Redox Unbalance: NADPH Oxidase as Therapeutic Target in Blood Pressure Control

Luiza A. Rabêlo1,2, Valéria Nunes de Souza1, Lucas José Sá da Fonseca1, Walkyria O. Sampaio2,3
Universidade Federal de Alagoas1, Maceió, AL; Universidade Federal de Minas Gerais2, Belo Horizonte, MG; Centro Universitário de Belo Horizonte - Uni-BH, Belo Horizonte, MG - Brazil

Abstract

Several studies refer to reactive oxygen and nitrogen species (RONS) as important agents in the pathogenesis of a number of heart diseases, including high blood pressure, arteriosclerosis and heart failure. Such species are highly bioactive molecules and a short life due chiefly to reduction of molecular oxygen. The enzyme complex of NADPH oxidase is the main source of these reactive species in vascular system. Under physiological conditions, formation and elimination of these substances seem balanced in vascular wall. During redox Unbalance, nonetheless, there is increase in NADPH oxidase activity and predominance of pro-oxidizing agents, surpassing the anti-oxidant capacity of the organism self-defense. Besides this, such enzyme hyperactivity reduces the bioavailability of nitric oxide, capital for vasodilation and maintenance of normal vascular function. In spite of NADPH oxidase being directly connected to the endothelial dysfunction, it was firstly described as for its expression in phagocytes, where its activity determines efficiency of organism defense mechanisms against pathogens. Slight differences between structural units of NADPH oxidases, depending on the type of cell which expresses it, may create therapeutic implications, allowing to selectively inhibiting redox unbalance triggered by NADPH oxidase, without compromising, however, its participation in physiological cellular signaling which make sure protection against micro-organisms.

Introduction

Several studies show that reactive oxygen and nitrogen species (RONS) effectively contribute to pathogenesis of cardiovascular disorders, such as high blood pressure, arteriosclerosis, hypertrophy and heart failure and restenosis, in addition to lesions arising out ischemia and reperfusion.

Key words

NADPH oxidase; NADPH oxidase inhibitors; reactive oxygen species; reactive nitrogen species; oxidation-reduction; cardiovascular diseases.

Multiple enzyme systems produce RONS and their derivatives in the vascular system, including cyclo-oxigenase, lipoxigenase, P450 cytochrome, xanthine oxidase (XO), myeloperoxidase (MPO), nitric oxide synthase (NOS) and NADPH oxidase - this latter one of the most important sources of this substance, both in endothelial cells as in smooth muscle cells1-22.

Since the findings of Baehner et al22 40 years ago - which allowed the discovery of NADPH oxidase24 - several studies addressed the relation between said enzyme complex and the redox unbalance (oxidative stress). Increase in production of superoxide anion (•O2-) and other RONS is implied in arteriosclerosis, arterial high blood pressure, cell proliferation and hypertrophy. However, the role of NADPH oxidase in such processes remain unknown, which is mainly attributable to occurrence of multiple isoforms of Nox (subunits which form the NADPH oxidase) and their vehicles, as well as to the lack of specific inhibitors25,26. The enzyme complex act as electron donor for reduction of O2 into •O2-; following the reaction 2O2 + NAD(P)+H → 2O2- + NAD(P)+ + H+ 10,16,22,25,27.

•O2-; production by NADPH oxidase and its relation with the •NO

Groundbreaking experiments of Furchgott and Zawadzki28 first demonstrated the existence of endothelium-derived relaxing factor which was subsequently identified as “NO”29, produced based on L-arginine by the action of endothelial nitric oxide synthase (eNOS)29-32 in the presence of co-factors, mainly the tetrahydrobiopterin (H4B; Figure 1). The “NO diffuses into vascular smooth muscle cells and activates the guanylate cyclase, promoting cyclic GMP mediated vasodilation29,31. In normal conditions, the “NO performs key role in maintenance of vascular wall in quiescent status by inhibition of inflammation, cell proliferation and thrombus, reducing vascular tone, activation of platelet and leukocytes, proliferation of smooth muscle cells, extracellular matrix deposition and death of endothelial cells34-36. “NO is also a free radical and, when produced jointly with O2, react in an extremely swift way to form a highly reactive proinflammatory species: the peroxynitrite anion (“ONOO-”; vasodilator less powerful than “NO”). Thus, most of the cytotoxicity attributed to “NO comes from the “ONOO-”37. This last substance is an important lipid peroxidation mediator, including LDL oxidation, event of capital importance to atherogenesis38.

Physiologically, a certain amount of intracellular “O2” is required for normal signaling performed by “NO. Nevertheless, pathologically, the extracellular increase of the first species...
Figure 1 - Mechanisms of activation of NADPH oxidase and its relation with the NO metabolism.

Such unbalance is related to RONS due to the fact that the O₂ is germane to cell respiration, and there are several enzyme systems which use this substrate as an electron acceptor. Family of such species includes highly bioactive molecules with short life due chiefly to O₂ reduction. Physiologically, the formation and elimination of RONS are balanced in the vascular wall, whose key role is played by the endothelium in maintenance of vascular tone and in blood pressure by releasing vasoactive substances, such as the NO. The increase of RONS due to redox unbalance was found in elder rats’ and mice’s aorta, carotid, mesenteric and coronaries, implying that this unbalance is associated to ageing, with the increase of NADPH oxidase activity. Experimental evidences indicate that the oxidative damage induced by reactive species derives from the increase of O₂ production and its metabolism and/or reduction of NO bioavailability. However, in spite of the excess of RONS being toxic, physiological concentrations of these species may function as mediator signs of several responses, including cell migration and growth, once, under normal conditions and in most arteries, the production of NO is predominant, in such a way that this radical takes small amounts of formed O₂, making sure the maintenance of balance in organic oxidative status.

Non-selective inhibition of NADPH oxidase compromises physiological cell signaling

Better categorized NADPH oxidase is the phagocytic, present in macrophages and neutrophils, being a multimeric protein complex with components in cell membrane and cytoplasm. The component associated to the membrane is the cytochrome b558-oxidase, formed by a larger sub-unit, the gp91phox (the term “phox” comes from “phagocytic...
oxidase"), and a smaller sub-unit, the p22phox. Besides these, the enzyme comprises three cytoplasmatic subunits (p47phox, p67phox and p40phox) and a small regulatory protein G (Rac2). Activation of NADPH oxidase begins with serine phosphorylation of the cytoplasmatic subunit p47phox, triggering its migration to the membrane, where, jointly with Rac, gets associated with the cytochrome b558, beginning the enzyme’s catalytic activity\(^1,5,17,37,53-58\) (Figure 2).

NADPH oxidase expressed in vascular cells differs from those found in phagocytes, both for its biochemical structure and for its functions\(^5\). It was initially found in neutrophils, which are well-known as essential defenders against microorganisms released by a combination of several mechanisms, including the generation of \(\cdot\)O\(_2\) produced by endothelial subunits p40phox, p47phox and gp91phox of the NADPH oxidase\(^54\). Intriguingly, the catalytic groups gp91phox, p22phox, p47phox and p67phox were also found in adventitial fibroblasts\(^7,13,22,55\). Identification of subunits homologous to gp91phox resulted in the formation of Nox family (from “nonphagocytic NADPH oxidase”), currently consisting of seven members (Nox1, Nox2 [formally known as gp91phox], Nox3, Nox4, Nox5, Duox1 and Duox2 [dual oxidase]\(^56,57\). The main components of enzyme complex NADPH oxidase, Nox1 and Nox4, are highly expressed in vascular cells and increased during the vascular remodeling process, such as in high blood pressure and atherosclerosis\(^5\). These slight differences in structural expression facilitate development of specific inhibitors of the vascular NADPH oxidase which do not compromise the physiological signaling and the phagocytic functions mediated by RONS\(^5\).

Several characteristics of the NADPH oxidases are striking. First, the vascular enzyme produces \(\cdot\)O\(_2\) in low levels and for long periods, mostly intracellularly, where it plays the role of cell signalling\(^1,5,14,16,46,50,59\) (Figure 3). Second, gp91phox is usually substituted for homologous Nox1 or Nox4, particularly in smooth muscles. Third, while the homologous Nox and the subunit p22phox connected to the membrane are capital to maintaining a stable unit which supports the electron transfer for generation of \(\cdot\)O\(_2\), the role of cytosolic components in NADPH oxidase remain blurred, whose last aspect has implications on activation and specificities of the inhibitors of said enzyme\(^5,13,16,46\). Besides this, the expression of subunits seems to vary in smooth muscle cells of different vessels. Human vascular smooth muscle cells (HVSMC) seem to express a subunit gp91phox similar to neutrophilic\(^60\) subunit. The homologous of gp91phox, the Nox1, is expressed in rat vascular smooth muscle cells (RVSMC) and the ones of mice\(^61\) and aortic smooth muscle cells (ASMC), and do not appear in human vascular smooth muscle cells\(^60\). Nox1 is 56% homologous to human gp91phox and, jointly with p22phox, is the probable functional component of cytochrome b558 in ASMCs and RVSMC\(^62\). Nox4, another homologous of gp91phox, is expressed in RVSMC, ASMC and HVSMC. Endothelial cells are mainly expressed in subunits Nox2 and Nox4. Expression of Nox1 is less prominent in endothelium and may be increased during leukocyte adhesion process and shear stress. Nox2 and Nox4 seem to be the most important functional subunits in endothelial cells, contributing similarly in production of reactive species\(^63\).

There is also evidence that the NADPH oxidase is found in the cell nucleus, acting in the gene expression. Several groups

![Figure 2 - Structure of phagocytic NADPH oxidase. The gp91phox is the NADPH binder with electron transport function in active NADPH oxidase. The extracellular production of \(\cdot\)O\(_2\) by reduction of an electron of O\(_2\) by gp91phox, using \(\beta\)-reduced nicotinamide adenine dinucleotide phosphate (NADPH). Adapted from Dusting et al, 2005.](image-url)
identified subunits of NADPH oxidase in internal membranes. However, consequences of the NADPH oxidase activation in between are not clear as of yet.

Enzyme activation: central role of subunits p47phox and gp91phox

The most relevant beginning event for the activation of NADPH oxidase involves the phosphorylation of p47phox and the consequent interaction with gp91phox. Bond between homologous domains of two structures Src produce self-inhibition of bond with p22phox. Such self-inhibiting interaction is lost in phosphorylation, allowing bond between the subunits p47phox and p22phox, and the activation of NADPH oxidase is made. Confirming this, a gene deletion of subunit gp91phox provides protection against ischemic stroke in mice, as it helps to prevent hematoencephalic barrier dysfunction.

NADPH oxidases are activated and regulated by several factors, as mechanical forces, hormones and cytokines, considering the thrombin, the Platelet-Derived Growth Factor (PDGF), the α Tumor Necrosis Factor (TNF-α), the lactosylceramide, the Interleukin-1 and the oxidized LDL stand out among these factors. The last one increases the production of RONS and the expression of Nox4 in human endothelium. Cultivated macrophages accumulate large amounts of cholesterol when exposed to modified LDL (receptors for LDL are abundant in these cells, indicating that such structures would be involved in the formation of atherosclerotic foam cells).

In vascular smooth muscle cells, the angiotensin II (Ang II) is a potent stimulator of NADPH oxidase activity. This angiotensinergic peptide is a known aspect in pathogenesis of most cardiovascular disorders, and it may induce formation of ‘O₂⁻, partly due to vascular NADPH oxidases. The activation mediated by Ang II was first demonstrated by Griendling et al., underscoring the importance of Ang II concentrations in the increase of NADPH oxidase’s activities in vascular smooth muscle cells. Despite the signaling by Ang II stimulating NADPH oxidase is not thoroughly studied, PLD, PKC, PLA2, PI3K and thiol oxireductases pathways seem to be involved. In vascular smooth muscle, Src tyrosine kinase regulates the NADPH oxidase activity stimulated by Ang II, inducing phosphorylation and translocation of subunit p47phox. Besides this, Src is capital for the effects of Ang II in the NADPH's subunits synthesis, stimulating the increase in the expression of gp91phox, p22phox, p47phox and p67phox. In addition to this, phosphorylation of Src precedes the activation of ERK1/2 and JNK cascades in vascular smooth muscle, leading to migration, cell growth, apoptosis and deposition of collagen, activating focal adhesion complexes, and has important participation in vascular inflammatory response.

These proliferative and inflammatory pathways provide positive feedback to the activation of NADPH oxidase in the vascular system. In vascular smooth muscle, the EGF receptor transactivation seems to be involved, leading to activation of PI3 kinase and small RacG protein minutes after the receptor AT1 of angiotensin is activated. Ang II acts by increasing the NADPH oxidase subunits expression in hours or days.

In the event of activation by Ang II, the H₂O₂ generated from the ‘O₂⁻ produced by NADPH oxidase, is capital for the hypertrophy of vascular smooth muscle cells, also acting as a vasoconstrictor (Figure 4). Nonetheless, in certain vascular
beds, both in human and mice, endogenous H$_2$O$_2$, in low concentrations, acts as an endothelium-dependent relaxing factor, promoting peripheral, coronary hyperpolarization and vasodilation, as well as in cerebral arteries. In cultivation of smooth muscle cells, Ang II induces hypertrophy mediated by H$_2$O$_2$, by means of activation of the expression of proto-oncogenes, MAP kinase and Akt/PKB. In mice with deficiency in gene which expresses the gp91phox, the Ang II fails to induce cardiac hypertrophy. Besides this, experimental evidences show that the "NO is a chemoattraction factor for angiogenesis, and inhibiting its production blocks vascular neoformation. In addition to this, Ang II also promotes mechanisms involved in inflammatory response, and it may simultaneously stimulate the production of "NO and "O$_2$ by endothelial cells, favorable situation to formation of "ONOO$^-$. Intriguingly, the activation of NADPH oxidase by Ang II is lessened by Ang-(1-7), and biologically active component of renin-angiotensin system. Ang-(1-7) seems to inhibit phosphorylation of Src induced by Ang II, promoting endothelium-dependent vasodilation due to induction of "NO release. As for the humoral activation mechanisms, the aldosterone regulates the MAP kinase and the generation of "O$_2$ by NADPH oxidase through c-Src-dependent mechanisms. Some therapeutic actions of hypotensor drugs (angiotensin receptor blockers and angiotensin converter enzyme inhibitors) are attributed to inhibitors of NADPH oxidase, with consequent reduction in production of RONS. However, there is no record that these therapeutic strategies which have as target such species may bring direct benefit to control of high blood pressure patients.

**Cardiovascular and neurodegenerative diseases are related to increased activity of NADPH oxidase**

Clinical studies show that RONS play a significant role in high blood pressure. The production of these species undergoes increase in patients suffering from essential high blood pressure, renovascular hypertension, malignant hypertension and preeclampsia hypertension. Besides this, increased production of "O$_2$ by NADPH oxidase in vessels is related to risk factors for arteriosclerosis (Fig. 1) and harm to endothelial function in coronary heart disease patients. Production of "O$_2$ appears increased in atherosclerotic blood vessels, contributing to the beginning of pro-inflammatory events, with gene transcription regulation of vascular cell adhesion molecules and chemoattractive proteins for monocytes. Patients suffering from arteriosclerosis present both endothelial dysfunction and redox unbalance. However, the accurate association mechanism for these two events in

**Figure 4 - Activation of NADPH oxidase by Ang II promotes increase of the inflammation and infiltration of macrophages in subendothelial space, mechanisms related to the progression of atheromatous plaque. Adapted from Schiffrin et all, 2003.**
human is unknown as of yet. The endothelial dysfunction is related to the increase in the NADPH oxidase activity (by increase of expression of its catalytic subunits13,15,17), ever since atherosclerotic lesions in human coronary show strong expression of gp91phox in the vulnerable area of the plaque. Besides this, Nox4 increases during the stage of formation of atheroma, whose appearance happens in a reduced form in advanced lesions. Great saphenous veins and internal mammary arteries of patients with diabetes mellitus present an increase in the NADPH oxidase activity and in decoupled eNOS (in this status, the enzyme acts as producer of O2 compared to NO) when compared to control group, evincing the relationship between NADPH oxidase and the atherosclerotic lesions14.

Vitamin supplementing may reduce vascular redox unbalance and improve total oxidative status. For instance, some animal models in which antioxidant properties of vitamins C and E are associated to decrease in activation of NADPH oxidase12,19 and increase in SOD activity (the largest system of cellular defense against is in vascular system). It may be advocated that hydrophobic vitamin E could inhibit the formation of enzyme complex of the NADPH oxidase subunits49. Despite these observations, clinical tests with antioxidant vitamins have not shown therapeutic benefits as for mortality in cardiovascular events24,56. Besides this, depending mainly on the posology, such substances may present oxidant properties with harmful organic interactions. As the data from major prospective clinical studies has failed to demonstrate beneficial effects of antioxidants25, a balanced diet without vitamin supplementing is commonly recommended, ever since potential harms of supplementing are still undefined.

In high blood pressure, the product of the activation of NADPH oxidase, the O2 produces the oxidation of H2O2 leading to an increase in the eNOS decoupling, and the levels of this type of oxygen increase, causing the bioavailability of NO6 to drop. Thus, H2O2 is believed to be deficient in condition associated with altered endothelial function, such as hypercholesterolemia, diabetes, high blood pressure and tobacco addiction. Stroes et al51 showed that the treatment with H2O2 increase vasodilation in human with hypercholesterolemia, insofar the removal of this biopertine implies in eNOS40 decoupling. In support to these findings, the eNOS superexpression prevents hypoxia-induced pulmonary vascular remodeling52, causing structural changes and alterations in cellular processes that result in increase of the median layer: vascular lumen ratio26,51.

Another factor implied in the redox unbalance is the endotoxemia. During its progress, release of lipopolysaccharides promotes increase of expression and activity of xanthine oxidase and of NADPH oxidase48. The increase of NADPH oxidase-dependent RONS in endosomes is important to pro-inflammatory immune response46. Activated neutrophils produce O2, hydroxyl radical (OH) and hypochlorous acid (HOCl), in addition to microbicide peptides and proteases44 responsible for the efficient immune response. Neutrophilic activation in phagocytosis of pneumococci induces the production of RONS by NADPH oxidase. In addition to this, the block of NADPH oxidase activation during acute bacterial meningitis in experimental animals provokes deficient defense against Streptococcus pneumoniae85.

As for the production of NO, clinical and experimental studies have shown that administering L-arginine recovers the synthesis of this radical and the vascular function in several cardiovascular diseases, implying that impairment of precursor amino acids availability is present in these disorders. Reduced levels of L-arginine also promote eNOS decoupling, resulting in increase of RONS46.

NO-donor drugs (nitrodilators) have been used to treat coronary heart diseases, high blood pressure and heart failure, having anti-inflammatory actions on vascular wall as suppressors of lipoprotein oxidation, inhibitors of migration and proliferation of vascular smooth muscle cells and platelet aggregation inhibitors. They act as suppressors of the expression of adhesion molecules and chemokines after the stimulation with proinflammatory factors. Such aspects prevent the inflammatory cell infiltration into the vascular wall, where there is also opposition to pro-inflammatory actions of RONS. In addition to this, it was recently found that NO seems to have anti-inflammatory effect of artery wall due to its suppression of RONS generator enzymes, particularly the NADPH oxidase4.

Nox proteins are also related to other countless organic disorders, including cancer, bone reabsorption and Alzheimer Disease, as they are expressed in several tissues80,86. Evidences demonstrate the role of Nox2 in neurodegenerative processes, such as Parkinson’s disease, stroke, cerebral trauma and meningitis, mainly due to neurotoxicity of RONS22,23. Thus, the Nox inhibitors present significant clinical potential, particularly if they do not inhibit the activity of phagocytes1.

Inhibitors of NADPH oxidase: potential therapeutic target

Pharmacological inhibitors of NADPH oxidase which block directly the activity of this enzyme have been described as consisting of peptide and non-peptide structures. The DIP-diphenyleneiodonium1,10 and 4-hydroxy-3-methoxyacetophenone (the apocynin, isolated from the medicinal plant Picrorhiza kurroa)50 has been broadly used to block the activity of the NADPH oxidase in vitro. Currently, the DPI-diphenyleneiodonium is known to be non-selective and act as non-specific oxidant4. In its turn, the apocynin has multiple biological actions in addition to antioxidant effects, being characterized as an inhibitor of NADPH oxidase since the 80’s4. Studies by Godbole et al59 have demonstrated that the apocynin recovers the production of nitrate and the vasodilation in ordinary femoral arteries of swines59. As the DIP do not stand for a specific inhibitor of the NADPH oxidase, the apocynin became very popular, with 692 records of publications on the PubMed database until January 15, 2009, 571 out of which are specific for the key “apocynin NADPH oxidase inhibition”, demonstrating strong correlation between the terms.

Leukocytes are capable of mediating the apocynin inhibiting effect. It may be suggested that myeloperoxidase (MPO), selectively expressed in leukocytes, is necessary for the occurrence of the inhibiting effect of this substance. In
HEK293 cells with no MPO, the formation of apocynin dimers does not occur even with superexpression of Nox isoforms. When human MPO is added, dimers may be identified in cells with superexpression of Nox isoforms or in cells co-incubated with H₂O₂. In absence of H₂O₂ or Nox, dimer production by HEK293 cells is inhibited. In stimulated leukocytes, dimers are promptly detectable, insofar as in stimulated vascular cells, there is not such production, even in presence of MPO. Thus, the authors suggest that the apocynin would act as anti-oxidant and not as inhibitor of NADPH oxidase in non-phagocytic cells, and the inhibiting action for the NADPH oxidase would be restricted to leukocytes which express MPO(Fig. 3). However, peroxidases other than the MPO may influence the apocynin activity, and it is possible that vascular cells have peroxidative enzymes capable of activating this inhibitor. Such fact is confirmed by findings on endothelial cells, in which apocynin dimers were identified, inhibiting, depending on concentration, the NADPH oxidase activity, the formation of RONS and the cell proliferation.

Pagano et al. developed the chimeric peptide gp91ds-Tat, comprised of nine amino acids of HIV protein coat and nine amino acids of gp91phox. This substance presents reaction with p47phox and interferes in bond of this subunit with the amino acids of gp91phox. The “Tat” portion allows the gp91ds-Tat to penetrate into the cell, and, thus, it is effective inhibitor of oxidases which contain gp91phox. However, despite the peptides as the one referred to are useful in experimental interventions, biotransformation processes render its oral administration difficult, limiting its potential in clinical therapeutics.

Peptidic inhibitors of NADPH oxidase are also described, such as the antibiotic PR-39, endogenously secreted by intestine cells and neutrophils of humans and swines. They act both as immunity cells and as inhibitors to the non-phagocytic NADPH oxidase activity. Initially, the PR-39 was seen as a specific inhibitor of the NADPH oxidase. However, recent studies have shown that the antibiotic presents several effects, partly because it binds to SH3 domains of other proteins and also because it interacts with membrane lipides.

Conclusion
This study identified that the NADPH oxidase emerged as one of the main structures involved in vascular redox unbalance. As the ‘NO is also capable of suppressing the NADPH oxidase activity, this event is believed to present direct implications not only in the development of vascular disease, but also acute recovery of ischemia and reperfusion lesion, in which NADPH-dependent unbalance is of major contribution.

The development of oxidase specific inhibitors, based on Nox units, may experimentally provide tools to enlighten on the role of these enzymes. It may also be useful in the treatment of several diseases, mainly when it comes to selective enzyme inhibition aiming to reduce the vascular damage arising out of excessive production of RONS without, nonetheless, compromising the cell signaling mechanism which depend upon said species.

Acknowledgements
The authors would like to thank the CNPq, the FAPEAL and the PPSUS-2006/Ministry of Health-FAPEAL for the financial support for this project.

Potential Conflict of Interest
No potential conflict of interest relevant to this article was reported.

Sources of Funding
This study was partially funded by CNPq, PROCAD-NF/ CAPES and FAPEAL.

Study Association
This study is not associated with any post-graduation program.

References


71. Arq Bras Cardiol 2010;94(5):643-651

Review Article

Role of NADPH oxidase in redox unbalance
