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Forum Mini Review

Role of NADPH Oxidases in the Control of Vascular Gene Expression

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ABSTRACT

All vascular cells, including endothelial cells and smooth muscle cells, express components of the leukocyte NADPH oxidase such as p22phox, p47phox, and Rac. Endothelial cells and fibroblasts also express the leukocyte NADPH oxidase subunit gp91phox/nox2, whereas in smooth muscle cells nox1 and nox4 are found. The different vascular NADPH oxidases represent important sources for the basal as well as the agonist-induced superoxide anion (O_2^-) generation in the vasculature. In vascular smooth muscle cells, activation of the NADPH oxidases and the subsequent formation of O_2^- has been demonstrated for various agents including angiotensin II, thrombin, lysophosphatidylcholine, and tumor necrosis factor α. By influencing the activity of p38 mitogen-activated protein kinase and AKT, NADPH oxidase-derived O_2^- increases the expression of several pro-arteriosclerotic genes, such as monocyte chemoattractant protein-1, tissue factor, and vascular endothelial growth factor. Thus, the vascular NADPH oxidases play an important role in mediating the signal transduction cascade of pro-arteriosclerotic stimuli. *Antioxid. Redox Signal.* 5, 803–811.

INTRODUCTION

OVER THE PAST 8 YEARS it has become evident that isoforms of the leukocyte NADPH oxidase, which generates the respiratory burst in granulocytes, are the predominant cellular source of oxygen-derived free radicals (reactive oxygen species [ROS]) in the vasculature.

In white blood cells, NADPH oxidase consists of several cytoplasmic subunits and two membrane-bound subunits, p22phox and gp91phox (the latter is now termed nox2), which form a cytochrome b558 complex in the presence of heme. Upon leukocyte activation the cytoplasmic subunits p47phox, p67phox, and p40phox as well as Rac-2 translocate to this membrane-bound protein complex and facilitate the transfer of electrons from NADPH to molecular oxygen, leading to the generation of the superoxide anion (O_2^-) (for review 2, 3).

As initially reviewed by Jones *et al.* (43) in 1995, different subunits of the leukocyte NADPH oxidase system are also present in nonphagocytic cells.

EXPRESSION OF NADPH OXIDASE IN VASCULAR CELLS

Particularly in the vasculature, our understanding about the isoforms of the leukocyte NADPH oxidase and their role in cellular O_2^- formation has greatly advanced since the initial reports of an angiotensin II-inducible p22phox-containing NADPH oxidase in vascular smooth muscle cells (VSMCs) by Griendling and co-workers (31, 90). Meanwhile, isoforms of the leukocyte NADPH oxidase have been identified in all types of vascular cells.

Since vascular endothelial cells are derived from hemangioblasts (73, 97), the common precursors of leukocytes and endothelial cells (14), several hematopoetic transcription factors (47) and, consequently, all subunits of the leukocyte NADPH oxidase (62) are found in this cell type (6, 44). As demonstrated using cells and vascular preparations from NADPH oxidase knock-out mice, the agonist-induced O_2^- formation

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in endothelial cells is critically dependent on the nox2 (29) and p47phox (55) subunit of the leukocyte NADPH oxidase.

In VSMCs the situation is more complex for several reasons. Homologues of gp91phox/nox2 have been cloned (50, 83) and two of these newly discovered proteins, nox1 and nox4, are expressed in VSMCs from conduit vessels (53, 63, 80, 85). In VSMCs derived from human arterioles, however, nox2 mediates O_2^- generation, whereas nox1 is undetectable (89). In addition to the nox homologues, p22phox has been detected by Western blot analysis and reverse transcription–polymerase chain reaction (RT-PCR) (29, 85, 89, 90) and shown to be involved in agonist-induced O_2^- formation of VSMCs (9, 90, 93). p67phox, although essential for the activity of the leukocyte NADPH oxidase (2), is undetectable in VSMCs (67). A homologue to p67phox will most probably be identified in VSMCs as in the absence of this subunit, no transfer of electrons from NADPH to oxygen can occur. With regards to p47phox, experiments carried out in vascular preparations and smooth muscle cells (SMCs) from p47phox −/− mice (5, 8, 39, 51, 54) as well as one study using p47phox neutralizing antibody (77) suggest that this subunit contributes to NADPH oxidase activity in SMCs. It should, however, be mentioned that conflicting observations regarding the expression of p47phox mRNA and protein have been made in cultured SMCs. p47phox was easily detected by Western blot in some studies (5, 67, 77), but Lavigne *et al.* (54) demonstrated that p47phox expression is rapidly lost after cell passage, and became undetectable in cells of higher passage (8). In this context, the very recent demonstration of p47phox and p67phox homologues in colon cells that form an oxidase complex with nox1 is exciting and may prompt towards a role of these proteins in the VSMC oxidase (4, 26). Although this aspect has not yet been extensively studied in vascular cells, mRNA for the mentioned p47phox homologue is detectable by RT-PCR in VSMCs (author's unpublished data).

In the adventitia, which is composed of fibroblasts, pericytes, nerves, and inflammatory cells such as mast cells, plasma cells, and monocytes and adipocytes, a leukocyte-type NADPH oxidase has been observed and demonstrated to be functionally active (for review see 70). A role of p67phox for vascular O_2^- generation has been demonstrated in this tissue, as well as in fibroblasts (15, 65, 66).

THE FUNCTION OF NADPH OXIDASE IN VASCULAR CELLS

Most enzymes capable of generating oxygen-derived radicals, such as cytochrome P450 monooxygenases (7, 23, 79), xanthine oxidase (81) and nitric oide (NO) synthase (NOS) (69, 99), usually do so under some kind of stress condition such as depletion of cofactors or partial oxidation of the enzyme (11). In contrast, the sole function of the leukocyte-type NADPH oxidase is the generation of O_2^-. In leukocytes, O_2^- plays an integral role in the bactericidal function of the cell as O_2^- helps to control the pH in the phagosomes to keep the proteolytic enzymes active (for review see 42). It also serves after dismuting to H_2O_2 as substrate for peroxidases, in particular myeloperoxidase, to form small bacteriotoxic molecules such as HClO or hydroxyl radicals (·OH). Finally, O_2^- also reacts with NO, leading to the formation of toxic peroxynitrite (for review see 34).

The question why nonphagocyte cells express an O_2^- generating enzyme is unanswered. It might be that the nonphagocytic NADPH oxidase is a remnant or precursor of the leukocyte-like defense system in cells not specialized in bacterial killing. In this context, it is certainly worth mentioning that osteoclasts (102) and epithelial cells such as colon cells (13, 83), gastric pit cells (48, 87), or tubulus cells in the kidney (25, 78) have relatively high levels of NADPH oxidases compared with mesenchymal cells and that expression of the enzyme appears to be increased by differentiating or inflammatory stimuli (45, 87).

Alternatively, the NADPH oxidase system has evolved to provide a system for uniform reactions in response to intracellular stress. Cells *in situ* are continuously exposed to potentially harmful environmental stress arising from different physico-chemical stimuli, such as radiation and oxygen toxicity. Therefore, antioxidative defense systems as well as redox-sensitive signaling cascades have developed [an issue extensively reviewed by others (for example, 22, 32, 40, 46, 60, 64, 88)]. As a consequence of the effects of redox modulation on cellular function, oxygen-derived free radicals at low, nontoxic concentration have to be considered as second messengers. The NADPH oxidase as a system generating O_2^- will affect the cellular redox milieu. Thus, one could speculate that the oxidase system serves as a convergent signaling pathway to activate the "stress" response program in reaction to hormonal stimuli. Therefore, it is imperative to realize that the non-leukocyte NADPH oxidase does not generate cytotoxic levels of ROS but rather modulates the cellular redox milieu. Moreover, compartmentalization of NADPH-dependent ROS formation will further direct the radicals to certain target structures, although our current methods for the measurement of oxidative stress do not allow us to address this aspect in detail.

Many hormones, such as angiotensin II (31, 90), platelet-derived growth factor (PDGF) (53, 54, 59), and thrombin (9) as well as cytokines like tumor necrosis factor (TNF) α (19, 24) have been demonstrated to activate NADPH oxidase in vascular tissue. It is, however, trivial that these substance not only activate NADPH oxidase but also multiple other elements inside and outside the cell. Angiotensin, for example, activates protein kinase C (PKC), extracellular signal-regulated kinase (ERK) 1/2, and p38 mitogen-activated protein (MAP) kinase and increases the intracellular calcium concentration (for review see 75). Although all of the above-mentioned elements are affected by oxidative stress (46, 88), only the activation of p38 MAP kinase is mediated by O_2^- derived from NADPH oxidase (91).

Consequently, for the understanding of the role of NADPH oxidase in cardiovascular pathophysiology and in order to identify the enzyme as a potential target for cardiovascular drug development, the effects specifically mediated by the oxidase have to be identified. This concern, however, is hampered by the lack of specific inhibitors.

STRATEGIES TO SPECIFICALLY ALTER NADPH OXIDASE ACTIVITY

The vast majority of the studies suggesting NADPH oxidase-mediated effects have been carried out using flavin inhibitors such as diphenylene iodonium (DPI) (16). Since this compound nonspecifically blocks almost all flavin-containing enzymes such as NOS (96, 100), cytochrome P450 monoxygenases (23, 61), xanthine oxidase (76), and enzymes of the respiratory chain (56), studies using DPI as the only approach to inhibit NADPH oxidase have to be interpreted with great caution. In addition to DPI, only the vanillinoid apocynin (86), which by some authors is considered to be an established NADPH oxidase inhibitor, is commercially available, and increasingly often used. The specificity of apocynin has, however, not been carefully studied, and very high concentrations of the compound (600 μmol/L) are required to reliably inhibit NADPH oxidase (82). Although nontoxic in animal experiments, it has to be expected that at such concentrations effects unrelated to the inhibition of NADPH oxidase occur (37). Indeed, apocynin has been shown to block the formation of thromboxane A_2 in porcine pulmonary macrophages (21). More importantly, apocynin enhanced γ-glutamylcysteine synthetase activity in A549 cells leading to increased glutathione synthesis and thus at least in chronic experiments to antioxidative effects unrelated to NADPH oxidase (52). Finally, in human VSMCs apocynin at a concentration of 600 μmol/L has direct effects on gene expression and MAP kinase phosphorylation (author's unpublished data, 2002). Because of these limitations, the effects of DPI and apocynin are usually compared with those of selective inhibitors of NOS, xanthine oxidase, and the respiratory chain. Although this approach is reasonable to pin down potential sources of O_2^-, one has to be aware of the fact that it will never be possible to exclude all relevant O_2^--generating enzymes. More importantly, because of the pronounced interference of DPI with many enzymes in the cell in addition to those generating O_2^-, the substance yields unpredictable results at the levels of signaling, transcription factor binding, and gene expression. For instance, incubation of resting vascular SMCs with DPI for 4 h leads to a pronounced induction of monocyte chemoattractant protein-1 (MCP-1), vascular endothelial growth factor (VEGF), and tissue factor mRNA (author's unpublished data), which is probably a consequence of the DPI-induced NADPH oxidase-independent activation of the transcription factor activator protein-1 (AP-1), as demonstrated recently (74).

Consequently, suppression of expression or blockade of different oxidase subunits using molecular biology tools appears to be the only way to specifically analyze the involvement of this enzyme in signaling and gene expression. In this respect, several different approaches have been taken so far: Phosphothioate-modified antisense oligonucleotides have frequently been used to attenuate the expression of p22phox, nox1, and gp91phox/nox2 in VSMCs, mesangial cells, cardiomyocytes, endothelial cells, and fibroblasts (9, 36, 89, 94, 98). Transfection of full-length antisense oligonucleotide against p22phox, nox1, and nox4 was performed in SMCs, fibroblasts, and renal cells (53, 78, 83, 90). SMCs and endothelial cells derived from p47phox −/− mice were used to study the role of this subunit in radical generation in cultured cells (5, 8, 54, 55). Moreover, p22phox and p47phox, respectively, were blocked by electroporation of neutralizing antibodies (9, 77). Finally, dominant-negative p47phox (101) and a peptide inhibitor (41, 57, 71) were developed to block the interaction of p47phox with the nox isoforms. As a consequence of these studies, an involvement of NADPH oxidase in agonist-induced O_2^- formation has been established for many scenarios.

REGULATION OF GENE EXPRESSION BY NADPH OXIDASE-ACTIVATING AGONISTS

The effects of NADPH oxidase-dependent O_2^- formation on signaling and gene expression, however, have been not exhaustively studied in vascular cells using these specific approaches. Most of the studies were performed using thrombin as an agonist. Thrombin increases the O_2^- generation in VSMCs (67) via a mechanism involving p22phox (9) and p47phox (5, 8). Using p22phox antisense oligonucleotides, as well as a p22phox-neutralizing antibody or VSMCs cultured from p47phox −/− mice, it could be demonstrated that the thrombin-induced expression of MCP-1 (8, 9), tissue factor (8, 38), and plasminogen activator inhibitor 1 (PAI-1) (30) involves NADPH oxidase-dependent O_2^- formation. Similar results were published concerning thrombin-induced VEGF expression (8, 30). Accordingly, overexpression of nox1 increases VEGF in fibroblasts (1). MCP-1 expression is controlled by NADPH oxidase not only in SMCs but also in endothelial cells, where serum starvation leads to the induction of this protein via a pathway sensitive to the NADPH oxidase-blocking peptide gp91ds-tat (58). The induction of MCP-1 by TNF α can also be mediated by NADPH oxidase. However, non–NADPH oxidase-induced ERK 1/2 activation in response to this strong stimulus is also sufficient to induce MCP-1 in VSMCs (17).

The potent mitogen PDGF is another strong agonist for SMC O_2^- formation (59). The involvement of NADPH oxidase for PDGF-induced radical generation has been demonstrated using nox1-antisense transfected cells (53), p22phox neutralizing antibody (49) and cells cultured from the aorta of p47phox −/− mice (54). Furthermore, the PDGF-induced expression of tissue factor (28) as well as of VEGF (8) is mediated by NADPH oxidase as demonstrated using antisense-plasmid (8) and SMCs cultured from p47phox −/− mice (8).

Although the role of NADPH oxidase in angiotensin II-induced O_2^- formation has been extensively demonstrated, very few studies have addressed the role of the oxidase in angiotensin II-induced gene expression, as most authors largely concentrated on the role of the oxidase in angiotensin II-induced hyperplasia and hypertrophy (5, 10, 83, 92). For this aspect, it is interesting to note that p22phox antisense oligonucleotides inhibit the angiotensin II-induced expression of the cell cycle regulator $p27^{Kip1}$ in mouse renal tubular cells, preventing hyperplasia (36). Furthermore, angiotensin II-induced interleukin-6 expression could be inhibited using a p47phox neutralizing antibody (77). Very recently, it has been demon-

strated that the specific NADPH oxidase peptide inhibitor gp91-ds-tat prevents angiotensin II-induced intercellular cell adhesion molecule-1 expression in the rat aorta (57). Angiotensin II-induced MCP-1 expression is also likely to be dependent on NADPH oxidase-mediated O_2^- formation, so far only DPI and apocynin have been used to address this issue (12).

SIGNALING PATHWAYS UNDERLYING NADPH OXIDASE-INDUCED GENE EXPRESSION

Alterations in gene expression are primarily a consequence of altered transcription factor binding. To date, no study has addressed the effect of specific inhibition of NADPH oxidase on transcription factor binding, except for those carried out using DPI. Nevertheless, it might be speculated based on the known oxidase-activated kinases that at least the transcription factors nuclear factor κB (NF κB) and AP-1 are partially controlled by radicals generated from NADPH oxidase. In this context it has been demonstrated very recently that SMCs from p47phox −/− mice exhibit an attenuated activation of NF κB DNA binding in response to angiotensin II and AT1-receptor autoantibodies (18). In addition, we have previously reported that the thrombin-induced expression of the transcription factor hypoxia-inducible factor-1α (HIF-1α) is mediated by NADPH oxidase, which controls expression of VEGF and PAI-1 (30).

With regard to the signaling upstream of transcription factors but downstream of NADPH oxidase, most work has focused on the different MAP kinase pathways as well as on Akt/protein kinase B. Angiotensin II as well as thrombin activate ERK 1/2, p38 MAP kinase, and Akt in VSMCs. Although ERK 1/2 phosphorylation can be elicited by exogenous oxidative stress, induced by H_2O_2, inhibition of NADPH oxidase using the antisense technique had no effect on thrombin- as well as angiotensin II-induced ERK 1/2 phosphorylation. In contrast, agonist-induced activation of p38 MAP kinase and Akt was suppressed by NADPH oxidase inhibition (9, 10, 91, 93). In line with this observation, transfection of nox1 antisense also blocked angiotensin II-induced p38 MAP kinase and AKT phosphorylation, but not that of ERK 1/2 (53). Similar results were obtained in rat cardiomyocytes, using antisense oligonucleotides against nox1 and p22phox, which also inhibited p38 MAP kinase activation.

In contrast to these observations, lysophosphatidylcholine (LPC)-induced ERK 1/2 phosphorylation in a VSMC line was sensitive to dominant negative p47phox (101). Nevertheless, the upstream pathways of LPC and angiotensin II are very different, since LPC signaling occurs mainly via PKC, a well-characterized redox-sensitive group of enzymes (for review see 27). Thus, an NADPH oxidase-mediated activation of PKC rather than ERK 1/2, which are downstream of PKC, may underlie the LPC-induced ERK 1/2 phosphorylation.

Agonist-induced activation of c-Jun N-terminal kinase (JNK) also appears to involve NADPH oxidases, as antisense oligonucleotides against p22phox prevented angiotensin II-mediated activation of JNK in VSMCs (93). In addition, a role of the oxidase has been suggested for TNF α-induced JNK activation (33), but this study was performed in ECV-304 bladder carcinoma cells, erroneously assumed to be an endothelial cell line (20).

MECHANISMS OF NADPH OXIDASE-INDUCED SIGNALING

The activation of downstream targets in agonist-induced signaling of the NADPH oxidase is sensitive to antioxidants

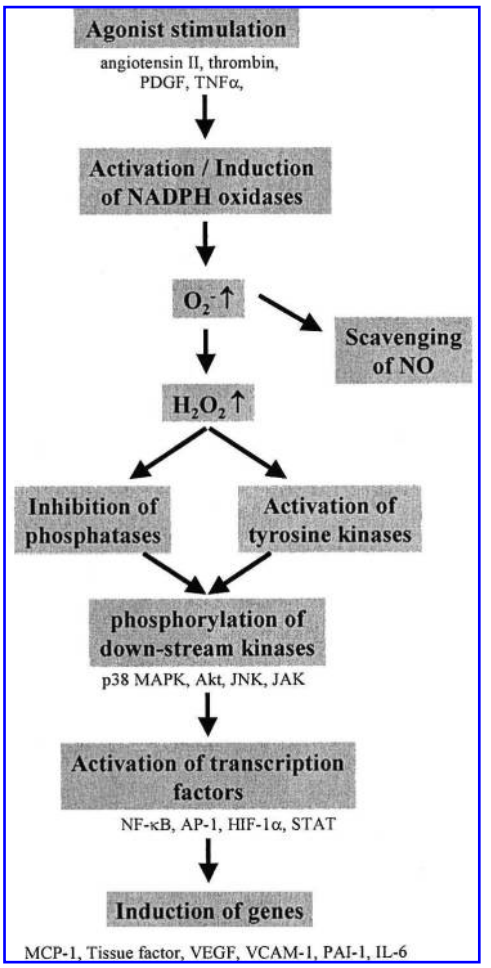

FIG. 1. Scheme of the signaling pathways leading to NADPH oxidase-mediated gene expression in VSMCs. Agonist stimulation with angiotensin II, thrombin, PDGF, or TNF α leads to the induction and/or activation of NADPH oxidases and the subsequent formation of O_2^-, which scavenges NO or rapidly dismutates to hydrogen peroxide (H_2O_2). H_2O_2 activates tyrosine kinases and inhibits phosphatases, a process that leads to an enhanced phosphorylation of downstream kinases such as p38 MAP kinase (p38 MAPK), Akt, JNK, or Janus kinases (JAKs). One consequence of this process is the activation of transcription factors such as NF-κB, AP-1, HIF-1α, and signal transducers and activators of transcription (STATs), which leads to alterations in cellular gene expression. Via this pathway, agonist-induction activation of NADPH oxidase increases the expression of MCP-1, tissue factor, VEGF, vascular cell adhesion molecular 1 (VCAM-1), PAI-1, and interleukin 6 (IL-6).

such as tiron, vitamin C, and catalase, which indicates that the downstream effects are a consequence of NADPH oxidase-dependent oxygen radical formation. To address the question how oxygen radicals affect signaling would certainly go beyond the scope of this review, and this topic will be addressed or has been addressed (35) in detail by other review articles published in this forum. On the level of signaling cascades, oxygen radicals, in particular hydrogen peroxide, have been shown to inhibit activity of specific phosphatases, leading to the activation of protein tyrosine kinases, tyrosine kinase receptors, and serine–threonine kinases. This, however, is only one aspect of ROS-mediated signaling and changes in the redox milieu of numerous other signaling elements, including GTPases, lipids, and transcription factors, will alter cellular gene expression (22, 32, 35, 40, 46, 60, 64, 88).

Particularly in the vasculature another important aspect is the NADPH oxidase-mediated scavenging of endothelial NO, which leads to the formation of peroxynitrite. Numerous studies have demonstrated that the NADPH oxidase limits NO bioavailability (29, 51, 72, 95) and controls peroxynitrite formation (41, 95). There is no doubt that NO has an important influence on vascular gene expression (68), but the specific question whether NADPH oxidase indirectly via this pathway affects signaling has not been studied.

CONCLUSION

NADPH oxidase plays an important role in modulating the cellular redox state and in mediating agonist-induced gene expression (Fig. 1). Nevertheless, the exact mechanism underlying the oxidase-induced effects and those leading to proliferation and hypertrophy in response to NADPH oxidase activation are still incompletely understood. Future work will identify transcription factors selectively activated by NADPH oxidase-dependent mechanisms and will yield insight into the differential effects of the novel gp91phox/nox2 homologues on gene expression and cellular function.

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ABBREVIATIONS

AP-1, activator protein-1; DPI, diphenylene iodonium; ERK, extracellular signal-regulated kinase; HIF-1α, hypoxia-inducible factor-1α; LPC, lysophatidylcholine; MAP, mitogen-activated protein; MCP-1, monocyte chemoatractant protein-1; NF κB, nuclear factor κB; NO, nitric oxide; NOS, NO synthase; O_2^- superoxide anion; PAI-1, plasminogen activator inhibitor 1; PDGF, platelet-derived growth factor; PKC, protein kinase C; ROS, reactive oxygen species; RT-PCR, reverse transcription– polymerase chain reaction; SMC, smooth muscle cell; TNF, tumor necrosis factor; VEGF, fascular endothelial growth factor; VSMC, vascular smooth muscle cell.

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