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Forum Review

NADPH Oxidase in Endothelial Cells: Impact on Atherosclerosis

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ABSTRACT

An elevated vascular superoxide anion formation has been implicated in the initiation and progression of hypertension and atherosclerosis. In this review, we would like to discuss the generation of superoxide anions by an NADPH oxidase complex in vascular cells. Special focus is on the induction of endothelial NADPH oxidase by proatherosclerotic stimuli. We propose a proatherosclerotic vicious cycle of increased NADPH oxidase-dependent superoxide anion formation, augmented generation and uptake of oxidatively modified low-density lipoprotein, and further potentiation of oxidative stress by oxidized low-density lipoprotein itself, angiotensin II, and endothelin-1 in endothelial cells. Furthermore, novel homologues of NADPH oxidase subunit gp91^{phox} are summarized. Future directions of research for a better understanding of the role of NADPH oxidase in the pathogenesis of atherosclerosis and clinical implications are discussed. *Antioxid. Redox Signal.* 5, 171–180.

INTRODUCTION

REACTIVE OXYGEN SPECIES (ROS) are oxygen-derived radicals. An important ROS is superoxide anion ($O_2^{\cdot -}$). It can be converted into other ROS including hydrogen peroxide (H_2O_2) and hydroxyl radical ($HO^{\cdot}$). Under physiological conditions, the cellular ROS formation can be balanced by antioxidative processes that include scavenging of ROS (*e.g.,* by α-tocopherol, β-carotene, ascorbate, glutathione) or enzymatic degradation (*e.g.,* by superoxide dismutase, catalase, glutathione peroxidase). Disturbance of this equilibrium by increased ROS formation or reduced antioxidative capacity is thought to be involved in the pathogenesis of several diseases, including cardiovascular diseases, diabetes mellitus, and cancer. Increasing evidence supports an important role of ROS as second messengers. This issue has been reviewed recently (18). In this review, we would like to discuss the generation of $O_2^{\cdot -}$ by an NADPH oxidase complex in vascular cells. Special focus will be on the NADPH oxidase in endothelial cells and its putative impact on atherosclerosis.

ROS AND ATHEROSCLEROSIS

An elevated vascular $O_2^{\cdot -}$ formation has been implicated in the initiation and progression of hypertension and atherosclerosis (Fig. 1) (30, 49). Increased superoxide generation by NADPH oxidase has been associated with endothelial dysfunction and clinical risk factors of atherosclerosis (32). Expression of NADPH oxidase subunits has been associated with the severity of atherosclerosis (75). $O_2^{\cdot -}$ rapidly reacts with nitric oxide ($NO^{\cdot}$) forming peroxynitrite ($ONOO^-$) (31). As $NO^{\cdot}$ is an important mediator of endothelium-derived relaxation, a reduced $NO^{\cdot}$ availability by $ONOO^-$ formation results in endothelial dysfunction and development of atherosclerosis (9). The negative impact of $ONOO^-$ on endothelial function is further potentiated by inhibited synthesis of the vasodilator prostacyclin (90). In addition, $NO^{\cdot}$ is known to mediate antiatherosclerotic effects. $NO^{\cdot}$ inhibits thrombocyte aggregation, endothelial adhesion molecule expression, and smooth muscle cell proliferation (25, 40, 63). Recently, chronic treatment with $NO^{\cdot}$-releasing aspirin has been shown to re-

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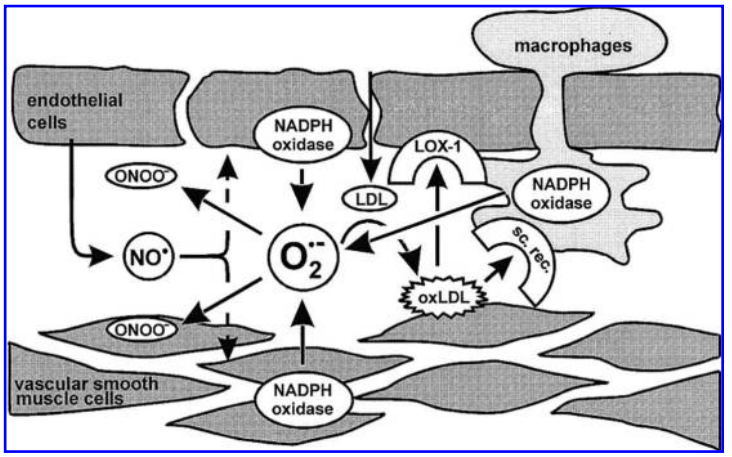

FIG. 1. Proatherosclerotic role of NADPH oxidase in the vessel wall. The NADPH oxidase complex has been described in all cells of the vessel wall and generates superoxide anions ($O_2^{\cdot -}$). $O_2^{\cdot -}$ can reduce nitric oxide ($NO^{\cdot}$) availability by peroxynitrite ($ONOO^-$) formation. Furthermore, $O_2^{\cdot -}$ could oxidize low-density lipoprotein (LDL) to form oxidized LDL (oxLDL). OxLDL is taken up by classical scavenger receptors (sc. rec.) or the endothelial oxLDL receptor LOX-1, thus promoting atherosclerosis.

duce low-density lipoprotein (LDL) oxidation, oxidative stress, and atherosclerosis in hypercholesterolemic animals (56). The antiatherosclerotic effects of $NO^{\cdot}$ are diminished by inactivation with $O_2^{\cdot -}$, too.

Another proatherosclerotic potential of augmented vascular $O_2^{\cdot -}$ formation is the increased oxidative modification of LDL (15). In several ways, oxidized LDL (oxLDL) contributes to the pathogenesis of atherosclerosis. It interferes with the endothelium-dependent relaxation by reducing expression of endothelial nitric oxide synthase. It induces chemotactic factors and expression of adhesion molecules, as well as the expression of scavanger receptors on macrophages (87). Thus, oxLDL promotes infiltration of macrophages into the intima and unlimited uptake of oxLDL by these macrophages via scavenger receptors. This process known as foam cell formation contributes substantially to the development of atherosclerotic plaques (85). In addition, oxLDL stimulates vascular smooth muscle cell proliferation (4). This intimal thickening further reduces the lumen of blood vessels, leading to further potentiation of hypertension and atherosclerosis.

Because of its unique localization between the circulating blood and the vessel wall, the endothelium has been suggested to play a crucial role in development and progression of atherosclerosis. Endothelial $O_2^{\cdot -}$ formation is suggested to contribute to $NO^{\cdot}$ inactivation and oxLDL formation. $O_2^{\cdot -}$ can be generated by a variety of enzymatic mechanisms. Enzymes of the respiratory chain, xanthine oxidase, nitric oxide synthase, cyclooxygenase, lipoxygenase, cytochrome P450 monooxygenase, as well as NADPH oxidase, have been shown to generate $O_2^{\cdot -}$ under physiological or certain pathophysiological conditions (39). In endothelial cells, an NADPH oxidase, similar to the NADPH oxidase of granulocytes, was shown to be a main source of $O_2^{\cdot -}$ formation (52).

STRUCTURE AND FUNCTION OF ENDOTHELIAL NADPH OXIDASE

In granulocytes, NADPH oxidase is involved in the antimicrobial defense. It resides in the plasma membrane and becomes part of the phagolysosome membrane when phagocytosis of pathogens takes place (73). The NADPH oxidase consists of several subunits (Fig. 2). Two of them, $p22^{phox}$ (phox: phagocytic oxidase) and the glycoprotein $gp91^{phox}$, are integral membrane proteins. Together, they bind the components of the electron transport chain, flavine and heme, thus forming the cytochrome b_{558} (17). The subunits $p47^{phox}$ and $p67^{phox}$ are located in the cytosol. Upon activation, $p47^{phox}$ is phosphorylated by protein kinase C, and in complex with $p67^{phox}$ it translocates to the cytochrome b_{558} inducing $O_2^{\cdot -}$ formation (12). Small G proteins (rac/rap) are also involved in the assembly of the NADPH oxidase complex (21, 34). Later, a third cytosolic subunit, $p40^{phox}$, was identified that reduces $O_2^{\cdot -}$ formation by binding to the NADPH oxidase complex (71). Using cell-free reconstitution assays, it was shown that a complex of $p22^{phox}$, $p47^{phox}$, $p67^{phox}$, and $gp91^{phox}$ with a stoichiometry of 1:1:1:1 was sufficient to induce $O_2^{\cdot -}$ formation (81).

The existence of functionally active endothelial NADPH oxidase complexes had been questioned, because expression of one of the essential subunits, $gp91^{phox}$, could not be detected in endothelial cells. Later, expression of this missing component could be confirmed on the mRNA (38) and protein level (50). Thus, a functionally active NADPH oxidase complex has been shown to be present in all vascular cell types, including endothelial cells. Functionally, differences between the NADPH oxidase in endothelial cells and granulocytes have been described. This has led to the conclusion that NADPH oxidases of both cell types were similar, but not identical. First, the activity of this enzyme complex in granulocytes is strongly inducible by agents that stimulate protein kinase C activity (*e.g., N*-formyl-methionyl-leucyl-phenylalanine or phorbol 12-myristate 13-acetate). This induction is known as "oxidative burst." In contrast, NADPH oxidase-dependent endothelial $O_2^{\cdot -}$ formation is rather constitutive on a much lower level (35). This lower NADPH oxidase activity seems to be mediated by the ~100-fold lower expression of subunit $gp91^{phox}$ in endothelial cells compared with granulocytes (69). Furthermore, granulocytes were reported to use primarily NADPH as electron donor, whereas endothelial cells were described to prefer NADH (14, 52). In recent studies, the primary structure of putative substrate-binding subunits $gp91^{phox}$ and $p67^{phox}$ was shown to be identical between granulocytes and endothelial cells (28, 69). Furthermore, under modified experimental conditions, a pref-

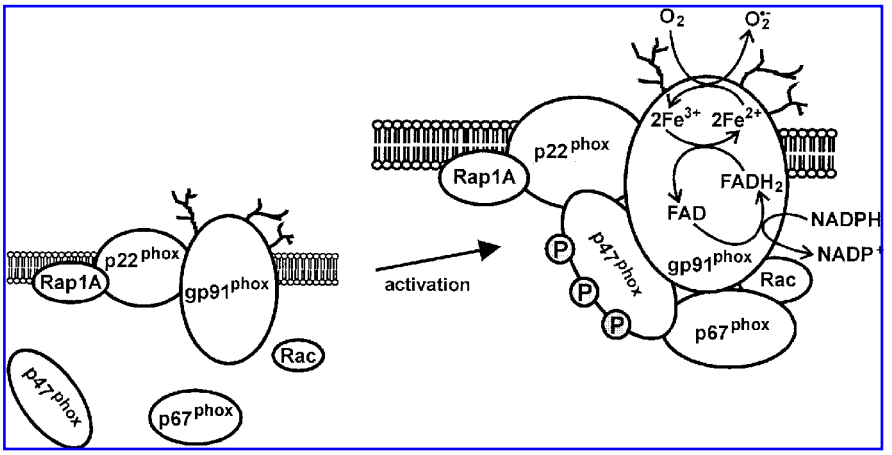

FIG. 2. Activation of the NADPH oxidase complex. The NADPH oxidase subunits p22^{phox} and gp91^{phox} are located in the plasma membrane. They bind flavin and heme as components of electron transport chain and form cytochrome b_{558}. Subunits p47^{phox} and p67^{phox} are located in the cytosol. Upon activation, p47^{phox} is phosphorylated by protein kinase C and translocated with p67^{phox} to the cytochrome b_{558}, forming an active $O_2^{\cdot -}$ generating enzyme. Small G proteins (Rac/Rap) are involved in the assembly of NADPH oxidase. The complex shows structural similarities between granulocytes and endothelial cells.

erence for NADH or NADPH oxidase in vascular smooth muscle cells and endothelial cells could not be confirmed (30, 47).

PROATHEROSCLEROTIC INDUCTION OF ENDOTHELIAL NADPH OXIDASE BY OXLDL

Hypercholesterolemia is an important risk factor for the development of atherosclerosis (67). An increased vascular $O_2^{\cdot -}$ formation could be demonstrated in animal models of hypercholesterolemia (60, 83), which might contribute to endothelial dysfunction. Recently, it has been shown in patients that hypercholesterolemia is associated with reduced endothelium-dependent relaxation due to increased vascular NADPH oxidase-dependent $O_2^{\cdot -}$ formation (32). Even native LDL is able to increase $O_2^{\cdot -}$ formation in vascular smooth muscle cells (48). Oxidative modification of LDL to oxLDL, e.g., by ROS, has been shown to potentiate this response. Furthermore, endothelium-dependent relaxation of rabbit coronary arteries was reported to be reduced by incubation with oxLDL, but not native LDL (10). OxLDL was shown to induce oxidative stress in endothelial cells, as well as in aortic segments of rabbits and rats (23, 24). The increased $O_2^{\cdot -}$ formation of oxLDL-treated endothelial cells was due to an induction of NADPH oxidase activity (33, 69). The augmented NADPH oxidase activity in response to oxLDL seems to be mediated by an increased expression of subunit gp91^{phox} in endothelial cells (69). Formation of oxLDL was suggested to take place primarily in the intima, because oxLDL could only hardly be detected in the blood, but readily found in the intima of atherosclerotic vessels (86). Furthermore, in hypercholesterolemia, the arterial influx of LDL proportionally increases with increasing plasma levels of LDL (59). Thus, in hypercholesterolemic vessels, more LDL infiltrates the intima where it can be oxidatively modified by $O_2^{\cdot -}$ generated by vascular NADPH oxidase. Furthermore, all vascular cell types can oxidize LDL in vitro by radical formation (11, 55). Because oxLDL itself is able to further potentiate endothelial gp91^{phox} expression and NADPH oxidase activity, we propose a vicious cycle involving augmented oxidative stress in the pathogenesis of atherosclerosis (Fig. 3). Therapeutic options to slow down this vicious cycle could involve lowering LDL plasma levels. This could reduce infiltration and oxidative modification of LDL and subsequently vascular gp91^{phox} expression. Indeed, lipid-lowering therapy with hydroxymethylglutaryl (HMG) CoA reductase inhibitors down-regulates gp91^{phox} expression in internal mammary arteries of patients with coronary artery disease undergoing selective coronary artery bypass grafting surgery (69). Furthermore, HMG CoA reductase inhibitors reduce expression of p22^{phox} and p47^{phox}

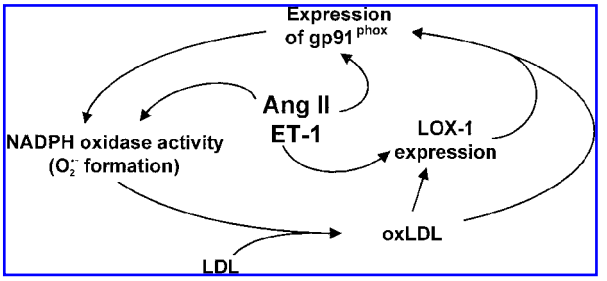

FIG. 3. Vicious cycle involving oxidative stress in the pathogenesis of atherosclerosis. The figure presents proatherosclerotic vicious cycle of locally increased angiotensin II (Ang II) and endothelin-1 (ET-1) levels, augmented oxidative stress, increasing oxidative modification of low-density lipoprotein (LDL) to oxidized LDL (oxLDL), augmented uptake of oxLDL by the endothelial oxLDL receptor LOX-1 in response to Ang II and ET-1, and further potentiation of oxidative stress in response to oxLDL in human endothelial cells.

in cultured endothelial cells (37) and interfere with subunit assembly by inhibition of small G proteins (80). Together, these antioxidative effects on vascular NADPH oxidase could contribute to the improvement of endothelium-dependent relaxation in patients with coronary artery disease and HMG CoA reductase inhibitor therapy (1).

REGULATION OF NADPH OXIDASE BY ANGIOTENSIN II (ANG II) AND ENDOTHELIN-1 (ET-1)

Increased systemic and tissue Ang II levels resulting from an activated renin angiotensin system have been implicated in the development of hypertension and atherosclerosis (36). A variety of studies suggest that induction of vascular $O_2^{\cdot-}$ formation by Ang II substantially contributes to its proatherosclerotic potential. Chronic infusion of Ang II into rats results in increased vascular $O_2^{\cdot-}$ formation with subsequent development of endothelial dysfunction and hypertension (64). In addition, activation of the renin angiotensin system induces vascular $O_2^{\cdot-}$ formation and endothelial dysfunction in rabbits (61). An essential role of increased vascular $O_2^{\cdot-}$ formation in Ang II-mediated hypertension is supported further by reduced blood pressure in Ang II-infused hypertensive rats after treatment with superoxide dismutase (44). Increased vascular $O_2^{\cdot-}$ formation in response to Ang II seems to be mediated mainly by induction of vascular NADPH oxidase activity (22, 61, 64). *In vitro,* Ang II was shown to induce NADPH oxidase activity in all vascular cell types (29, 43, 62, 89). In vascular smooth muscle cells, Ang II-mediated induction of $p22^{phox}$ expression was reported to be crucial for Ang II-induced NADPH oxidase activity (78). In fibroblasts, induction of $p67^{phox}$ expression by Ang II was suggested to account for the induced NADPH oxidase activity in fibroblasts (62). In human endothelial cells, an induction of NADPH oxidase subunits $gp91^{phox}$, $p22^{phox}$, $p67^{phox}$, and $p47^{phox}$ expression in response to Ang II has been found (70). *In vitro* and *in vivo* stimulation of vascular NADPH oxidase activity by Ang II involves Ang II receptor type 1 (AT_1) receptors (8, 43, 64, 70, 83). Interestingly, further increased Ang II levels subsequently reduce augmented NADPH oxidase activity in endothelial cells and fibroblasts (43, 62, 70, 89). In human endothelial cells, this seems to be mediated by AT_1-dependent induction of $gp91^{phox}$ expression at lower Ang II levels, but by Ang II receptor type 2 (AT_2)-dependent down-regulation of $gp91^{phox}$ (in contrast to the other subunits) at higher Ang II concentrations (70). The finding that Ang II infusion does not induce vascular NADPH oxidase activity in $gp91^{phox}$ knockout mice (82) further supports an essential role of $gp91^{phox}$. Thus, differential stimulation of Ang II receptor subtypes results in contrary effects on endothelial $gp91^{phox}$ expression and NADPH oxidase activity. As both receptor subtypes have been reported to have a similar affinity to Ang II (77), higher threshold of AT_2-mediated repression might result from a lower expression of AT_2 receptors compared with AT_1 receptors (46). Therefore, vessel-specific ratio of endothelial AT_1 and AT_2 receptors could determine $gp91^{phox}$ expression and NADPH oxidase activity at a certain Ang II concentration. This might have clinical implications. Treatment of patients with AT_1 receptor blockers improves endothelium-dependent relaxation (27, 72). While the expression of $gp91^{phox}$ in internal mammary arteries from patients with coronary artery disease was analyzed, preoperative therapy with AT_1 receptor blockers was found to down-regulate $gp91^{phox}$ in contrast to angiotensin-converting enzyme (ACE) inhibitors (70). This blood pressure-independent effect could be due to the retrospective determined rather low doses of ACE inhibitors (~30% of target dosages in recent megatrials) prescribed by the referring physicians. This might have resulted in Ang II levels below threshold of AT_2-mediated repression, but above threshold of AT_1 receptor-mediated induction of $gp91^{phox}$ expression. In patients receiving similar ACE inhibitor dosages, Ang II-induced expression of endothelial oxLDL receptor LOX-1 was reduced in internal mammary arteries (53). Therefore, prescribed ACE inhibitor dosage seems to be crucial in reducing proatherosclerotic oxidative stress and uptake of oxLDL.

The potent vasoconstrictor ET-1 has been shown to be implicated in the development and progression of atherosclerosis (7). ET-1 induces NADPH oxidase and oxLDL uptake in human endothelial cells as well (19, 54).

Therefore, the proposed vicious cycle of vascular $O_2^{\cdot-}$ formation, oxidative modification of LDL, endothelial oxLDL uptake by LOX-1, and subsequent oxLDL-mediated induction of $gp91^{phox}$ expression can be potentiated further by Ang II and ET-1 (Fig. 3). Furthermore, Ang II might activate the cytochrome b_{558} complex by protein kinase C-dependent $p47^{phox}$ phosphorylation, thus increasing directly NADPH oxidase activity. As proatherosclerotic effects of Ang II are mediated by AT_1 receptors, additional mechanisms might be involved. AT_1 receptor expression has been induced by high levels of LDL *in vitro* and reduced by HMG CoA reductase inhibitor therapy *in vivo* (57, 58). Therefore, LDL not only serves as a substrate for oxidative modification, but also potentiates Ang II-mediated effects by induction of AT_1 receptor expression in the proposed vicious cycle. In addition, $NO^{\cdot}$ was shown to repress AT_1 receptor expression (79). As increased NADPH oxidase-dependent $O_2^{\cdot-}$ formation could additionally reduce $NO^{\cdot}$ availability by $ONOO^-$ formation, this mechanism could further promote proatherosclerotic effects of Ang II mediated by the AT_1 receptor.

NOVEL HOMOLOGUES OF $gp91^{phox}$

During the last years, several novel homologues of NADPH oxidase subunit $gp91^{phox}$ have been cloned and characterized. These homologues have been designated as Nox1–5 and Duox1–2 (Table 1) (42). Nox is the abbreviation of NADPH oxidase and Duox of Dual oxidase. In this new nomenclature, $gp91^{phox}$ has been renamed as Nox2. Nox1–3 proteins have six transmembrane domains with four histidines constituting the conserved heme-binding residues of cytochromes. Intracellular C-termini have binding sites for FAD and NADPH. These conserved regions are essential to generate $O_2^{\cdot-}$. The deduced molecular mass of the corresponding protein is 65–66 kDa for Nox1–4, 85 kDa for Nox5, and 175–177 kDa for Duox1–2, respectively (Fig. 4).

TABLE 1. HUMAN NOX ISOFORMS

Name	*Gene locus*	*Sequence motifs*	*Tissue*	*Function*
Nox1	Xq22	6 transmembrane domains, 4 histidines for heme binding, binding sites for FAD and NAD(P)H	Colon, uterus, prostate, colon carcinoma cells, and VSMC*	Cell proliferation and trigger for angiogenic switch
		Short form: NOH1S with 3 transmembrane domains	Colon and colon carcinoma cells	Voltage-dependent H^+ currents
Nox2	Xp21.1	6 transmembrane domains, 4 histidines for heme binding, binding sites for FAD and NAD(P)H	Phagocytes	Immune defense
			Endothelial and adventitial cells	Oxygen sensor, regulation of blood pressure
Nox3	6q25.1-q26	6 transmembrane domains, 4 histidines for heme binding, binding sites for FAD and NAD(P)H	Fetal kidney and cancer cell line HepG2	Development of fetal kidney
Nox4	11q14.2-q21	6 transmembrane domains, 4 histidines for heme binding, binding sites for FAD, NAD(P)H, and ATP or GTP	Kidney	Regulation of erythropoietin synthesis
			Heart, skeletal muscle, brain, endothelial cells, fibroblasts, and VSMC	Oxygen sensor
Nox5	15q22.31	6 transmembrane domains, 4 histidines for heme binding, binding sites for FAD and NAD(P)H, 3EF-hand motifs	Testis	Sperm activation
			Spleen and lymph nodes	Activation of B-cell and T-cell receptors, proliferation and differentiation of B- and T-lymphocytes
Duox1 and 2	15q15.3-q21 independent genes in close vicinity	7 transmembrane domains, 4 histidines for heme binding, binding sites for FAD and NAD(P)H, 2 EF-hand motifs	Thyroid cells	Biosynthesis of thyroid hormones

*VSMC, vascular smooth muscle cells.

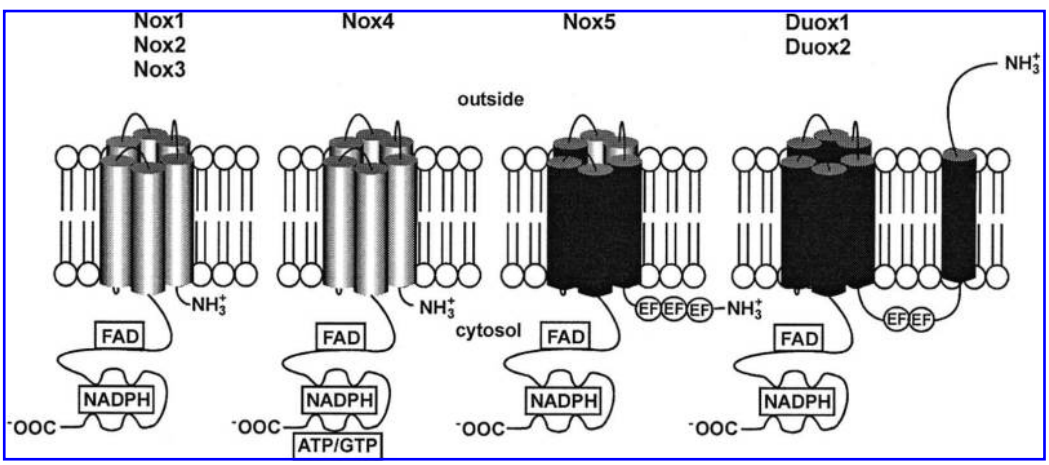

FIG. 4. Structure of novel gp91^{phox} homologues. The structures of novel homologues (Nox1–5, Duox1–2) of gp91^{phox} (Nox2) are summarized. The transmembrane domains containing the heme-binding residues of cytochromes and cytosolic binding sites for FAD, NADPH, or ATP/GTP at the C-terminal part of the protein are indicated.

Nox1

The Nox1 was detected in human and rat tissues (76). The Nox1 gene is localized on chromosome Xq22. Mitogenic oxidase [Mox1 (76)], NADPH oxidase homologue 1 [NOH1 (5)], and gp91-2 (41) are alternative expressions for Nox1. Nox1 reveals 58% identity of amino acids with CYBB, including all essential motifs to generate $O_2^{\cdot -}$ (41). Nox1 is expressed in colon, uterus, prostate, colon carcinoma cells, and vascular smooth muscle cells. The vascular NADPH oxidase complex containing Nox1 seems to control proliferation in nonphagocytic cells. An increased Nox1 mRNA expression stimulates platelet-derived growth factor in vascular smooth muscle cells. Furthermore, cells overexpressing Nox1 have increased $O_2^{\cdot -}$ generation, cell proliferation, and tumor development in athymic mice (76). In addition, increased H_2O_2 levels deduced from dismutation of $O_2^{\cdot -}$ have been found in Nox1-expressing cells. This H_2O_2 could act as an intracellular signal of the genetic program related to cell growth (3). Furthermore, ROS generated by Nox1 complexes trigger the angiogenic switch (2). This process involves induction of vascular endothelial growth factor, the corresponding receptors, and matrix metalloproteinase activity in vascular cells of Nox1-expressing tumors (2).

By alternative splicing of Nox-1, a shorter isoform (NOH1S, short) forming a H^+ channel has been described (5). NOH1S consists of exons 1–5 and exon 14 and was detected in colon and colon carcinoma cells. The NOH1S protein includes the first three transmembrane domains of gp91^{phox}-like proteins and contains a distinct fourth and a short intracellular C-terminus. These histidine-rich transmembrane motifs are postulated to generate voltage-dependent H^+ currents and can be blocked by zinc, a H^+ channel inhibitor. The H^+ channels are thought to play an important role in cellular defense against acidic stress (5). The existence of the NOH1S transcript *in vivo* has recently been questioned, because it could not be detected by northern blotting and the Nox1 genomic sequence does contain nonconserved splice sites (45). Future studies are needed to clarify this issue.

Nox2

Nox2 is the first Nox isoform discovered in human tissues (68). The Nox2 gene (CYBB) is localized on human chromosome Xp21.1. Gp91^{phox} is the original name of the catalytic subunit of the NADPH oxidase. Following new nomenclature, gp91^{phox} has been renamed as Nox2 (42). The Nox2-containing phagocyte NADPH oxidase produces high levels of $O_2^{\cdot -}$ to kill ingested microorganisms. An inherited failure to generate $O_2^{\cdot -}$ renders individuals with chronic granulomatous disease highly susceptible to infection (66). However, Nox2 is also expressed in nonphagocytic cells like endothelial cells (38) and adventitial cells (61). The vascular NADPH oxidase has been proposed to be an oxygen sensor and to regulate the blood pressure by consuming $NO^{\cdot}$ (51). The putative involvement of Nox2 in the pathogenesis of atherosclerosis has been discussed previously in this review already.

Nox3

By genomic sequence analysis, the Nox3 or gp91-3 gene has been mapped to chromosome 6q25.1-q26 (41). The Nox3 protein is 56% identical to CYBB. Nox3 expression has been detected in fetal kidney and in cancer cell line HepG2 so far, but not in adult tissues. The function of the Nox3 containing NADPH oxidase is currently not clear. Nox3 might play a role in the development of fetal kidney.

Nox4

Nox4 was identified as the gp91^{phox} homologue RENOX in the human and mouse kidney (26). Nox4 is expressed in heart, skeletal muscle, brain (74), endothelial cells, smooth muscle cells, and fibroblasts (75). The Nox4 gene is localized on human chromosome 11q14.2-q21. The Nox4 protein reveals 39% identity to CYBB (74) and contains all conserved regions essential to form an active enzyme complex. Furthermore, Nox4 has a nucleotide-binding sequence motif in the C-terminal region (P-loop) usually found in ATP- or GTP-binding proteins. Overexpression of Nox4 in cultured cells leads to increased $O_2^{\cdot -}$ production and decreased cell growth (74). Nox4-transfected fibroblasts developed signs of cellular senescence and showed a significant decreased rate of proliferation and increased apoptosis. Nox4 mRNA expression using an *in situ* hybridization technique has been described in renal cortex, especially in proximal convoluted tubule cells. Therefore, Nox4 has been suggested to act as an oxygen sensor regulating oxygen-dependent gene expression (26). Recently, Nox 4 has been shown to be highly expressed in endothelial cells and in the media of atherosclerotic arteries (75). Furthermore, Nox4 mRNA expression is induced by Ang II *in vitro* and in hypertensive animals *in vivo* (84). Therefore, Nox4 might contribute to increased intracellular stress in human coronary atherosclerosis.

Nox5

The gp91^{phox} homologue Nox5 was detected in the human testis, spleen, and lymph nodes (6). The Nox5 gene is localized on human chromosome 15q22.31. Comparison of the Nox5 amino acid sequence with gp91^{phox} revealed conserved NADPH-, FAD-, and heme-binding motifs. In addition, the Nox5 protein has three EF-hand motifs for calcium binding. The Nox5 protein shows some identity to the gp91^{phox} (27%) and Nox1 (56%) protein. In testis and spleen, two Nox5 isoforms of 737 and 719 amino acids were found: Nox5A and Nox5B. Exons 3–19 generate the Nox5A mRNA, whereas exons 1, 2, and 4–19 encode Nox5B mRNA. The Nox5 containing NADPH oxidase produced large amounts of $O_2^{\cdot -}$ in response to elevated cytosolic calcium. Furthermore, Nox5 has a H^+ channel function, presumably to compensate electron transport-dependent charge and pH alterations. Nox5 might couple elevations of cytosolic calcium concentration during sperm activation to spermatozoa effector function. In addition, Nox5 could play a role in activation of B-cell and T-cell receptors and in proliferation and differentiation of B- and T-lymphocytes.

Duox1–2

The Duox group consists of two novel gp91^{phox} homologues: Duox1 and 2. Duox proteins have seven transmembrane domains, characteristic motifs of flavoproteins including NADPH- and FAD-binding domains, four specific

histidines for heme binding. They also contain two intracellular EF-hand motifs involved in direct activation of the enzyme by calcium. The N-terminal domain contains a peroxidase function. Their topology suggests an NADPH oxidase complex able to generate $O_2^{\cdot -}$ and to dismutate H_2O_2 by their peroxidase function (20). Duox1 and 2 proteins display strong similarity to each other (83%) and are related to CYBB (53% and 47% similarity). The genes are localized on human chromosome 15q15.3-q21. Independently, the Duox1 and 2 genes have been discovered as Thox1 and 2 (16) and $p138^{Thox}$ (20). $P138^{Thox}$ is a short version of Duox2 lacking three out of four sequences involved in the interaction with $p47^{phox}$ (20). The proteins were detected in the apical membrane of human thyroid cells (16). The Duox proteins seem to play a role in the biosynthesis of the thyroid hormone. Duox1 and 2 mRNAs are up-regulated by cyclic AMP antagonists in cultured human thyrocytes. Thyrotropin induces the expression of the thyroid NADPH oxidase through a cyclic AMP-dependent pathway in thyrocytes of animal models (65). The finally generated H_2O_2 could serve as electron acceptor for biosynthesis of thyroid hormone catalyzed by thyroperoxidase of thyrocytes.

In summary, several novel $gp91^{phox}$ homologues have been described recently. The impact of these isoforms on $O_2^{\cdot -}$ formation and the pathophysiological relevance in different diseases is the subject of ongoing studies.

CONCLUSIONS

An elevated vascular $O_2^{\cdot -}$ formation by increased NADPH oxidase expression seems to be involved in the development and progression of endothelial dysfunction and atherosclerosis. The investigation of novel NADPH oxidase subunits will give further insight into the regulation of superoxide-forming complexes in different *in vitro* and *in vivo* models of atherosclerosis. Even though the treatment with vitamins did not improve cardiovascular mortality in recent megatrials (13, 88), therapy with ACE inhibitors, AT_1 receptor blockers, HMG CoA reductase inhibitors, and the recently approved endothelin receptor antagonists might have an antioxidative and antiatherosclerotic potential by reducing tissue NADPH oxidase expression and $O_2^{\cdot -}$ formation in the vessel wall.

ABBREVIATIONS

ACE, angiotensin-converting enzyme; Ang II, angiotensin II; AT_1, angiotensin II receptor type 1; AT_2, angiotensin II receptor type 2; ET-1, endothelin-1; HMG, hydroxymethylglutaryl; H_2O_2, hydrogen peroxide; $HO^{\cdot}$, hydroxyl radical; LDL, low-density lipoprotein; $NO^{\cdot}$, nitric oxide; $O_2^{\cdot -}$, superoxide anion; $ONOO^-$, peroxynitrite anion; oxLDL, oxidized low-density lipoprotein; ROS, reactive oxygen species.

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