-dependent superoxide production in various cell lines that express Nox4. Mechanism of action is unknown | Napthoquinone structure may impart ROS-scavenging effects | Fan et al. (2005); Rossary et al. (2007) |
| VAS-2870 (non-peptide) | Undefined mechanism of action. Inhibits NADPH oxidase activity in Nox2-containing HL-60 cell line and in vascular endothelial cells containing Nox2 and Nox4. Does not scavenge superoxide | No other reported effects | Stielow et al. (2006); ten Freyhaus et al. (2006) |

Adapted from Selemidis et al. 2008

*DPI* diphenyleneiodonium chloride, *AEBSF* 4-(2-aminoethyl)-benzenesulphonyl fluoride, *S178341*,4-dimethyl-2,3,5,6-tetraiodobenzene, *VAS-2870* -3-benzyl-7-(2-benzoxazolyl)thio-1,2,3-triazolo(4,5- d) pyrimidine, *SKF 525A* -2-diethylaminoethyl 2:2-diphenylvalerate hydrochloride, *PR-39* proline-arginine-rich antimicrobial peptide, *SDS* sodium dodecyl sulphate, *TPA* 12-O-Tetradecanoylphorbol-13-acetate

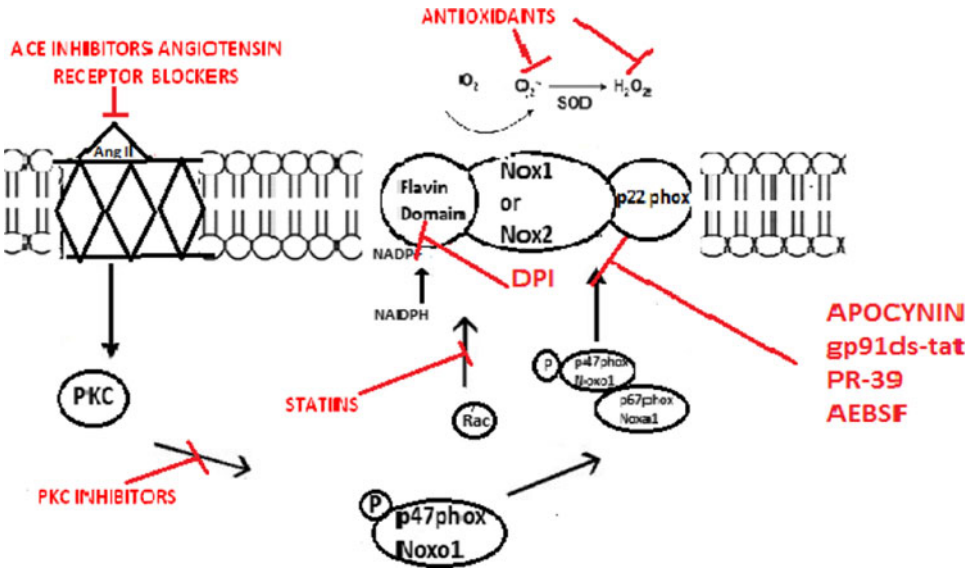


**Fig. 2** Mechanisms of NADPH oxidase inhibition. Most currently available specific inhibitors act by interfering with this translocation. Non-specific inhibitors target the flavin-containing subunit (DPI), major activators of the oxidase (ACE inhibitors and angiotensin receptor blockers), upstream kinases (PKC inhibitors) or act to scavenge the reactive oxygen species produced by NADPH oxidases (antioxidants). $O_2^-$: superoxide anion. Based on Williams and Griendling 2007

such as endothelium-dependent relaxation. A major source for vascular ROS is a family of nonphagocytic NADPH oxidases, including the prototypic Nox2 homolog-based NADPH oxidase, as well as other NADPH oxidases, such as Nox1 and Nox4. Other possible sources include mitochondrial electron transport enzymes, xanthine oxidase, cyclooxygenase, lipoxygenase, and uncoupled nitric oxide (NO) synthase.

It has been now recognized that Nox family of NADPH oxidases may play a key role in ROS generation, especially during vascular injury (Sedeek et al. 2009). In primary hypertension, Nox-derived superoxide decreases vascular NO-released from the endothelium resulting in peroxynitrite formation (Dai and Dai 2010). Moreover, vascular gene expression of the NADPH oxidase subunits p22phox, g p91phox, Nox1 and Nox4 are significantly attenuated during hypertension (Takai et al. 2011). A recent study also highlights the role played by three single-nucleotide polymorphisms (–930A/G, A640G and C242T) of the p22phox subunit in hypertension (Xaplanteris et al. 2010); it was found that the –930A/G, but not the A640G and C242T polymorphism of p22phox is a determinant of peripheral and central pressures in normotensive individuals (Xaplanteris et al. 2010).

One widely tested anti-hypertensive agent is apocynin, which inhibits the release of superoxide anion by inhibiting NADPH oxidase activity and blocking the migration of p47phox and p67phox to the mitochondrial membrane. Furthermore, it was shown to attenuate aldosterone-induced hypertension (Xue et al. 2011) and to decrease superoxide anion production in the coronary arteries of low salt-diet dogs where upregulation of gp91phox and p47phox was observed (Huang et al. 2010). Moreover, apocynin administration to mice with hereditary hypertension normalized blood pressure, catecholamines, $H_2O_2$, isoprostane, and NO concentration (Gayen et al. 2010). Similar results were obtained by Tsai and Jiang (2010), where apocynin and VAS-2870 decreased phenylephrine-induced superoxide anion production.

In the kidney, all NADPH oxidase subunits and Nox1, Nox2 and Nox4 are expressed, especially in the renal arterioles, glomeruli and distal nephron. Moreover, it is speculated that renal p22phox expression is important in the development of hypertension, as silencing its RNA attenuated angiotensine II and caused an increase in blood pressure (Modlinger et al. 2006).

Angiotensin II is an important, potent, regulator of cardiovascular NADPH oxidase activated via AT1 receptors and stimulation of numerous signaling pathways like c-Src p21Ras, PKC, phospholipase D and phospholipase A2 (Sedeek et al. 2009). Nox1 may play an important role in Ang II induced hypertension. Dikalova et al. (2005) have indicated that smooth muscle-specific Nox1 overexpression augments the oxidative, pressor, and hypertrophic responses to Ang II, supporting the concept that medial Nox1 participates in the development of cardiovascular pathologies. Angiotensine II pressor response is blunted in Nox1 deficient mice probably via reduction in vascular superoxide anion production.

Also superoxide produced by NADPH oxidase enhances transport in the loop of Henle and sodium reabsorption in spontaneously hypertensive rats, as demonstrated by apocynin or small interfering RNA for p22phox normalizing proximal tubule fluid reabsorption (Panico et al. 2009). Apocynin was shown to reduce the proximal tube flow in the kidney, probably due to inhibition of NADPH oxidase free radical generation (Ciarcia et al. 2010). However, Heumüller et al. (2008) recently reported that

apocynin is an antioxidant which reduces ROS bioavailability rather than an agent which inhibits vascular NADPH oxidase. This is in line with the work of Miller et al. (2010), where apocynin treatment reversed collateral growth impairment during aging in rats by decreasing free radical generation.

Systemic hypertension is an age-related disease characterized by an overexpression of Nox1 and Nox2 in the aortic wall; consequently, it is not surprising that VAS-2870 and NADPH oxidase inhibitor, but not the xanthine oxidase inhibitor oxypurinol, have been shown to improve vessel relaxation spontaneously in hypertensive rat aorta (Wind et al. 2010). The recently-developed NADPH inhibitor gp91ds-tat, which interferes with Nox and subunit assembly, is successful in promoting superoxide anion generation and blood pressure reduction (Rey et al. 2001). It has also been shown to attenuate cereblovascular dysfunction caused by ROS and increased blood pressure (Capone et al. 2011). Moreover, DPI, a NADPH oxidase inhibitor, inhibited the augmented proliferation of vascular smooth muscles cells (VSMCs) in spontaneously hypertensive rats (Li et al. 2010).

Despite numerous in vitro and in vivo experiments illustrating the possible role of NADPH oxidases in the origin and development of hypertension, a recently performed clinical trial showed that in patients with hereditary deficiency of gp91phox (Nox2), enhanced arterial dilatation is not correlated with decreases in blood pressure (Violi et al. 2009). Therefore, this may suggest that Nox2 may not play an important role in human hypertension.

### Nox Inhibitors in Atherosclerosis

Atherosclerosis is one of the most common diseases in which excessive ROS generation in vascular cells is observed (Bouloumie et al. 1999; Hennig et al. 1996). Therefore, endothelial and VSMCs dysfunction is closely associated with an increased risk of atherosclerosis (Lee et al. 2010; Sorescu et al. 2002). Moreover, excessive ROS production leads to enhanced expression of proinflammatory compounds (e.g. cytokines, chemokines, adhesion molecules) in endothelial cells, whose activation is an early step in atherosclerotic plaque development (Bouloumie et al. 1999).

It has been reported that all subunits of the prototype neutrophil NADPH oxidase, including gp91phox (now termed Nox2), p22phox, p47phox, p67phox and the small G-protein Rac1, as well as the Nox2 isoforms Nox1, Nox4, and Nox5, are expressed in human vascular cells and involved in superoxide anion generation (Brandes and Schröder 2008; Selemidis et al. 2008). However, Nox1 and Nox2, are expressed at low levels in the vasculature during normal physiology but are upregulated in cardiovascular risk settings such as hypertension, diabetes and hyperlipidemia (Judkins et al. 2010). Furthermore, recent findings indicate that atherosclerosis development is associated with interleukin (IL)-4-induced monocyte chemoattractant protein (MCP)-1 activation and that inhibition of Nox by apocynin or DPI successfully prevents these changes (Lee et al. 2010). This is in line with Walch et al. (2006), where gp91ds-tat, Nox-2 containing oxidase blocker, successfully decreased IL-4 upregulated MCP-1 expression and NO bioavailability.

Judkins et al. (2010) indicated that Nox-2-containing NADPH oxidase plays a direct role in vascular superoxide production and the early development of atherosclerotic lesions in apolipoprotein E-null ($ApoE^{-/-}$) mice. Moreover, it takes part in lipoprotein oxidation by macrophages (Vendrov et al. 2007) and ox-LDL has been shown to increase Nox-2 and p22pox mRNA expression (Bai et al. 2009). VAS-2870, a novel Nox-2 inhibitor, has been found to decrease superoxide production in phagocytic cells and ox-LDL-dependent ROS production in VSMCs (Stielow et al. 2006). Similarly, DPI decreased ox-LDL-dependent ROS formation and the proliferation of vascular cells acting on Rac-1 (Kaneyuki et al. 2007). Also, chronic subcutaneous infusion of gp91ds-tat attenuated total aortic ROS production, vascular inflammation and medial hypertrophy in cases of increased blood pressure induced by Ang II (Selemidis et al. 2008) and also decreased ROS production acting on Rac-1 activation in endothelial cells (Krötz et al. 2007). Similar results were seen with chronic apocynin treatment (Park et al. 2004; Zhang et al. 2005).

Recently it has been indicated that adhesion protein CD44 plays an important role in ROS generation in VSMCs and atherosclerotic lesions. It has been shown that NADPH oxidase deficiency decreased atherosclerosis, while increased levels of p22phox mRNA are correlated to endothelial cell proliferation (Kou et al. 2009). Vendrov et al. (2010) showed that GKT136901, a specific inhibitor of Nox1 and Nox4-containing NADPH oxidase activity, inhibits ROS generation and atherosclerosis. It also decreased CD44 expression in atherosclerotic lesions, thus preventing vascular alterations.

## Conclusion

This review has presented the most important information about recent studies concerning structure and function of the NADPH oxidase family, Nox inhibitors and their role in the treatment of disorders with endothelial damage.

Typical phagocytic NADPH oxidase consists of five subunits: p67phox, p47phox, p40phox, p22phox and the catalytic subunit gp91phox. In recent years, a whole family of nonphagocytic NADPH oxidases has been identified.

The new homologues, along with gp91phox, are now designated the Nox family of NADPH oxidases and have participated in the production of ROS in the vasculature. These enzymes are involved in many physiological and pathological processes. They are very important for correct function host defense, post-translational processing of proteins, cellular signaling regulation of gene expression and cell differentiation. In nonphysiological concentrations contribute to many disorders. Low concentration of these enzymes lead to immunosuppression or hypothyroidism, while increased their activity leads to a large number of pathologies in particular cardiovascular diseases. Specific inhibitors of Nox may have contributed to the development of new therapeutic strategy for treating diseases whose pathology is associated with oxidative stress induced by NADPH oxidases.

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