Pathophysiological Roles of NADPH Oxidase/Nox Family Proteins in the Vascular System

– Review and Perspective –

Tetsuro Ago, MD, PhD; Junya Kuroda, MD, PhD; Masahiro Kamouchi, MD, PhD; Junichi Sadoshima, MD, PhD; Takanari Kitazono, MD, PhD

It has been established that oxidative stress plays a crucial role in the development and progression of vascular diseases. Besides the mitochondria, the NADPH oxidase/Nox family proteins are now thought to be important origins of the reactive oxygen species that underlie various vascular disease states, such as hypertension, atherosclerosis, angiogenesis, and ischemia/reperfusion injury. This review summarizes the basis of vascular Nox proteins and discusses their pathophysiological roles in the vascular system. (Circ J 2011; 75: 1791–1800)

Key Words: NADPH oxidase/Nox; Reactive oxygen species; Vascular diseases

Oxidative Stress in the Cardiovascular System

Cardiovascular cells, such as endothelial cells and cardiomyocytes, are metabolically active even under physiological conditions in which abundant ATP is constitutively generated to maintain their cellular functions. A large amount of ATP is generated by oxidative phosphorylation in the mitochondrial electron transport chain under normoxic conditions. During this process, superoxide (O$_2^-$) is inevitably produced in the mitochondria as a byproduct (Figure 1).$^1$

The O$_2^-$ produced in the mitochondria is immediately converted to H$_2$O$_2$ by mitochondrially localized manganese-superoxide dismutase (also known as SOD2). The importance of SOD2 in cellular function has been shown particularly in the cardiovascular system.$^2$ There are 2 other SODs in mammals, cytosolic copper-zinc SOD (SOD1) and extracellular SOD (SOD3).$^3$ The presence of cytosolic SOD1 may be a backup system to dismute O$_2^-$ that has overflowed from the mitochondria, or may imply that a significant amount of O$_2^-$ is produced outside the mitochondria. Because decreased activity of SOD3 leads to endothelial dysfunction, O$_2^-$ derived from circulating white blood cells and smooth muscle cells plays an important role in the development of endothelial dysfunction (Figure 1).$^5$

Compared with O$_2^-$, H$_2$O$_2$ is a stable and diffusible molecule, and is catalyzed to water by catalase and various types of peroxidases. However, H$_2$O$_2$ beyond the antioxidant capacity can function as a signaling molecule or be converted to hydroxyl radical (·OH). The highly reactive ·OH oxidizes lipid and DNA, thereby impairing cellular functions and causing mutagenesis. Oxidative modification of critical proteins, such as disulfide bond formation, can affect intracellular signaling, thereby leading to cardiovascular diseases (Figure 1).$^5,7$

Under physiological conditions in which small amounts of reactive oxygen species (ROS) are constantly produced within cells, the intracellular environment is maintained in a reductive state by the presence of abundant antioxidant proteins. In contrast, under pathological conditions, ROS production is greatly, and sometimes rapidly, enhanced and may be beyond the antioxidant capacity, thereby causing cardiovascular diseases.$^1,8$

It is believed that the mitochondria primarily contribute to increased production of ROS under physiological and pathological conditions. However, there exist various enzymes that can generate ROS and these enzymes may also play important roles under both physiological and pathological conditions.$^9,10$

ROS-Generating Enzymes in the Cardiovascular System

Xanthine oxidase (XO) and (uncoupled) nitric oxide synthase (NOS) are well-known ROS-generating enzymes that are highly expressed in endothelial cells, and may be relevant to vascular diseases (Figure 2).$^8$

Xanthine oxidoreductase can exist in 2 forms: xanthine dehydrogenase (XDH) and XO. Although XDH and XO catalyze the final 2 steps in purine catabolism by converting hypoxanthine to uric acid, hypoxic conditions trigger the conversion of NAD-reducing XDH to the O$_2^-$-producing XO. Thus, XO can produce abundant O$_2^-$ during ischemia/reperfusion (Figure 2).$^{11,12}$

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Department of Medicine and Clinical Science, Graduate School of Medical Sciences, Kyushu University, Fukuoka (T.A., J.K., M.K., T.K.), Japan; Department of Cell Biology and Molecular Medicine, University of Medicine and Dentistry of New Jersey, New Jersey Medical School, Newark, NJ (U.S.A.)
Mailing address: Tetsuro Ago, MD, PhD, Department of Medicine and Clinical Science, Graduate School of Medical Sciences, Kyushu University, 3-1-1 Maidashi, Higashi-ku, Fukuoka 812-8582, Japan. E-mail: agou@intmed2.med.kyushu-u.ac.jp
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Figure 1. ROS-generating and antioxidant enzymes in vascular cells. ROS, reactive oxygen species.

Figure 2. Central role of NADPH oxidases in RIRR in vascular (endothelial) cells. ROS-induced ROS release; ROS, reactive oxygen species.
Endothelial NOS (eNOS) produces NO using tetrahydrobiopterin (BH4) as a cofactor and NADPH as its electron donor, which is crucial for endothelium-dependent vasodilation. Decreased activity of eNOS or elimination of NO by O2− leads to vascular constriction and hypertension. Increased oxidative stress causes the oxidation of cofactor BH4 and its degradation. In these situations, eNOS cannot use BH4 efficiently (ie, a state of uncoupled eNOS), thereby generating O2− instead of NO. ROS production by these enzymes may be important as a ROS-induced ROS release.
In contrast to these enzymes, the NADPH oxidase/Nox family proteins are unique in their purposeful production of ROS. It is evident that the Nox family proteins are important for redox-mediated signaling in various cell types. Thus, these proteins are now receiving much research attention.

**Nox Family Proteins**

The Nox family proteins are membrane-spanning proteins with NADPH- (or NADH) and FAD-binding domains in their C termini, and they produce \( \text{O}_2^- \) by transferring an electron from NADPH (or NADH) to \( \text{O}_2 \) \cite{15}.

In humans, NADPH oxidase had been thought to be a phagocyte-specific enzyme (its catalytic unit: gp91\(^{phox}\)) that plays a crucial role in mediating bacterial killing by producing a burst of \( \text{O}_2^- \). \cite{10} p22\(^{phox}\), a membrane protein, forms a heterodimer with gp91\(^{phox}\), thereby stabilizing gp91\(^{phox}\) and enhancing its \( \text{O}_2^- \)-producing activity. \cite{10,14,15} The ubiquitous expression of p22\(^{phox}\) in non-phagocytic cells was one of the enigmas in this area of research. The identification of Nox1, a homolog of gp91\(^{phox}\), in non-phagocytic cells \cite{16} has prompted researchers to identify other Nox proteins. Finally, 7 NADPH oxidase family proteins (ie, Nox1–Nox5 and Duox1 and Duox2) have been identified. gp91\(^{phox}\) has been renamed Nox2 in the current nomenclature. \cite{10,14,15}

**Expression of Nox Family Proteins in the Vascular System**

It is believed that Nox1, Nox2 and Nox4 are expressed significantly in the vascular system (Figure 3). \cite{10,17,18} Although it has been speculated that each Nox protein plays a specific role according to its unique expression profile, intracellular localization, activation mechanism, and ROS-producing activity, it is difficult to identify their specific roles because multiple Nox proteins often exist simultaneously in one cell-type.

In humans, genetic abnormalities of Nox1 and Nox4 have not been reported to date, whereas lack of Nox2 is known to cause chronic granulomatous disease, a rare disease with a recurrent and life-threatening infection. \cite{19}

**Regulation of ROS Production in Vascular Nox Proteins**

**Nox2/gp91\(^{phox}\)** Detailed activation mechanisms are documented in the prototype phagocyte NADPH oxidase Nox2. Although phagocytes express large amounts of Nox2 and p22\(^{phox}\), even in the resting state, they do not produce \( \text{O}_2^- \) without stimulation, because the purposeless \( \text{O}_2^- \) production by the Nox2 injures tissues (Figure 3). In phagocytes, p47\(^{phox}\) forms a constitutive complex with p67\(^{phox}\) in the cytosol. Upon stimulation, p47\(^{phox}\) is phosphorylated at its C terminus, thereby translocating to the membrane and interacting with p22\(^{phox}\). Independently of these molecules, Rac2 translocates to the membrane upon stimulation and interacts with p67\(^{phox}\). Nox2 becomes able to produce \( \text{O}_2^- \) after full complex formation in phagocytes (Figure 3).

Nox2 also contributes to ROS production in endothelial cells. The activation mechanism of endothelial Nox2 is slightly different from that of phagocyte Nox2 (Figure 3). Although the expression levels of Nox2 are much less than those in phagocytes, endothelial Nox2 exists as a pre-assembled intracellular complex that is associated with the cytoskeleton, and produces a low but significant amount of ROS constitutively in endothelial cells. Instead of Rac2, Rac1 plays a central role in activating Nox2 in endothelial cells. Angiotensin II (Ang II) may augment the complex formation and ROS production in endothelial cells.

**Nox1** It is thought that Nox1 is highly expressed in vascular smooth muscle cells (VSMCs), and less in endothelial cells. In VSMCs, Nox1 is upregulated by vasoactive and growth factors, such as Ang II and platelet-derived growth factor (PDGF), thereby participating in the hypertrophy and proliferation of VSMCs.

\[ \text{(RIRR) mechanism (Figure 2).} \]

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The activation mechanism of Nox1 is somewhat different from that of Nox2 (Figure 3). Nox1 and Nox1a, homologs of p47\textsuperscript{phox} and p67\textsuperscript{phox} respectively, have been identified as cytosolic regulators for Nox1, although the classical p47\textsuperscript{phox} and p67\textsuperscript{phox} can partially replace them for Nox1 activation. Nox1 consists of almost the same domain structure as p47\textsuperscript{phox}, except that it lacks the autoinhibitory region, and can bind constitutively to p22\textsuperscript{phox}. Thus, Nox1 activation seems to be regulated by Rac1. Furthermore, phosphorylation of Nox1 induces dissociation between Rac1 and Nox1a, thereby inhibiting hyperactivation of Nox1 (Figure 3). Nox1-derived ROS activate nuclear factor (NF)-\(\kappa\)B, a transcription factor for Nox1, and further increase Nox1 expression and ROS production, as a positive feedback mechanism. Nox4 has been reported to produce a small amount of ROS constitutively without cytosolic activators, in contrast to Nox1 and Nox2. p22\textsuperscript{phox} stabilizes the Nox4 structure and enhances its ROS-producing activity. It is still controversial whether Rac1 increases Nox4 activity (Figure 3). There are still some issues to be elucidated regarding the activity of Nox4. First, it is suggested that Nox4 produces H\(_2\)O\(_2\) directly, but not O\(_2\) (Figure 4). A functional SOD-like domain has not been identified in Nox4. One possibility is that the catalytic activity of Nox4 is very low, which may be associated with slow dissociation of Nox4–O\(_2\)– complexes, allowing a second electron transfer to produce H\(_2\)O\(_2\). Alternatively, SOD activity may be tightly associated with Nox4.

Substrate specificity and intracellular localization of Nox4 are also debated. In contrast to Nox2, which uses only NADPH as its substrate, it has been suggested that Nox4 can use NADH as well as NADPH. Because NADH is produced mainly by the TCA cycle, it would be interesting if Nox4 were localized in the mitochondria. Consistently, the mitochondrial localization of Nox4 has been suggested, although it may also exist in the cytosol, nucleus, and endoplasmic reticulum (Figure 4). The different localizations may depend on cell type or cellular conditions. The fact that Nox4 induces significant oxidation of various mitochondrial proteins in vivo and in vitro may support its mitochondrial localization (Figure 4).

Because the amount of Nox4-mediated ROS production seems to be determined by its expression level, its transcriptional regulation has been investigated extensively. Hypoxia inducible factor (HIF)-1\(\alpha\) is an important transcriptional factor for Nox4, whereas the Nox4-mediated ROS stabilize HIF-2\(\alpha\). Thus, Nox4 could function upstream and downstream of HIFs, and account in part for hypoxic responses mediated by HIFs (Figure 4). It has been reported that other transcription factors related to hypoxia and oxidative stress, such as NF\(\kappa\)B and Sp3, also increase Nox4 expression.

**Central Role of NADPH Oxidases in RIRR**

Accumulating evidence has suggested that an increase in intracellular ROS further induces ROS production, which is called RIRR in cardiovascular cells. In the original study, ROS-induced mitochondrial permeability transition (MPT) played a crucial role in RIRR (Figure 2). MPT is a phenomenon showing an increase in the permeability of the mitochondrial membranes that is caused by opening of the MPT pore (MPTP). ROS may cause the oxidation of the components comprising MPTP, thereby leading to MPT. Although this concept was introduced in cardiomyocytes, it could be expanded further to vascular cells, because ROS are produced intracellularly by Nox proteins and a close association between Nox and the mitochondria has been shown in vascular cells. In cardiomyocytes, Nox4 seems to participate in RIRR through the oxidation of MPTP components (Figures 2, 4).

In addition, other types of RIRR have been reported: NADPH-oxidase-derived ROS induce conversion of XDH to XO, and degradation of BH4, which leads to uncoupling of eNOS, thereby increasing ROS production. Taken together, the NADPH oxidase may play a central role in several types of RIRR in vascular cells (Figure 2).

**Roles of Nox-Mediated ROS in the Vascular System**

The roles of Nox2 in vivo have been extensively investigated in various cardiovascular disease models. Nox2-deficient mice are apparently normal and fertile if they are bred in pathogen-free environments. There are emerging in vivo data about transgenic or knock out mice with regard to Nox1 and Nox4. Surprisingly, baseline phenotypes of Nox1-deficient and Nox4-deficient mice are also apparently normal. One possibility is that intact Nox proteins partially compensate for the functional roles of the deleted Nox protein under physiological conditions. Alternatively, Nox proteins are dispensable for ontogenesis and for physiological functions after birth. Transgenic or knockout mice for each Nox protein seem to show interesting phenotypic changes in various disease models. The roles of Nox proteins under pathological conditions have been emphasized in the vascular system.

**Hypertension**

It is thought that ROS, particularly O\(_2\) and hypertension are closely related: O\(_2\) contributes to the development of hypertension by decreasing NO bioavailability (Figure 5A), whereas increased shear stress by hypertension augments vascular O\(_2\) production in endothelial cells (Figure 5B). ROS are a cause and a consequence of hypertension, and can form a vicious cycle. It is thought that Ang II plays an essential role in O\(_2\)–mediated hypertension. Ang II increases the expression of Nox1 in VSMCs (Figure 5A). The crucial role of Nox1 in Ang II-induced hypertension in vivo has been proved by using Nox1-overexpressing and deficient mice. Endothelium-dependent vasorelaxation is preserved in Nox1-deficient mice infused with Ang II, whereas SMC-specific upregulation of Nox1 significantly impairs the relaxation, by causing uncoupling of eNOS, thereby inducing vasoconstriction.

The role of Nox2 in Ang II-dependent hypertension seems complicated. It has been reported that Ang II-dependent hypertension is unaffected in Nox2-deficient mice, although increased activity of the Nox2-mediated oxidase in vascular walls is found in other chronic hypertension models. On the other hand, mice with endothelial cell-specific overexpression of Nox2 showed an Ang II-induced greater increase in ROS production in vascular walls and hypertension, although basal Nox activity and blood pressure were unaltered in the mice. These findings indicate that the upregulation of Nox2 in vascular walls (endothelial cells) may be a secondary response to hypertension or Ang II-independent hypertension.
However, Nox2 upregulation in vascular walls (endothelial cells) may enhance the response to Ang II and induce hypertension (Figure 5B). It has been reported that Nox4 is upregulated in vascular cells during chronic hypertension, although the causative role of Nox4 in hypertension is still uncertain, in contrast to Nox1 and Nox2.

Atherosclerosis
Atherosclerosis is characterized by the following 4 steps: endothelial dysfunction; plaque formation following subendothelial accumulation of low-density lipoprotein (LDL)-cholesterol and monocyct infiltration; migration, hypertrophy and proliferation of VSMCs in plaques; and plaque rupture and thrombosis.

Hypertension is the most important risk factor for atherosclerosis. As mentioned earlier, hypertension itself causes endothelial dysfunction by increasing shear stress and inducing O$_2^-$ production by Nox2 (and/or Nox4) in endothelial cells (Figure 5B). Ang-II-mediated Nox1 upregulation and subsequent O$_2^-$ production in VSMCs can lead to endothelial dysfunction before causing hypertension (Figure 5A).

Figure 5. ROS-mediated mechanisms that underlie hypertension and atherosclerosis. (A) Crucial roles of Nox1 in Ang II-induced hypertension and hypertrophy of SMCs. (B) Roles of Nox2 and Nox4 in endothelial dysfunction during increased shear stress by hypertension. Ang II, angiotensin II; ROS, reactive oxygen species; SMC, smooth muscle cell.
Due to formatting issues, the text is not visible in the image. It appears to be discussing NADPH oxidases in vascular diseases, focusing on endothelial dysfunction, angiogenesis, and ischemia/reperfusion injury. The text refers to various NADPH oxidase (Nox) proteins and their roles in these processes, including their upregulation in response to hypoxia, diabetes, and dyslipidemia. It also mentions the role of other growth factors like PDGF and VEGF in angiogenesis and ischemia/reperfusion injury.
and/or severe ischemia, which is usually found under experimental conditions, the Nox-mediated angiogenic responses may not work sufficiently and may rather function adversely because of a burst of ROS production. Indeed, it has been reported that experimental acute tissue damage associated with ischemia/reperfusion is attenuated particularly in the brain in Nox1-19, Nox2-100 and Nox4-101 deficient mice. A possible explanation is that the Nox proteins activated during hypoxia/ischemia produce considerable amounts of ROS by the reoxidation of O2 during the reperfusion phase, which may simply cause deterioration of ischemic tissue damage. Alternatively, the Nox-mediated ROS may affect crucial intracellular signaling pathways, thereby leading to expansion of cell death during ischemia/reperfusion. Taken together, Nox-mediated ROS may function as double-edged swords for ischemia/reperfusion injury and angiogenesis, which are also relevant to the development of atherosclerosis.

Conclusions

In the past decade, a new family of highly regulated ROS-producing enzymes has been identified, and named the Nox family proteins because of their structural similarity to the phagocyte NADPH oxidase. It is evident that these proteins are crucial in various biological events. Among 7 Nox family proteins, Nox1, Nox2 and/or Nox4 are expressed significantly in vascular cells, and account for ROS production in vascular walls. As mentioned in this review, these 3 Nox proteins seem to contribute cooperatively to vascular pathophysiological events, such as hypertension, atherosclerosis, angiogenesis, and ischemia/reperfusion injury. ROS can function both adversely and beneficially. Thus, we have to judge correctly the significance of ROS depending on the situation, and thus correctly manipulate ROS production. To develop novel therapeutic approaches via redox regulation and to overcome vascular diseases, we should elucidate in more detail the molecular mechanisms that underlie Nox-mediated vascular diseases.

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