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Published in:
Antioxidants & Redox Signaling

Document Version:
Publisher's PDF, also known as Version of record

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Comprehensive Invited Review

NADPH Oxidases in Cardiovascular Health and Disease

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Reviewing Editors: Aron B. Fisher and Sashwati Roy

ABSTRACT

Increased oxidative stress plays an important role in the pathophysiology of cardiovascular diseases such as hypertension, atherosclerosis, diabetes, cardiac hypertrophy, heart failure, and ischemia–reperfusion. Although several sources of reactive oxygen species (ROS) may be involved, a family of NADPH oxidases appears to be especially important for redox signaling and may be amenable to specific therapeutic targeting. These include the prototypic Nox2 isoform-based NADPH oxidase, which was first characterized in neutrophils, as well as other NADPH oxidases such as Nox1 and Nox4. These Nox isoforms are expressed in a cell-
and tissue-specific fashion, are subject to independent activation and regulation, and may subserve distinct functions. This article reviews the potential roles of NADPH oxidases in both cardiovascular physiological processes (such as the regulation of vascular tone and oxygen sensing) and pathophysiological processes such as endothelial dysfunction, inflammation, hypertrophy, apoptosis, migration, angiogenesis, and vascular and cardiac remodeling. The complexity of regulation of NADPH oxidases in these conditions may provide the possibility of targeted therapeutic manipulation in a cell-, tissue- and/or pathway-specific manner at appropriate points in the disease process. Antioxid. Redox Signal. 8, 691–728.

I. INTRODUCTION

All aerobic organisms generate reactive oxygen species (ROS), oxygen-based molecules that are characterized by their high chemical reactivity. ROS include free radicals (species with one or more unpaired electrons) such as superoxide (\(O_2^{•−}\)) and hydroxyl radicals (\(\text{OH}^{•}\)), and non-radical species such as hydrogen peroxide (\(\text{H}_2\text{O}_2\)). In health, ROS generation is counteracted by the activity of enzymatic and nonenzymatic antioxidant systems that scavenge or reduce ROS levels, thereby maintaining an appropriate redox balance in cells and tissues. Perturbation of this normal balance due to increased ROS production and/or reduced antioxidant reserve leads to a state of oxidative stress, namely an enhanced susceptibility of biological molecules and membranes to reaction with ROS.

Increased oxidative stress is recognized to play an important role in the pathophysiology of numerous diseases, which in the cardiovascular system include hypertension, atherosclerosis, diabetes, cardiac hypertrophy, heart failure, and ischemia–reperfusion. A huge number of experimental studies have defined mechanistic pathways through which oxidative stress impacts on these diseases, and have shown that manipulation of oxidative stress may have therapeutic potential. Plentiful evidence also implicates a role for oxidative stress in human cardiovascular disease, and oxidative stress has been shown to be an independent risk marker for future cardiovascular disease (172, 299). Nevertheless, clinical trials of antioxidant vitamins in patients at risk of cardiovascular disease have not shown benefit in reducing cardiovascular events or mortality (163). It is increasingly evident, however, that the situation is much more complex than might initially be imagined. ROS have a wide range of potential actions that are influenced by the specific moiety generated, its localization, amount, and proximity to other radicals, enzymes, and signaling molecules. A key determinant of the biological consequences of cellular ROS generation in specific biological settings is likely to be the enzymatic source of ROS generation, particularly with regard to redox signaling (see later). Potential sources of ROS in the cardiovascular system include mitochondria, NADPH oxidases, uncoupled nitric oxide (NO) synthases, xanthine oxidase, cytochrome P450-based enzymes, and infiltrating inflammatory cells. This article focuses on the roles of NADPH oxidases, a family of enzymes first described in phagocytes but now known to be expressed much more widely. NADPH oxidases appear to be especially important for redox signaling and may be amenable to specific therapeutic targeting as opposed to the nonspecific ‘antioxidant’ approaches utilizing vitamin E and/or vitamin C, which have been disappointing in clinical trials to date. Several recent studies have provided confirmatory evidence of important pathophysiological roles for NADPH oxidases in human cardiovascular disease (128, 137, 224, 316).

II. REACTIVE OXYGEN SPECIES IN CARDIOVASCULAR BIOLOGY

Traditionally, oxidative stress was considered to be universally deleterious as a result of free radical-induced oxidation and damage of macromolecules, membranes, and DNA. For example, the restoration of \(O_2\) supply during myocardial reperfusion after prolonged ischemia is accompanied by a burst of free radical production that has damaging consequences such as the acceleration of cell death through apoptosis and necrosis. More recently, however, it has been appreciated that ROS can exert more subtle modulatory effects. First, tightly regulated ROS production can modulate the activity of diverse intracellular molecules and signalling pathways and thereby induce highly specific acute and chronic changes in cell phenotype—a mechanism commonly termed “redox signaling.” Second, the interaction of \(O_2^{•−}\) with the signaling molecule nitric oxide (NO) leads both to a reduction in NO bioavailability and the generation of another reactive species, peroxynitrite (\(\text{ONOO}^{•−}\)), which itself has biological activity. The inactivation of NO by ROS is a key mechanism underlying the development of endothelial dysfunction, which in turn is an important contributor to cardiovascular disease pathophysiology. Therefore, ROS can exhibit a wide spectrum of biological activity with at one extreme being signaling molecules that may subserve useful physiological functions and at the other being harmful species responsible for oxidative damage.

As an example, consider the \(O_2^{•−}\) radical (generated by a one-electron reduction of molecular \(O_2\)) which is quite unstable and has a half-life of only a few seconds in aqueous solution. It is poorly cell membrane-permeable and therefore usually restricted to the cell compartment in which it is produced. When \(O_2^{•−}\) is produced in relatively low amounts (picomolar range) it is rapidly dismutated to \(\text{H}_2\text{O}_2\), especially in the presence of superoxide dismutase (SOD) enzymes. \(\text{H}_2\text{O}_2\) is considerably more stable, diffusible, and cell membrane-permeable than \(O_2^{•−}\) and may therefore be responsible for redox signaling effects attributed to \(O_2^{•−}\) in many settings. The reaction of \(O_2^{•−}\) with NO (rate constant \(\sim 7 \times 10^9\) \text{mol}^{-1}\text{L.s}^{-1}\)) occurs at a significantly faster rate than with SOD (125), so that in the presence of high nanomolar NO there may be significant generation of \(\text{ONOO}^{•−}\). When \(O_2^{•−}\) levels are higher still, it may react with iron–sulfur centers in
proteins and release iron which reacts with H₂O₂ to produce highly reactive OH radicals.

III. REDOX SIGNALING

Transduction of the chemical ROS signal into a biologically relevant event is mediated by posttranslational covalent modification of specific amino acid residues on proteins, resulting in a change in protein function. This can be an acute alteration (over seconds to minutes) in function of the target molecule (an ion channel or contractile protein), or may result in chronic changes in cell phenotype (over hours and days) when the target protein is a signaling molecule such as a protein kinase or a redox-sensitive transcription factor. For the ROS-mediated posttranslational modification to succeed in biologically relevant signaling, the modification should proceed at a physiologically significant rate, be chemically reversible under physiological conditions, and/or be enzymatically catalyzed. A classical example is the progressive oxidation of thiol residues by, for example H₂O₂, to give rise to reaction products such as sulfenic acid, sulfenic acid, and sulfonic acid derivatives (265). Alternatively, oxidation may promote the formation of cysteine disulfide bonds within a protein or mixed disulfide bonds between a cysteine-containing protein and a low molecular weight thiol such as glutathione (265). Interestingly, different modifications to cysteine residues within a protein, in terms of either the source of ROS or the oxidation form, can deliver discrete and diverse regulatory outcomes. Once formed, intramolecular and mixed disulfide linkages can be removed by thiol disulfide exchange reactions and the activities of protein disulfide reductase, glutaredoxin, and thioredoxin reductase. A large number of proteins are known to be regulated by S-thiolation, including structural proteins (43, 83), metabolic enzymes (252), ion translocators (82), DNA isomerases (345), and signaling proteins (182). The specificity that is essential for pathophysiologically relevant redox signaling is effected through several mechanisms, including ligand-dependent stimulation of ROS production, the colocalization of ROS with specific substrates or downstream targets, and stimulus-coupled regulation of thiolation within the confines of a signaling molecule [for a detailed discussion of this topic, see recent reviews (102, 106, 265)]. Within the above general scheme, NADPH oxidases have several attributes that position them as prime candidates to be enzymes specifically designed to facilitate cellular redox signaling.

IV. PHAGOCYTIC AND NONPHAGOCYTIC NADPH OXIDASES

The NADPH oxidase was first described in professional phagocytes of the innate immune system (e.g., neutrophils and macrophages) where it is responsible for generating a large burst of O₂⁻ (the “oxidative burst”), using NADPH as an electron donor, during the process of phagocytosis (185). This high level ROS generation is largely generated within phagocytic vacuoles (i.e., within the “extracellular” compartment) and is pivotally involved in the killing of ingested pathogens, although not necessarily directly. The significance of phagocytic NADPH oxidase in host defence is clearly demonstrated in a rare disorder known as chronic granulomatous disease (CGD), in which genetic defects in essential oxidase components result in an inactive enzyme and a predisposition to recurrent life-threatening infections in affected children (76, 327). Considerable information on the structural requirements for a fully functional phagocyte NADPH oxidase derives from studies in CGD patients. The phagocyte NADPH oxidase comprises a membrane-associated low-potential heterodimeric flavocytochrome b₅₅₆ composed of one 22 kDa p22phox (for phagocyte oxidase) subunit and one gp91phox subunit which has a core molecular weight of ~65 kDa but migrates on SDS-PAGE with an apparent mass of ~91 kDa due to its heavy glycosylation state. Interaction between p22phox and gp91phox appears to be necessary for stability of the flavocytochrome complex. Although the flavocytochrome contains all the catalytic machinery required for electron transfer from NADPH to molecular O₂, activation of the phagocyte oxidase requires the translocation of several cytosolic regulatory subunits (p47phox, p40phox, p67phox, and the small G protein Rac1 or Rac2) to the membrane and their association with cytochrome b₅₅₆ (Fig. 1).

Over the last 10–15 years, it became evident that a rather similar, albeit lower-level, NADPH or NADH-dependent ROS-generating activity exists in numerous nonphagocytic cell types. In the cardiovascular system, these include vascular smooth muscle cells (VSMC) (118, 336), endothelial cells (EC) (24, 25, 111, 166), adventitial and cardiac fibroblasts (44, 263), and cardiomyocytes (29, 203, 355, 362). In general, nonphagocytic cells appear to generate low-level ROS continuously even in the absence of extrinsic stimulation (unlike neutrophils) but could increase their ROS production in response to specific stimuli. The use of relatively specific inhibitors (e.g., diphenylene iodonium [DPI] and apocynin) suggested that the source of this activity might be an NADPH oxidase enzyme, whereas other studies found that the p22phox oxidase subunit, but not gp91phox, was expressed in almost all cell types. These observations prompted a search for homologues of gp91phox, and resulted in the identification of a new family of homologous gp91phox isoforms, each encoded by distinct genes. These are now termed Noxs (for NADPH oxidase), with gp91phox known as Nox2 in the new terminology.

The first new member of the Nox family, Nox1, was originally cloned from a human colon cDNA library, and was shown to be expressed additionally in prostate, uterus, and cultured vascular smooth muscle cells (VSMC) (318). Subsequently Nox3, -4, and -5 were all cloned from human fetal kidney cDNA (49). Nox3 is primarily expressed in fetal tissues and the adult inner ear (18, 49). Nox4 (also known as Renox) was independently cloned by three separate groups, and is widely expressed in many adult tissues including pancreas, placenta, heart, vessels, ovary, testis, skeletal muscle, and, in particular, kidney (49, 103, 303). Nox5 is highly expressed in fetal tissue, and also in adult testis, spleen, ovary, placenta, and pancreas (49). All the novel Noxs encode predicted proteins of around 65 kDa, and show 21%–59% identity to Nox2, with Nox3 being the most similar and Nox5 the most divergent; all catalyze electron transfer from a reduced
substrate to molecular $O_2$ in a similar manner to Nox2 although their requirements for other subunits may differ (49). Two longer proteins with predicted molecular weights of $\sim 177$ kDa, namely Duox1 and Duox2, were cloned from human thyrocyte cDNA libraries and show 53% and 47% homology, respectively, to Nox2 within their C-terminal regions (64). However, the Duoxs also contain an N-terminal extension with no counterpart within the other Nox isoforms (see Fig. 2). Strong expression of both Duoxs was initially identified in the thyroid, with additional weak expression of Duox2 observed in the stomach (64). The extended family of Nox isoforms can be classified into three groups, according to the presence of specific domains: (a) Noxs1–4 have similar predicted general structures with six transmembrane $\alpha$-helices, containing conserved histidines implicated in heme binding, and putative flavin- and NADPH-binding domains towards the carboxyl termini (184) (Fig. 2); (b) Nox5 builds on the basic structure of Nox2 with an additional N-terminal calmodulin-like EF domain that contains four Ca$^{2+}$-binding sites, allowing its activation by elevated cytosolic Ca$^{2+}$ (20, 21), and demonstrates similarities with some plant oxidases (20, 21, 49); (c) The Duox enzymes further extend the Nox5 structure to include an N-terminal peroxidase-homology domain that is separated from the calcium-binding domain by an additional transmembrane segment (64, 81, 84, 185). Recently, isoforms of the regulatory subunits $p47^{phox}$ and $p67^{phox}$ have also been discovered in some nonphagocytic cells although in cardiovascular cells the classical isoforms appear to be more important. Colon epithelial cells express an $\sim 41$ kDa $p47^{phox}$ isoform termed NoxO1 (for Nox Organizer) and an $\sim 51$ kDa $p67^{phox}$ isoform termed NoxA1 (for Nox Activator). NoxO1 and NoxA1 substitute for $p47^{phox}$ and $p67^{phox}$ respectively in some cell types and may specifically function to activate Nox1 in vivo (17, 50, 105, 326). NoxO1 differs from $p47^{phox}$ in that it lacks phosphorylation sites that disinhibit an autoinhibitory region in the latter molecule, and therefore appears to be capable of supporting constitutive Nox1 activity (unlike $p47^{phox}$ which generally requires phosphorylation to facilitate oxidase activity). NoxA1 seems to be broadly similar to $p67^{phox}$ apart from lacking an N-terminal SH3 domain and a $p40^{phox}$ binding site. NoxO1 and NoxA1 have also been detected in liver, pancreas, and testis (326). Finally, the expression of Rac isoforms also varies among different cell types with Rac2 being the main isoform found in phagocytes, whereas Rac1 is the predominant isoform in most nonphagocytic cells.

**FIG. 1. Schematic diagram of the structure of the classical Nox2 oxidase under basal and activated conditions.** Activation of the oxidase involves the stimulus-induced translocation of the cytosolic subunits $p47^{phox}$, $p67^{phox}$, and $p40^{phox}$ and GTP-bound Rac to the membrane-bound cytochrome $b_{558}$ composed of Nox2 and $p22^{phox}$. 

<table>
<thead>
<tr>
<th>Basal</th>
<th>Activated</th>
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<td>$O_2$</td>
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<th>Nox2</th>
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<td>$p40^{phox}$</td>
<td>Rac</td>
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Colon epithelial cells express an $\sim 41$ kDa $p47^{phox}$ isoform termed NoxO1 (for Nox Organizer) and an $\sim 51$ kDa $p67^{phox}$ isoform termed NoxA1 (for Nox Activator). NoxO1 and NoxA1 substitute for $p47^{phox}$ and $p67^{phox}$ respectively in some cell types and may specifically function to activate Nox1 in vivo (17, 50, 105, 326). NoxO1 differs from $p47^{phox}$ in that it lacks phosphorylation sites that disinhibit an autoinhibitory region in the latter molecule, and therefore appears to be capable of supporting constitutive Nox1 activity (unlike $p47^{phox}$ which generally requires phosphorylation to facilitate oxidase activity). NoxA1 seems to be broadly similar to $p67^{phox}$ apart from lacking an N-terminal SH3 domain and a $p40^{phox}$ binding site. NoxO1 and NoxA1 have also been detected in liver, pancreas, and testis (326). Finally, the expression of Rac isoforms also varies among different cell types with Rac2 being the main isoform found in phagocytes, whereas Rac1 is the predominant isoform in most nonphagocytic cells.
V. INTERACTIONS BETWEEN NADPH OXIDASES AND OTHER ROS SOURCES

While the current article focuses on NADPH oxidase-derived ROS, it is increasingly clear that there are complex interactions among different ROS sources such that in many pathological settings multiple sources may be involved. In many cases, NADPH oxidase-derived ROS may promote ROS production by other sources thereby amplifying the total levels of ROS (Fig. 3). Several studies have found that NADPH oxidase-derived ROS can promote the oxidative degradation of the essential NO synthase cofactor, H4B, thereby leading to NO synthase uncoupling and O2•− (rather than NO) generation. This phenomenon has been termed amplification via “kindling radicals” (188, 195). Secondly, the

![Diagram of NADPH oxidase and other ROS sources](image)

FIG. 3. Interplay between NADPH oxidase and other ROS sources. O2•− generated from NADPH oxidase can potentially influence ROS production by other enzymatic sources of O2•−. For example, xanthine dehydrogenase is converted to O2•−-generating xanthine oxidase through oxidation. Similarly, mitochondrial ROS generation can be increased by ROS derived from other sources. Finally, O2•− or ONOO− can degrade the essential NO synthase co-factor H4B, hereby promoting NOS uncoupling and further O2•− production (reproduced from Ref. 285 with permission).
oxidative conversion of xanthine dehydrogenase to xanthine oxidase (238) may also serve to increase \( \text{O}_2^{-} \) levels. This has been reported to be an important mechanism contributing to EC \( \text{O}_2^{-} \) production in response to oscillatory shear stress (237). Thirdly, mitochondrial ROS generation can be increased by ROS derived from other sources (381), while a recent study suggested that mitochondrial ROS generation in turn may lead to NADPH oxidase activation in EC (298).

In addition to the above interactions, NADPH oxidase activity is itself potentially subject to feedback or feedforward regulation. For example, in VSMC or fibroblasts, exposure to exogenous \( \text{H}_2\text{O}_2 \) caused NADPH oxidase activation and endogenous \( \text{O}_2^{-} \) generation, thereby amplifying the vascular injury process (214). On the other hand, Rac1-dependent EC NADPH oxidase activation and subsequent \( \text{O}_2^{-} \) production mediates a feedback loop leading to increased proteosomal degradation of Rac1, which may then downregulate enzyme activity (179).

VI. NADPH OXIDASE SUBUNIT EXPRESSION IN CARDIOVASCULAR CELLS AND TISSUES

The \( p22^\text{phox} \) subunit is readily detected at both mRNA and protein level in cardiovascular cells (i.e., VSMC, EC, cardiomyocytes, fibroblasts) of most species studied to date. The expression of the catalytic Nox subunits, however, varies among the different cell types with distinct and tissue-restricted expression patterns (Table 1). Individual cell types can coexpress more than 1 Nox subunit, implying distinct functions of different Nox subunit-based oxidases. VSMC in culture have generally been reported to express significant levels of Nox1 and Nox4 with isolated reports also of Nox5 expression (20, 111, 167, 191, 313, 356), and one report of Duox1 in human aortic media (167). Significant Nox2 rather than Nox1 expression was found in human resistance artery VSMC (330), while low levels of Nox2 are also detectable in rat VSMC. In EC, a large number of studies in several different species have reported the expression of Nox2 mRNA and protein (192). Nox4 appears to be expressed at higher level than Nox2 in EC (7, 8, 338). Cardiomyocytes are generally reported to express both Nox2 and Nox4, but not Nox1 (38). Nox2 expression is also documented in adventitial fibroblasts (217), whereas cardiac fibroblasts reportedly express Nox4 rather than Nox2 (55). Nox5 was reported to be present in EC and cardiac fibroblasts in some studies (8, 20, 57, 111, 348) but there are no reports to date of either Nox3 or Duox2 expression in cardiovascular cells.

It should be noted that currently available data regarding the expression patterns of the Nox isoforms (see Table 1) are often contradictory, at least in part due to lack of suitable antibodies, species differences, and differences between cultured cells and tissue \textit{in situ}. Data regarding \textit{in vivo} expression in cardiovascular tissues and Nox isoform-specific functions remains extremely limited at present. Furthermore, the level of Nox mRNA expression does not necessarily correlate with oxidase activity. For example, recently, novel Nox4 splice variants have been discovered including two that have dominant negative characteristics for ROS generation (116). A novel Nox2 splice variant was also identified which is predicted to give rise to a truncated protein comprising only two transmembrane domains, together with a new C-terminal sequence, although the functional characteristics of this variant have not yet been established (133). A previously described Nox1 splice variant, however, subsequently proved to be an artifact (19, 104).

The cytosolic components of the classical NADPH oxidase (i.e., \( p47^\text{phox}, p40^\text{phox}, p67^\text{phox}, \) and Rac1) have generally been detected at both mRNA and protein level in most cardiovascular cells (reviewed in Ref. 192), apart from \( p67^\text{phox} \) which could not be detected in cultured VSMC (271). The cardiovascular expression of Nox1 and Nox4 has not been systematically studied but there is a report of low levels in rat basilar artery EC (7).

VII. NADPH OXIDASE ACTIVATION

A. Acute activation of phagocyte Nox2

NADPH oxidase

The vast majority of available information on the biochemical and molecular mechanisms underlying NADPH oxidase activation relates to the classical Nox2 oxidase of neutrophils, which we therefore consider first. Electron transfer in Nox2 occurs from NADPH, which binds to Nox2 at the cytosolic C-terminus, via FAD and two heme moieties (one towards the inner face and one towards the outer face of the membrane), to molecular \( \text{O}_2 \) in the interior of the phagocytic vacuole (i.e., the extracellular space). The initiation of electron transfer (oxidase activation) requires the recruitment of Rac as well as the cytosolic oxidase components \( p47^\text{phox}, p67^\text{phox}, \) and \( p40^\text{phox} \) to the cell membrane, and their association with flavocytochrome \( b_{558}^\text{c} \). Recruitment of Rac and the other components may be independent of each other and it remains unclear what the precise relative roles of these two events are. The \( p67^\text{phox} \) molecule contains a proline-rich activation domain which binds directly to an activation sequence in the C-terminal of Nox2 to initiate the process of electron transfer; thus, \( p67^\text{phox} \) is also known as the Nox activator. In resting neutrophils, \( p40^\text{phox}, p47^\text{phox}, \) and \( p67^\text{phox} \) may exist in a cytosolic complex stabilized by SH3 domain interactions. Intramolecular autoinhibitory interactions maintain \( p47^\text{phox} \) in a closed conformation that is unfavorable for binding to the flavocytochrome. During neutrophil activation, \( p47^\text{phox} \) becomes heavily serine phosphorylated at up to 11 sites, which relieves the above autoinhibitory interactions and elicits interaction with phosphoinositides on the cell surface (5, 6). In addition to phosphorylation, intracellular generation of arachidonic acid (AA) (and possibly phosphatidic acid) via phospholipase A2 appears to be necessary for recruitment to the cell membrane (59, 60, 62, 199, 304). Binding of SH3 domains of \( p47^\text{phox} \) to a proline-rich domain of \( p22^\text{phox} \) then allows interaction of \( p67^\text{phox} \) with Nox2 and oxidase activation. Thus, \( p47^\text{phox} \) plays an essential role in the assembly of the oxidase complex. The protein kinase C (PKC) isoforms \( \beta, \delta, \) and \( \zeta \) are suggested to be the major kinases responsible for \( p47^\text{phox} \) phosphorylation but recent studies suggest that other...
kinases such as Akt (PKC B), p38MAPK, and p21 activated kinase (PAK) can also be involved (46, 61, 72, 149, 178, 183, 256, 269). p67phox and p22phox also become phosphorylated during NADPH oxidase activation although the relevance of this remains unclear (31, 378). Likewise, the precise role of p40phox, which has significant homology to p47phox, in oxidase activation is poorly understood.

Rac binds to an N-terminal TPR domain in p67phox and this interaction may regulate electron transfer. However, recent evidence suggests that Rac-GTP also interacts directly with...

| Table 1. Expression of Nox Isoform mRNAs in Cardiovascular Cells and Tissue |
|----------------------------------------|-----------------|-----------------|----------------------------|
| Cardiomyocytes | Endothelial cells (EC) | Fibroblasts | Vascular smooth muscle cells (VSMC) |
| Nox1 | Isolated human coronary artery EC (313) | Isolated human cardiac fibroblasts (313) | Isolated human coronary artery SMC (313) |
| | Human umbilical vein EC (HUVEC) (8, 154) | Human umbilical vein EC (313) | Human aortic SMC (330, 272, 111) |
| | Rat aortic EC (8) | Rat aortic EC (8) | Rat VSMC from mesenteric arteries (330) |
| | Rat basilar artery EC (7) | Rat basilar artery EC (7) | Rat aortic VSMC (191, 114, 318, 350) |
| | P67phox (80, 111, 166, 289, 290, 343, 8, 154, 239) | P67phox (80, 111, 166, 289, 290, 343, 8, 154, 239) | Rabbit pulmonary arterial SMC (355) |
| | Isolated human coronary artery EC (313) | Isolated human cardiac fibroblasts (313) | Rabbit SMC from resistance arteries (353) |
| | Porcine pulmonary artery EC (147) | Porcine pulmonary artery EC (147) | Mouse aortic VSMC (124) |
| | Rat cardiac microvascular EC (24, 25) | Rat cardiac microvascular EC (24, 25) | A7r5 (rat aortic VSMC) (170, 356) |
| | Rat aortic EC (8) | Rat aortic EC (8) | Isolated human coronary artery SMC (313) |
| | Rat basilar artery EC (7) | Rat basilar artery EC (7) | HVSMC from resistance arteries (330) |
| | EA.Hy926 (transformed HUVEC) (111) | EA.Hy926 (transformed HUVEC) (111) | Human aortic intimal SMC (167) |
| | HUVEC (8, 154) | HUVEC (8, 154) | Adventitia of human coronary arteries (313) |
| Nox2 | Isolated human coronary artery EC (313) | Isolated human cardiac fibroblasts (313) | Adventitia of mouse aorta (348) |
| | Isolated human coronary artery EC (313) | Isolated human coronary artery EC (313) | Intimal cells of human coronary arteries (313) |
| | Porcine pulmonary artery EC (147) | Porcine pulmonary artery EC (147) | Rat aortic VSMC (191) |
| | Rat cardiac microvascular EC (24, 25) | Rat cardiac microvascular EC (24, 25) | |
| | Rat aortic EC (8) | Rat aortic EC (8) | |
| | Rat basilar artery EC (7) | Rat basilar artery EC (7) | |
| | EA.Hy926 (transformed HUVEC) (111) | EA.Hy926 (transformed HUVEC) (111) | |
| | HUVEC (20) | HUVEC (20) | |
| Nox4 | Isolated human coronary artery EC (313) | Isolated human cardiac fibroblasts (313, 37) | Intimal cells of human coronary arteries (313) |
| | Isolated mouse cardiomyocytes (273) | Isolated mouse cardiomyocytes (273) | Human aortic media (167) |
| | Mouse left ventricle (38) | Mouse left ventricle (38) | Media of human coronary arteries (313) |
| | Isolated mouse cardiomyocytes (273) | Isolated mouse cardiomyocytes (273) | A7r5 cells (356, 170) |
| | Isolated adult rat cardiomyocytes (55) | Isolated adult rat cardiomyocytes (55) | Isolated human coronary artery SMC (313) |
| | Isolated adult rat cardiomyocytes (55) | Isolated adult rat cardiomyocytes (55) | Human VSMC from resistance arteries (330) |
| | Isolated adult rat cardiomyocytes (55) | Isolated adult rat cardiomyocytes (55) | Human aortic SMC (330, 272) |
| | Isolated adult rat cardiomyocytes (55) | Isolated adult rat cardiomyocytes (55) | Rat VSMC from mesenteric arteries (330) |
| | Isolated rat cardiomyocytes (26) | Isolated rat cardiomyocytes (26) | Rat aortic VSMC (191) |
| | Isolated rat cardiomyocytes (26) | Isolated rat cardiomyocytes (26) | Medial smooth muscle within rat carotid arteries (323) |
| | Isolated rat cardiomyocytes (26) | Isolated rat cardiomyocytes (26) | Mouse aortic VSMC (124) |
| | Isolated rat cardiomyocytes (26) | Isolated rat cardiomyocytes (26) | Human VSMC (20) |
| | Isolated rat cardiomyocytes (26) | Isolated rat cardiomyocytes (26) | Human aortic SMC (272) |
| | Isolated rat cardiomyocytes (26) | Isolated rat cardiomyocytes (26) | Human aortic media (167) |
| | Isolated rat cardiomyocytes (26) | Isolated rat cardiomyocytes (26) | Intimal SMC within human atherosclerotic lesions (167) |

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the flavocytochrome b558 to regulate electron transfer (74). Rac-GTP has also been reported to be capable of initiating signaling pathways leading to translocation of cytosolic oxidase subunits in COS-7 cells (278, 279, 295). Rac translocation requires geranylgeranyl modification of its C-terminal (175), and this process is regulated by membrane-bound guanine nucleotide exchange factors (GEFs) which catalyze conversion of Rac-GDP to Rac GTP. A newly identified GEF, P-Rex1, which is activated either by phosphatidylinositol or G_{	ext{pr}} subunits (141, 354), appears particularly important (354), while other GEFs that may be involved include Tiam-1, Trio, and Vav-1 (244, 278). With regard to potential therapeutic manipulation of oxidase activity, it is relevant that the synthesis of geranylgeranyl groups is inhibited by HMG-CoA reductase inhibitors (statins); therefore, some of the pleiotropic effects of statins may be mediated through inhibition of Rac translocation.

B. Mechanisms underlying acute activation of cardiovascular NADPH oxidases

The continuous low-level NADPH oxidase-derived ROS production in cardiovascular (and other nonphagocytic) cells, even in the absence of agonist activation, has no parallel in the neutrophil oxidase. In EC, studies from our laboratory and others found that a significant proportion of the Nox2-based NADPH oxidase exists as fully preassembled and functional ROS-generating complexes associated with the perinuclear intracellular cytoskeleton, even in unstimulated cells (Fig. 4) (24, 94, 202, 330). This observation may provide a potential explanation for the continuous low-level activity in these cells. Intriguingly, experiments with \( p47^{\text{phox}} \) depletion and transfection in some of these studies have suggested that unphosphorylated \( p47^{\text{phox}} \) may act to modestly inhibit basal oxidase activity in unstimulated EC or aorta (208). However, more recent studies have also suggested that the continuous activity seen in the absence of agonist stimulation may be Nox4-oxidase-based (8, 11, 229, 303). Knockdown of Nox4 reduced basal ROS production in cultured EC and VSMC (8, 90), while in transfected HEK cells, EC, and VSMC, Nox4 oxidase activity was unaffected by the cytosolic subunits \( p67^{\text{phox}}, p47^{\text{phox}}, \) NOXA1 or NOXO1—suggesting that it does not require binding to these oxidase components for its activation but may be constitutively active (11, 103, 229, 303). Some studies have also suggested that Rac1 may regulate basal oxidase activity based on the finding that statin withdrawal after chronic treatment in animals stimulates endothelial \( O_2^- \) generation through Rac1-dependent activation of NADPH oxidase (339).

In addition to basal ROS production, NADPH oxidase activity in cardiovascular cells is acutely upregulated by a large number of stimuli (Fig. 5). In many cases, however, the Nox isoform that is responsible has not been definitively identified and it remains unclear whether activation is isoform-specific. There may also be significant variations in the responses to similar stimuli among different cell types, at least in part due to the heterogeneity in Nox isoform expression. While upstream signaling events leading to cardiovascular Nox2- and Nox1-based oxidase activation have been quite well studied for some agonists (54, 98, 337), the molecular events involved in oxidase activation at the level of the enzyme itself are relatively poorly characterized. Data on activation of Nox4-based oxidases are also extremely scanty. The precise location of ROS production (either basally or after agonist-induced activation) remains a matter of some debate (206, 363), largely because current methods for imaging ROS lack sufficient spatial resolution, but appears to be both intracellular and extracellular.

In general, the key features of Nox2 oxidase activation in cardiovascular cells are similar to the phagocytic enzyme insofar as the roles of \( p47^{\text{phox}} \) phosphorylation and Rac1...
translocation are concerned. Thus, Rac translocation is implicated in oxidase activation and response to altered shear stress (334, 368), phorbol esters (344), vascular endothelial growth factor (VEGF) (335), tumor necrosis factor α (TNFα) (48, 65, 68, 107, 258), hypoxia-reoxygenation (173), ischemia–reperfusion (173), depolarization (310), and nutrient deprivation (219) (Fig. 5). Similarly, the phosphorylation of p47phox and its translocation and association with cytochrome b558 is involved in oxidase activation in response to angiotensin II (Ang II) (208), TNFα (207), VEGF (360), chronic oscillatory shear (153), and other stimuli. PKC-dependent phosphorylation was implicated in the responses to Ang II and TNFα (207, 208), whereas the response to hyperoxia of human pulmonary artery EC (52) or to VEGF in human umbilical vein endothelial cells (HUVEC) (360) appears to involve tyrosine phosphorylation of p47phox. In contrast to Nox2, the roles of p47phox phosphorylation and translocation and Rac translocation in the activation of Nox1-based activity in cardiovascular cells remain to be definitively demonstrated but appear likely (e.g., in cultured VSMC).

Interestingly, recent data indicate that p47phox may have additional roles in nonphagocytic cells. It has been suggested that protein–protein interactions involving p47phox and other nonoxidase factors may play an important role in the spatial confinement of NADPH oxidase-derived ROS signals and thereby in local redox signaling (206, 363). A yeast two-hybrid screen of lung and EC libraries for interaction partners of p47phox by Xu et al. recovered several different proteins including the TNF receptor-associated factor 4 (TRAF4) (363). In HUVEC, Wu et al. (360) reported that VEGF-induced translocation of p47phox to membrane ruffles involved a direct interaction with WAVE1, an important regulator of cytoskeleton, which may act as a scaffold to recruit the NADPH oxidase to a complex involved with both cytoskeletal regulation and downstream JNK activation; the WAVE1-dependent complex also contained Rac1 and the kinase PAK1. Similarly, in human microvascular EC, we showed that the association of phosphorylated p47phox with TRAF4 was critical for TNFα-induced ROS-dependent activation of ERK1/2 (206) (Fig. 6).

In WEHI 231 lymphomas, CD40-induced NADPH oxidase activation required TRAF3 (129). Analogous to these roles of p47phox, protein–protein interactions involving Rac1 or Rac-GEFs may also be important in targeted redox signaling. Thus, in VSMC, AT1 receptor-dependent Rac1 and NADPH oxidase activation and EGF-receptor transactivation required caveolin-1-dependent GEF phosphorylation and trafficking into lipid rafts (382). Interaction with cytoskeletal elements also appears to have an important regulatory role in NADPH

**FIG. 5. Schematic diagram illustrating known activators of the Nox2 oxidase.** A diverse range of signals activate the oxidase including G-protein coupled receptor (GPCR) agonists such as angiotensin II (Ang II) and endothelin-1 (ET-1), mechanical forces, ischemia-associated factors, metabolic factors, and growth factors such as vascular endothelial growth factor (VEGF), platelet-derived growth factor (PDGF), and endothelial growth factor (EGF). AGE, advanced glycation end-products; LDL, low density lipoprotein.
oxidase activation and downstream redox signaling. The translocation of p47phox to the membrane is reported to be assisted by interactions with the cytoskeleton mediated by moesin (374). In VSMC, Ang II-stimulated ROS production and the phosphorylation of p38MAP kinase and JNK were attenuated by treatment with cytochalasin B, which disrupts the cytoskeleton; p47phox colocalized with the actin cytoskeleton in Ang II-stimulated cells in this study (331). In human EC exposed to human immunodeficiency virus type 1 Tat, p47phox becomes phosphorylated and rapidly redistributed to membrane ruffles; this response is associated with stress fiber disassembly and peripheral retraction and is mediated by PAK (359). In EC exposed to arsenic, NADPH oxidase activation and ROS production were shown to involve Cdc42-mediated actin filament reorganization; either overexpression of a dominant negative Cdc42 mutant or pretreatment with an actin filament stabilizing reagent, jasplakinolide, abrogated arsenic-induced NADPH oxidase activation (282). Taken together, these data suggest that interactions of p47phox and other oxidase components with cytoskeletal proteins and with other signaling molecules may play an important role in spatially confined redox signaling in response to specific agonists. This could take place either around specialized regions of the plasma membrane (such as caveolae) or in the vicinity of intracellular membranous compartments.

C. Activating stimuli for cardiovascular NADPH oxidases

Cardiovascular NADPH oxidase activity may be acutely upregulated by a wide variety of (patho)physiological stimuli which include (a) G-protein coupled receptor agonists such as Ang II and endothelin-1 (ET-1) (29, 80, 91, 187, 189, 210, 330, 332); (b) growth factors such as VEGF (54, 335), thrombin (135), platelet-derived growth factor (PDGF) (231), and EGF (113); (c) cytokines such as TNFα (107), interleukin 1 (IL-1), and transforming growth factor β (TGFβ) (113, 126); (d) “metabolic” factors such as elevated glucose (53, 152, 156), insulin (168), free fatty acids (156), and advanced glycation end products (AGE) (351, 377); (e) oxidized LDL, lysophosphatidylcholine, and hypercholesterolemia; (100, 134, 259, 261, 262); (f) mechanical forces such as oscillatory shear stress (144, 145); and (g) ischemia-related stimuli such as nutrient deprivation, membrane depolarization, flow cessation, hypoxia–reoxygenation, and ischemia (9, 218, 219) (Fig. 5). For detailed reviews of the signaling pathways upstream of

FIG. 6. Requirement of p47phox phosphorylation and TRAF4 for acute TNF-α induced redox signaling in endothelial cells. (A) Time course of TNF-α-induced p47phox phosphorylation, p47phox–TRAF4 binding, p47phox–p22phox binding (top panels, immunoblots) and NADPH-dependent SOD-inhibitable O₂⁻ production (bottom panel) in human microvascular endothelial cells (HMEC-1). Immunoblots demonstrate that TNF-α-induced p47phox phosphorylation and the association of p47phox with TRAF4 was detectable after 5 min, with a peak at 15–30 min. Along with p47phox phosphorylation, the amount of p22phox which co-immunoprecipitated with p47phox rapidly increased after TNF-α stimulation, being maximal at ~60 min. TNF-α also induced a significant increase in NADPH-dependent O₂⁻ production which peaked at ~30 min. (B) Role of p47phox and TRAF4 in TNF-α-induced ERK activation. Top panel: Representative immunoblots showing ERK-1/2 phosphorylation in wild-type and p47phox⁻/⁻ coronary microvascular endothelial cells. No ERK phosphorylation was detected in the absence of p47phox. Bottom panel: Effect of siRNA-mediated knockdown of TRAF4 on acute TNF-α-induced ERK1/2 activation. TRAF4 protein expression was substantially reduced after siRNA treatment, and TNF-α induced-ERK1/2 phosphorylation was concomitantly inhibited. Adapted from Ref. 206 with permission.
NADPH oxidase activation that may be involved, the reader is referred to several excellent recent publications (34, 118, 133). Here, we discuss what is known about the Nox isoforms that are activated in response to the above stimuli.

Ang II was first reported to upregulate NADPH oxidase activity in cultured VSMC in a seminal study by Griendling and colleagues (117). It has subsequently been shown to have similar effects in EC (204, 208, 375), cardiomyocytes and fibroblasts (122, 254, 264). Ang II-induced acute activation of NADPH oxidase in VSMC appears to involve Nox1, since antisense Nox1 cDNA inhibited this response (191). In EC (348) and cardiac tissue (29), however, Ang II-induced oxidase activation is critically dependent upon Nox2. Ang II is reported to induce ROS production with a biphasic time-course in VSMC, with initial very rapid NADPH oxidase activation occurring via PKC, whereas subsequent maintained activation involves EGFR receptor transactivation and robust Rac activation (301).

The Nox isoform involved in PDGF-induced oxidase activation in VSMC (231) and fibroblasts is unclear but the requirement for cytosolic subunits suggests that it is probably Nox1 rather than Nox4 (3, 196, 292). In Caco-2 and HEK293 cells, EGF stimulates Nox1-dependent radical generation (315). Similarly in Caco-2 cells, the inflammatory mediator IFN-γ induced an increase in Nox1 mRNA levels (274, 369). In rat cardiomyocytes, we have recently shown that NADPH oxidase activation induced by glycated albumin is dependent on Nox2 (377).

Mechanical stimuli, in particular cyclic stretch, induce NADPH oxidase activation in several cardiovascular cell types, including human coronary artery VSMC and human aortic EC (144, 145). A role for p47phox has been demonstrated in stretch-induced ROS formation and MMP2 activation in cultured VSMC (124) and in high pressure-induced ERK activation (260). Mechanically-induced oxidase activation is likely to be pathophysiologically important in many conditions (e.g., hypertension), but the Nox isoform(s) involved remain unclear (162).

D. Transcriptional regulation of oxidase subunits

In addition to acute activation of NADPH oxidase in cardiovascular cells, enzyme activity is also modulated by the transcriptional upregulation of oxidase subunits which presumably increases the pool of enzyme complexes available for activation. Indeed, chronic increases in oxidase activity (over hours or days) either in vitro in response to specific stimuli or in vivo in a variety of pathological contexts (see later sections) correlate in many cases with an increase in mRNA expression level of one or more oxidase subunit (including the Noxs) (290). However, in the in vivo setting it has not always been possible to determine which cell type is mediating the increase. It remains unclear whether simultaneous increases in all oxidase subunits are required to allow an increase in activity. In the case of Nox-based oxidase activity in EC, isolated increases in Nox2 may be sufficient since Nox2 mRNA expression level is low compared to other oxidase subunits (290). In the case of Nox4 oxidase, changes in Nox4 expression level may be the major mechanism responsible for modulating activity; p22phox is also required for activity but other subunits do not appear to be required (8, 229).

Available evidence indicates that distinct signaling pathways and/or effectors may be involved in the regulation of expression of different Nox isoforms. In VSMC, Nox1 mRNA expression is upregulated by serum, Ang II, PGF2α, LDL, phorbol ester, and mechanical stretch (124, 170, 191, 318, 356), downregulated by atorvastatin (350), and unaffected by endothelin-1, lipopolysaccharide, interleukin-1β, thrombin, or the oxysteroid 7-ketocholesterol (90, 272, 356). By contrast, Nox4 mRNA is downregulated by thrombin and interleukin-1β (90) but upregulated by human urotensin II (77) and 7-ketocholesterol (272), whereas the effects of Ang II and serum are conflicting (356, 191). In EC, Nox2 expression is reportedly upregulated by Ang II, endothelin-1, oxidized LDL, and shear stress (80, 155, 289, 290), while statins, estrogens, BMP-4, and pulsatile flow all cause a decrease in expression (155, 289, 314, 343). Nox4 expression is also reportedly upregulated by Ang II and shear stress (155, 365) and to be downregulated by pulsatile flow, although in another report, shear stress acted to decrease Nox4 mRNA (155, 314). In contrast to the Nox isoforms, increased expression of regulatory oxidase subunits in response to several agonists often appears to occur in a coordinated fashion. For example, in VSMC, chronic exposure to Ang II upregulates the expression of p22phox as well as p40phox, p47phox, and p67phox (65, 77, 112, 330). Likewise, in EC, Ang II upregulates p22phox, p47phox, and p67phox (290). The expression of p22phox or regulatory subunits may also be specifically downregulated by various agents, such as dexamethasone (230), activation of PPAR-α or −γ (157, 158) or statins (157, 158).

Taken together, the above data clearly indicate that the regulation of individual Nox isoforms and oxidase subunits is quite different and potentially complex even within a single cell type. It is clearly therefore of importance to determine the molecular mechanisms which effect the agonist-induced changes in transcription of NADPH oxidase subunits, as these may inform therapeutic strategies to target expression. The identification of cis-acting regulatory elements within the gene loci which mediate the agonist-induced transcriptional changes, and identification of the trans-acting factors that bind to these elements would begin to elucidate the pathways involved. To date, the gene whose regulation has been best characterized at the molecular level is Nox2. The minimal Nox2 promoter region required for monocye/macrophage expression was identified as a 450 bp region proximal to the transcription initiation site (309), which includes binding sites for both positive and negative regulators of transcription (86, 87, 161, 222, 233, 341). In terminally differentiated phagocytic cells, Nox2 expression is induced by the immune mediator interferon-γ (IFNγ), and this response involves the hematopoietic-lineage specific transcription factor PU.1. The latter binds to an element within the proximal Nox2 promoter and can form a complex with interferon regulatory factor-1, interferon consensus sequence binding protein and CREB binding protein (85, 87). In addition, eosinophil-specific regulation of Nox2 transcription was shown to be dependent upon activation by the direct binding of GATA-1, and competitive inhibition by the binding of GATA-2 to the same site (366). Point mutations within the Nox2 promoter...
have also been identified which act to specifically repress expression within neutrophils, but do not affect Nox2 transcription in the patients' eosinophils (352). However, the functional Nox2 promoter within cardiovascular cells has not as yet been characterized. The cis-acting elements that regulate expression of other Nox isoforms in any cell type are only just beginning to be studied. In the case of Nox1, we have recently identified the promoter sequences that drive expression in colon epithelial cells and shown that maximal expression is dependent upon binding of a GATA factor (35).

The regulation of the promoters of p47phox and p67phox has also thus far only been studied in myeloid cells. As was found to be the case for Nox2, both were dependent upon binding of PU.1 (171, 212). In the case of p67phox, cooperation between PU.1, IRF-1, ICSBP, and CBP was also required for full myeloid expression, as with Nox2 (171). In addition, the protein tyrosine phosphatase, SHP1, was shown to decrease the interactions of these proteins with the promoter elements, and so downregulate expression of both p67phox and Nox2 (171). Functional binding sites for the ubiquitous transcription factors Sp1 and AP-1 were also characterized within the p67phox promoter (213), but their significance in cardiovascular cells is unknown. In the case of p22phox, the identification of five polymorphisms present within the sequence of the p22phox promoter in spontaneously hypertensive rats (SHR) is of potential interest. In a transient transfection assay, these polymorphisms were shown to significantly increase promoter activity in rat VSMCs (372); however, the factors that potentially bind to these regions have not yet been characterized.

VIII. PHYSIOLOGICAL ROLES OF CARdioVASCULAR NADPH OXIDASEs

Whether NADPH oxidase-derived ROS have physiological (in addition to their well-recognized pathophysiological) roles in the cardiovascular system is an interesting question that is open to debate. A physiological role would provide at least a teleological explanation for the existence of these enzymes. As a minimum, the effects of NADPH oxidases on cell growth, migration, proliferation, activation, etc. which have been documented in pathological settings (see later) could clearly also serve important physiological functions during development or reparative processes. NADPH oxidase-derived ROS could also be relevant to the physiological regulation of vascular smooth muscle tone and in oxygen sensing.

A. Effect on vascular tone

In most vascular beds, the local production and activity of NO is pivotaly involved in the endothelial regulation of vasomotor tone in health (266). NO-dependent regulation is rapidly sensitive to alterations in local stimuli (such as increased shear stress) and appropriate local vasodilator actions are central to the achievement of integrated increases and/or redistribution of blood flow among specific vascular beds. The local levels of O2•− (together with molecules such as hemoglobin and antioxidants such as the SODs, which all influence NO bioactivity) are potentially important in the spatial restriction of NO action, even in health. In this regard, it is of interest that increased flow is a potent stimulus for the release of O2•− (as well as NO) in vessels (193). The involvement of NADPH oxidases remains to be demonstrated but oxidase activity is known to be increased by shear stress (155).

In addition to indirect effects through inactivation of NO, O2•− may also exert direct effects on vascular tone following dismutation to H2O2. Indeed, recent studies suggest that H2O2 released from the endothelium may account for endothelium-derived hyperpolarizing factor (EDHF) vasodilator activity in murine and human mesenteric arteries and in human coronary arterioles, where it is involved in flow-induced dilatation (232, 233, 243, 247). Studies by Matoba et al. (233) have suggested that NO synthases are responsible for the EDHF-like activity attributed to H2O2. To date, the specific involvement of NADPH oxidases in this response has not been demonstrated. However, these findings were not supported by those of Ellis et al. (88) who reported that catalase had minimal effects on endothelium-dependent relaxations in both aorta and small mesenteric arteries. It should be noted that H2O2 may also has vasodilator actions that are independent of hyperpolarization. In one report, the myogenic constrictor response of arteriolar vascular smooth muscle to increases in transmural pressure was found to be NADPH oxidase-dependent as it was inhibited either by pharmacological inhibition of the oxidase or in vessels from p47phox- deficient mice (257).

B. Role in oxygen sensing?

Maintenance of O2 homeostasis is paramount for survival and consequently a number of different mechanisms have evolved to safeguard and mitigate deleterious reductions in O2 tension. In mammals, hypoxia is acutely sensed by the glomus cells of the carotid bodies, which throughafferent regulatory pathways influence appropriate central nervous system responses, for example, increases in alveolar ventilation (37, 117). The equivalent counterparts in the airways are the neuroepithelial bodies (NEB). At a local level, reflex hypoxic pulmonary vasoconstriction allows regulation and optimization of ventilation-perfusion matching whilst in systemic vascular beds such as the coronary circulation, hypoxic vasodilation serves to maintain O2 delivery (342). Chronic hypoxia also evokes many adaptive changes in gene expression in cardiovascular cells, for example, genes involved in angiogenesis, energy metabolism, cell proliferation, and vascular remodelling (37, 109).

The precise configurations of the O2-sensing pathways that regulate the above processes in different cells and tissues remains a hotly debated subject despite considerable advances in understanding several components of these pathways, for example, the role of the ROS-sensitive transcription factor hypoxia-inducible factor-1 (HIF-1) in regulating O2-dependent gene expression (see Refs. 109, 164, and 300, for detailed reviews). It is likely that the detailed configurations will differ among different cells and tissues. An involvement of ROS-generating proteins in the proximal part of the O2-sensing pathways has been suggested in many cell types and a possible role of NADPH oxidases has been speculated upon (37). In keeping with a possible role of NADPH oxidase, the enzyme is suggested to generate ROS in a dose-
dependent manner in response to variations in local O₂ tension (2).

Evidence from studies in Nox2-deficient mice suggested that the oxidase is integral to O₂ sensing in NEBs, through interactions with K⁺ channels (95). However, Nox2 was not essential for O₂-sensing in the carotid bodies of these mice (131, 288), nor was it required for the hypoxic pulmonary vasoconstriction response (12). On the other hand, mice lacking p47^{phox} had potentiated respiratory responses to a hypoxic stimulus, leading to suggestions that other Nox homologues may be involved (293). Recently, Nox4 has been proposed to act as an oxygen sensor in conjunction with the potassium channel TASK-1 in transfected HEK293 cells (198). Furthermore, in a cell culture model using human lung adenocarcinoma A549 cells, an increase in Nox1 mRNA and protein and in ROS generation were observed in response to hypoxia (115). Cells stably transfected with Nox1 showed significant accumulation of HIF-1α, which increased further on exposure to hypoxia. HIF-1-dependent gene transcription was attenuated by either catalase or the NADPH oxidase inhibitor, DPI, suggesting a link between Nox1 and HIF-1 activation.

IX. NADPH OXIDASES IN ENDOTHELIAL CELL ACTIVATION AND INFLAMMATION

Inflammation describes the stereotyped response of vascularized tissues to injury and various stresses, and mainly involves vascular leak and leukocyte extravasation at the level of the microvasculature. In addition to being a major part of immune responses, components of this process are also fundamental in the initiation and perpetuation of diseases such as atherosclerosis. An early step in the process of inflammation is EC “activation,” which involves the regulated expression of cell surface adhesion molecules and cytokines that enable the recruitment and adhesion of circulating leukocytes, and is accompanied by an increase in endothelial permeability, allowing the transmigration of inflammatory cells into the affected tissue. Expression of adhesion molecules, such as intercellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1), endothelial leukocyte adhesion molecule-1 (E-selectin), and P-selectin, is induced by several stimuli including pro-inflammatory cytokines (e.g., IL-1β, TNFα, and IFN-γ) (228), altered vascular wall shear stress (45), hypercholesterolemia, oxidized low density lipoprotein (ox-LDL), and ischemia–reperfusion (221).

Intracellular ROS production and redox signalling are implicated in the induction of EC adhesion molecule expression and associated changes and several studies suggest an important role for NADPH oxidases as sources of the ROS, for example, in the context of increased oscillatory shear stress, ischemia–reperfusion, activation of the renin–angiotensin system, or exposure to AGEs (150, 281, 324, 351). The NADPH oxidase-derived ROS may emanate from leukocytes and inflammatory cells (169) as well as EC themselves (234, 249). A contribution from other ROS sources such as xanthine oxidase is also reported (324). TNFα-induced NF-κB-dependent VCAM-1, E-selectin, and ICAM-1 gene expression in human aortic EC is inhibited by adenosine overexpression of dominant negative Rac1 or SOD, consistent with an involvement of NADPH oxidase (47). In another study, the involvement of NADPH oxidase was clearly demonstrated in TNFα-induced increases in endothelial permeability, which involved a ROS-dependent, JNK-mediated phosphorylation of VE cadherin (258). Stokes et al. (317) clearly demonstrated that leukocyte–endothelial adhesion in response to a high cholesterol diet involved NADPH oxidase in that it was attenuated in p47^{phox}−/− mice compared to wild type. Furthermore, with the use of bone marrow chimeras to disect out the contributions of the vessel wall versus bone marrow-derived cells, these authors demonstrated an important role for NADPH oxidase in both cell types (Fig. 7). The same group also showed that P-selectin-dependent adhesion of platelets and leukocytes in the cerebral microcirculation in response to hypercholesterolemia was blunted in Nox2−/− mice (160).

X. VASCULAR CELL GROWTH AND APOPTOSIS

Inherent to the understanding of vascular disorders such as atherosclerosis, restenosis, and hypertensive vascular remodeling, is an appreciation of the processes involved in the proliferation and/or apoptosis of vascular cells (i.e., VSMC, EC, and fibroblasts). It is now clear that ROS may significantly modulate cellular growth, proliferation, and death. Low concentrations of H₂O₂ stimulate VSMC proliferation and hypertrophy (370), whereas high concentrations initiate growth arrest and cell death (69, 211). H₂O₂ may also have a role in cell survival, as exemplified by the finding that rat aortic VSMC exhibit reduced proliferation and an increased rate of apoptosis following adenosine-mediated overexpression of catalase (36). Transfection of NIH 3T3 fibroblast cells with Nox1 induced an increase in O₂⁻ and to an even greater extent H₂O₂ levels, and increased cell growth and tumorigenicity and upregulated a battery of genes critical to cell growth and neoplasia. Subsequent overexpression of catalase in these cells reduced ROS levels and partially normalized a range of cell growth parameters (13). Ang II increases VSMC growth and hypertrophy and this process is dependent on NADPH oxidase-derived ROS, probably mainly Nox1-derived (370). Thus, Ang II-induced VSMC hypertrophy is attenuated by pharmacological inhibitors of the oxidase, depletion of p22^{phox}, or catalase overexpression (Fig. 8) (117, 336, 370). Furthermore, hypertrophy of cultured VSMCs following stimulation by either thrombin or serum was found to be p47^{phox}-dependent but did not require Nox2 suggesting the involvement of the Nox1 isoform (22).

EC growth and survival are also influenced by NADPH oxidases. The proliferation of EC induced by VEGF is inhibited by three structurally unrelated NADPH oxidase inhibitors but not by xanthine oxidase or NOS inhibitors (1). Furthermore, Usorio-Fukai et al. (335) showed that VEGF-mediated proliferation is inhibited by dominant negative Rac1 or antisense Nox2 oligonucleotides. Other stimuli for EC proliferation, such as oxidized LDL (ox-LDL) (289), Ang II (298), ET-1 (78), altered shear stress (241), and hypoxia (298), also appear to signal these effects via NADPH oxidase-derived ROS (298).
In sufficiently high doses, ROS predictably lead to irreversible cell damage and programmed cell death (apoptosis). In EC, TNFα-induced apoptosis was prevented by dominant negative Rac1 (70). Furthermore, EC apoptosis induced by other stimuli that activate NADPH oxidase, namely Ang II, oxidized LDL, and hyperglycemia, is attenuated by antioxidants (79, 99, 201). Finally, endothelial cell anoikis, a process in which cell detachment from the extracellular matrix induces cell death, is associated with rapid increases in intracellular ROS which appear to at least partly emanate from NADPH oxidase although mitochondria are also involved (200).

XI. EC MIGRATION, REGULATION OF EXTRACELLULAR MATRIX, AND ANGIOGENESIS

EC migration is important in inflammation, vascular injury, angiogenesis, and other disorders. Early studies showed that migration of cultured EC upon exposure to Ang II, oxLDL, hypoxia, or VEGF necessitated NADPH oxidase-derived ROS (1, 289). Several different ROS-dependent processes probably contribute to this promigratory effect. The initial polarization of the cell towards the direction of intended migration involves significant reorganisation of the cytoskeleton and has been shown to require Rac1 (357) and ROS production (e.g., in EC monolayer wounding assays) (245). Actin filament reorganisation following exposure of EC to hypoxia–reoxygenation is also ROS-dependent (56).

In sufficiently high doses, ROS predictably lead to irreversible cell damage and programmed cell death (apoptosis). In EC, TNFα-induced apoptosis was prevented by dominant negative Rac1 (70). Furthermore, EC apoptosis induced by other stimuli that activate NADPH oxidase, namely Ang II, oxidized LDL, and hyperglycemia, is attenuated by antioxidants (79, 99, 201). Finally, endothelial cell anoikis, a process in which cell detachment from the extracellular matrix induces cell death, is associated with rapid increases in intracellular ROS which appear to at least partly emanate from NADPH oxidase although mitochondria are also involved (200).

FIG. 7. Hypercholesterolemia-induced endothelial activation and leukocyte adhesion require NADPH oxidase both in the vessel wall and in circulating bone marrow-derived cells. Mean baseline leukocyte adhesion in postcapillary venules of cremaster muscle was quantified in wild-type (WT) and p47phox-deficient mice subjected to hypercholesterolemic diet (HCD) or normal diet (ND) for 2 weeks. Bone marrow chimeras were generated to produce animals lacking p47phox either in marrow cells alone or in all cells except marrow cells. HCD increased adhesion in WT and heterozygous p47phox mice (p47phox+/−) but not in homozygous p47phox−/− mice (p47phox−/−). Leukocyte adhesion after HCD was reduced either in animals with p47phox−/−-deficient marrow (and therefore, presumably leukocytes) (p47phox−/−→WT) or in animals with intact marrow but p47phox−/− deficiency elsewhere (WT→p47phox−/−). *p < 0.01 vs. WT ND mice; †p < 0.05 vs. WT HCD and p47phox+/− HCD mice; ‡p < 0.005 vs. p47phox−/−→WT HCD mice; †p < 0.05 vs WT→WT HCD mice. Reproduced from Ref. 317 with permission.
angiogenesis. These authors reported that VEGF-induced angiogenesis involved a Nox2 oxidase since it was inhibited by transfection of antisense Nox2 oligonucleotides, DPI, or dominant negative Rac1 (Fig. 10). Furthermore, in an in vivo sponge implant assay, angiogenesis was significantly inhibited in Nox2−/− mice or in wild-type mice treated with antioxidants (335). The same group subsequently showed that ischemia-induced neovascularization in a hind-limb ligation model was significantly diminished in Nox2−/− mice (328).

XII. IMPAIRED ENDOTHELIUM-DEPENDENT VASODILATATION

Endothelial dysfunction is a broad term that describes an alteration in normal vascular homeostasis towards a state characterized by reduced endothelium-dependent vasodilation and proinflammatory and prothrombotic tendencies (108). The most widely studied aspect of endothelial dysfunction is impaired endothelial-dependent vasodilatation which is commonly the result of a reduction in NO bioavailability. Importantly, the severity of endothelial dysfunction in conditions such as atherosclerosis, hypertension, chronic heart failure, and diabetes mellitus is a strong predictor of future cardiovascular morbidity and mortality (39, 297). The reduction in NO bioavailability arises through its scavenging by excess O2•− radicals (73), a decline in NO production due to reduced eNOS expression, a deficiency of eNOS substrate (L-arginine), or cofactors (BH4), and/or NOS inhibition by endogenously generated antagonists such as asymmetrical dimethylarginine (ADMA) (240, 250, 347). Excess O2•− production (presumably extracellular) may emanate from multiple cell types including EC, VSMC, adventitial fibroblasts, and infiltrating inflammatory cells. An important role for NADPH oxidases in the genesis of endothelial dysfunction has now been reported by a large number of studies in experimental hypercholesterolemia, hypertension, diabetes, atherosclerosis, and heart failure (39, 142, 205), as well as in human arteries and veins from subjects with these conditions (Fig. 11) (127, 316). In addition to NADPH oxidases, other sources of O2•− relevant to endothelial dysfunction include xanthine oxidase and uncoupled eNOS. As discussed earlier, in many settings, NADPH oxidase-derived ROS may promote or augment O2•− production by these enzymes (Fig. 12) (188, 195, 237).
Increased vessel wall NADPH oxidase-derived $\text{O}_2^•$ is an important determinant of endothelial dysfunction in experimental Ang II-induced hypertension, renovascular hypertension, DOCA-salt hypertension, and genetic hypertension (188, 190, 194, 209, 210, 371, 379). Consistent with an important role for activation of the renin–angiotensin system in human hypertension, treatment of hypertensive subjects with an AT1 receptor antagonist improved endothelial function assessed by forearm flow-mediated dilator response to hyperaemia, and reduced markers of inflammation and oxidant stress (177). In patients with renovascular hypertension, impaired endothelium-dependent vasodilatation was correlated with excessive oxidative stress and both improved after surgery to correct renal artery stenosis (138). On the other hand, an important driver for vascular NADPH oxidase activation and endothelial dysfunction in low renin hypertension (often studied experimentally using unilateral nephrectomy and administration of deoxycorticosterone acetate [DOCA] plus salt) appears to be endothelin-1 (210, 379).

Endothelial NADPH oxidase activation at least partly driven by Ang II appears to also be largely responsible for the endothelial dysfunction found in models of early atherosclerosis, such as heritable Watanabe hypercholesterolemic rabbits or cholesterol-fed normal rabbits (349). Similarly, diet-induced atherosclerosis and endothelial dysfunction in primates is associated with increased NADPH oxidase-derived $\text{O}_2^•$ (130). In human coronary arteries from patients with coronary artery disease, Spiekermann et al. (316) found that endothelial dysfunction was attributable to increased $\text{O}_2^•$ from both NADPH oxidase and xanthine oxidase.

In diabetes, most evidence suggests an involvement of both NADPH oxidase and uncoupled eNOS in the genesis of endothelial dysfunction, for example, in aorta from streptozotocin-treated rats (142) and mice (10) as well as in arteries.
from human diabetic patients undergoing coronary artery bypass surgery (127). Increased NADPH oxidase-derived O$_2^•$ may also contribute to endothelial dysfunction in heart failure, where the ensuing vascular dysfunction may contribute to increased loading of the heart and reduced exercise tolerance (186). In experimental heart failure induced by coronary ligation in rats, aortic endothelial dysfunction was attributable to increased O$_2^•$ production from NADPH oxidase (23). Similarly, our own group showed that NADPH oxidase-derived ROS contributed to impaired endothelium-dependent (NO-dependent) enhancement of left ventricular relaxation in experimental pressure overload cardiac hypertrophy and failure (225).

XIII. HYPERTENSION

Although the pathogenesis of hypertension is complex and multifactorial, a role for increased ROS generation has been suggested by many studies, especially in relation to Ang II-dependent hypertension (194). For example, vascular NADPH oxidase activity is increased in rats made hypertensive by chronic Ang II infusion (283), together with increases in the expression of Nox1, 2, and 4 (246) and p22$^{phox}$ mRNA (96). Similarly, Nox1 and Nox4 transcript levels were found to be higher in aortae of transgenic hypertensive rats overexpressing the Ren2 gene, compared to wild-type controls (356). Nox2 but not Nox4 mRNA levels were increased in artery ring segments of rabbits after aortic banding (268). In cerebral arteries of SHR, however, an increase in NADPH oxidase activity correlated with an upregulation in Nox4 but not Nox1 or Nox2 expression (267). It should be noted, however, that a causative relationship between increased oxidative stress and hypertension is much more contentious than that between oxidative stress and the endothelial dysfunction that often accompanies hypertension. In short-term Ang II-induced hypertension in mice, the infusion of a peptide inhibitor of NADPH oxidase attenuated the rise in blood pressure (286). Likewise, studies of p47$^{phox}$ knockout mice showed reduced levels of hypertension in response to chronic Ang II infusion (187). However, Touyz et al. (329) reported that the crossing of transgenic mice expressing human renin, which normally have an an-

**FIG. 10. Involvement of Nox2 and Rac in VEGF-induced endothelial cell migration and proliferation.** (A) Transfection of HUVEC with Ad.N17Rac1 (dominant negative Rac1) significantly reduced cell migration when compared with control β-galactosidase transfected cells (Ad.LacZ). (B) Involvement of Nox2 in VEGF-induced cell migration and proliferation. HUVEC were transfected with reagent alone (control), Nox2 antisense or sense oligonucleotides. Cells were then stimulated with vehicle, VEGF or sphingosine 1-phosphate (S1P) and cell migration and proliferation assessed. Reduction of Nox2 abolished the increase in cell migration and proliferation in response to VEGF but not S1P. Adapted from Ref. 335 with permission.
giotensin II-dependent hypertensive phenotype, with Nox2−/− mice did not prevent the development of hypertension. Alternative Nox isoforms may therefore be involved in short-term Ang II-driven hypertension. Dikalova et al. (75) recently reported that in a transgenic mouse with VSMC-specific overexpression of Nox1, Ang II-induced hypertension, and VSMC remodeling were significantly greater than in wild-type mice. In DOCA-salt hypertension in rats, where endothelin-1-dependent increases in NADPH oxidase activity seem to be important, a selective ETα antagonist significantly reduced both ROS generation and hypertension (210).

The precise mechanism(s) involved in NADPH oxidase (ROS)-dependent hypertension remain to be established and could involve vascular or nonvascular pathways (such as altered regulation in the kidneys and brain). A large number of studies have implicated NADPH oxidase in vascular remodeling induced by hypertensive stimuli, for example, in response to Ang II infusion (340, 346, 373) (discussed earlier sections). Furthermore, stretch-induced MMP-2 activation (and therefore potentially remodeling) in VSMCs was reported to be NADPH oxidase-dependent, being absent in p47phox−/− cells (124). However, several studies also show a dissociation between altered vascular \( \mathrm{O}_2^- \) and blood pressure (210, 311). Interestingly, recent studies suggest that cerebrovascular NADPH oxidase-derived ROS may contribute to the development of Ang II-induced hypertension (380).

XIV. ATHEROSCLEROSIS

A detailed discussion of the complex pathogenesis and pathophysiology of atherosclerosis is beyond the scope of this review but many aspects of this process are known to be redox-sensitive, for example, endothelial activation (discussed earlier), oxidative modification of lipids, the recruitment of immune and VSMC into atherosclerotic plaques, and VSMC proliferation (discussed earlier). Here, we focus specifically on evidence that implicates NADPH oxidase-derived ROS in one or more of these processes.

The accumulation of LDL at sites of atheromatous lesion predilection is crucial in the evolution of atherosclerosis. OxLDL is a potent stimulus for NADPH oxidase activation in EC (134) which contributes to the expression of adhesion molecules and recruitment of monocytes and other cells. Furthermore, it has been demonstrated that NADPH oxidase-derived ROS contribute to macrophage-mediated oxidation of LDL potentially leading to a vicious cycle (14). Once monocytes traverse the EC monolayer into the vessel wall, they transform into macrophages that avidly take up oxLDL to become foam cells. OxLDL also activates NADPH oxidase within macrophages which contributes to further ROS generation and amplification of the steps described thus far (134, 289). The subsequent process of VSMC migration (e.g., in response to growth factors such as PDGF) may also involve NADPH oxidase-derived ROS (321). Likewise, NADPH oxidase may contribute to VSMC proliferation within the atherosclerotic plaque (22).

Taken together, the above observations provide circumstantial support for the potential of NADPH oxidases to be involved in atherogenesis. Nevertheless, more direct evidence for a role of NADPH oxidases remains relatively limited. Barry-Lane et al. (22) crossed the apolipoprotein E knockout (apoE−/−) mouse, which is predisposed to atherosclerotic lesions throughout the arterial tree but with a predilection for the aortic root, with p47phox−/− mice and found that lesion formation was significantly reduced in the descending aorta in p47phox−/− deficient animals. However, a separate study using this double knockout mouse found comparable levels of ath-
erosclerosis at the aortic root to wild-type animals, but importantly the descending aorta was not examined (151). Interestingly, apoE<sup>−/−</sup>/H<sup>11002</sup>/H<sup>11002</sup> mice are reported to have higher vascular expression of Nox1 relative to wild-type littermate controls (30). Nox1 mRNA was also found to be upregulated in both minimally and terminally diseased human coronary arteries (192), as was p22phox expression in atherosclerotic coronary arteries (15, 313). In more advanced lesions, it appears that an infiltration of macrophages contributes significantly to increased NADPH oxidase expression and activity (15, 167, 313, 323). Nox1, Nox2, and p22phox mRNAs were significantly increased within rat arteries during the early stages of restenosis after balloon injury (323). The latter could correspond to an upregulation in oxidase activity in VSMC and the adventitia respectively. By contrast, Nox4 expression in these experiments was not altered during the early stages of restenosis but increased in the neointima during the redifferentiation phase after cellular proliferation had ceased (323). The expression of p67phox and p47phox protein, assessed by immunocytochemistry, has also been suggested to increase in the adventitial fibroblasts of porcine coronary arteries after balloon-induced injury (302). In primates, Hathaway et al. (130) were able to correlate superoxide levels and expression of p22<sup>phox</sup> and p47<sup>phox</sup> subunits, with diet-induced atherosclerosis over a 4 year period and subsequent regression whilst on a normal diet over an 8 month period. These data have been further corroborated by human studies. Increased expression of the p22<sup>phox</sup> subunit was found throughout the wall of human coronary atherosclerotic vessels (15). Finally, not only was Nox subunit expression found to be associated with the severity of atherosclerosis in human coronary arteries, but also greater superoxide was detected in the plaque shoulder, suggesting a possible role of NADPH oxidase in plaque instability (313).

**XV. DIABETES MELLITUS**

A substantial body of evidence implicates oxidative stress as an important pathogenic factor in diabetic cardiovascular complications, in both Type I and Type II diabetes mellitus. The drivers of this oxidative stress include hyperglycemia,
hyperinsulinemia, and the elevated free fatty acids and lipids that are usually associated with diabetes. Hyperglycemia promotes the production of ROS and nitrogen species (RNS) in many cell types (142, 197, 280, 376) and when present chronically also promotes the formation of AGEs which themselves are capable of inducing ROS production (351, 358, 364, 367).

Hyperglycemia-induced ROS production undoubtedly emanates from several different sources, including mitochondria and uncoupled NOSs, but NADPH oxidases are important sources in many settings. An NADPH oxidase inhibitor, apocynin, as well as a PKC inhibitor inhibited vascular ROS generation in three different animal models of diabetes, namely streptozotocin-induced diabetes (a model of Type 1 diabetes), obese ob/ob mice, and Zucker fatty rats (both models of Type II diabetes) (312). In cultured aortic VSMC and EC, exposure to high glucose for 72 h also significantly increased ROS production, which was inhibited by either DPI or a PKC inhibitor (156). In isolated rat cardiomyocytes, incubation with high glucose for 24 h resulted in an enhanced free radical production and significant contractile dysfunction which was prevented by an AT\textsubscript{1} receptor antagonist, DPI or apocynin (280). In glomerular mesangial cells, however, high glucose-induced ROS production was effectively blocked by rotenone, an inhibitor of the mitochondrial electron transport chain complex I, as well as a PKC inhibitor, DPI or apocynin (197).

In bovine aortic EC, Nishikawa et al. (255) demonstrated that hyperglycemia-induced ROS production was prevented by an inhibitor of electron transport chain complex II, an uncoupler of oxidative phosphorylation, uncoupling protein-1 or manganese superoxide dismutase but not by rotenone (255). In contrast, Hink et al. (142) identified both uncoupled NOS and NADPH oxidase as \( \text{O}_2^- \) sources in aorta from rats subjected to streptozocin-induced diabetes, in association with a seven-fold increase in Nox2 mRNA (142). Consistent with an important role for Nox2 in mediating the effects of glycated proteins, macrophages retrieved from Nox2\textsuperscript{−/−} mice displayed a complete inhibition of AGE-induced tissue factor activity (351). Similarly, the induction of ROS and VCAM-1 expression by AGE in HUVEC was significantly inhibited by both apocynin and DPI (22). Apocynin also significantly inhibited AGE-induced NF-\kappaB translocation (22). Recently, we have shown that glycated proteins also induce Nox2 oxidase activation in isolated cardiomyocytes (377). The potential for such increased ROS production to promote abnormalities such as endothelial dysfunction or atherosclerosis was discussed in earlier sections.
NADPH OXIDASES IN CARDIOVASCULAR DISEASE

XVI. CARDIAC HYPERTROPHY

Chronic heart failure (CHF) occurs secondary to longstanding increases in heart workload, most commonly due to hypertension (pressure overload) or ischemic heart disease (322). The heart adapts to increased systemic pressure load through left ventricular hypertrophy (LVH), which involves alterations in cardiomyocyte, extracellular matrix, and coronary vessel structure and function. Persistent LVH usually progresses to contractile depression, cardiac dilatation, and CHF. Growing evidence supports an important role for oxidative stress and redox signaling in the pathophysiology of LVH. For example, hypertrophy of isolated cardiomyocytes induced by α-adrenergic agonists, Ang II, endothelin-1, TNF-α, or cyclic stretch has been shown to involve increased ROS production (143, 251, 275). In vivo, the development of pressure overload LVH in mice or the transition from compensated to decompensated pressure overload LVH in guinea pigs is inhibited by antioxidants (63, 71). Patients with CHF also have evidence of increased oxidative stress which has been correlated with myocardial and endothelial dysfunction and overall severity of heart failure (89, 148, 227, 236).

While the sources of ROS production and the mechanisms by which ROS exert pathophysiological effects remain under investigation, a significant role for NADPH oxidases has been suggested. Of interest, many stimuli that activate NADPH oxidases (e.g., cyclic stretch, Ang II, α-adrenergic agonists, endothelin-1 and TNF-α) (65, 66, 210, 336) are relevant to LVH and heart failure pathophysiology. In isolated rat cardiomyocytes (355, 362), hypertrophy induced by Ang II, endothelin-1, and norepinephrine may at least in part involve NADPH oxidases as evidenced by the use of oxidase inhibitors and the involvement of Rac1 (139, 277, 325). In experimental pressure overload LVH in guinea pigs, myocardial NADPH oxidase subunit expression and activity were increased in parallel with MAPK activation; oxidase expression was documented in both cardiomyocytes and EC in this study (203). Similar NADPH oxidase activation is observed in murine pressure overload LVH (38, 121, 235). Recently, it was confirmed that myocardium from end-stage human CHF patients demonstrated increased NADPH oxidase activity (137, 224).

More direct evidence for an involvement of NADPH oxidases in LVH comes from studies in Nox2−/− mice. In a model of short-term (7–14 days) subpressor Ang II infusion, both myocardial NADPH oxidase activation and the development of in vivo hypertrophy were significantly inhibited in Nox2−/− mice (Fig. 13) (29). In the setting of pressure overload induced by aortic banding, however, both morphological LVH and the associated rises in molecular markers such as ANF mRNA were similar in Nox2−/− and wild-type mice (38, 235). Interestingly, however, aortic banding significantly increased LV NADPH oxidase activity and in situ O2·− production not only in wild-type but also Nox2−/− mice, which was attributable to increased Nox4 expression in the banded Nox2−/− animals (38). Furthermore, chronic treatment of banded Nox2−/− mice with the antioxidant N-acetyl-cysteine significantly reduced the extent of LVH (38). These results suggest that, while Nox2 is pivotally involved in the development of Ang II-induced hypertrophy, LVH induced by pressure overload could be more dependent on Nox4. Nonetheless, further studies suggest that Nox2 plays an important role in the contractile dysfunction that accompanies pressure overload LVH even though it does not alter the extent of hypertrophy per se. Using pressure-volume analyses as well as echocardiography, we found that banded Nox2−/− mice were significantly protected against the LV systolic and diastolic dysfunction that occurred with banding in wild-type animals (Fig. 14) (120). Taken together, these data suggest distinct roles for Nox2 and Nox4 in different components of the overall hypertrophic response to pressure overload with the two isoforms exhibiting different activation as well as distinct downstream effects.
Potential redox-sensitive downstream signaling pathways that may be influenced by NADPH oxidase activation in the heart include RAS, the MAPKs (p38MAPK, ERK1/2, JNK), c-src, p90RSK, the PI3 kinase (PI3K)/Akt pathway, AP-1, NF-κB, HIF-1, and others (4, 77, 110, 136, 182, 191, 361). However, the involvement of NADPH oxidase in activating these pathways remains poorly characterized in cardiomyocytes. The small GTPase RAS has been suggested to be a redox-sensitive signaling switch in many cell types, and in NIH3T3 fibroblasts stably-transformed with a constitutively active isofrom of p21Ras, H-RasV12, it was suggested to mediate NADPH oxidase activation (159). In keeping with such a mechanism, it was recently reported that α-adrenergic receptor-induced hypertrophic signaling in rat cardiomyocytes involved a posttranslational oxidative modification of RAS, with downstream phosphorylation of MEK1/2, ERK1/2, and p90RSK (182, 361). Many studies have shown that activation of several members of the MAPK family is redox-sensitive (118, 136, 174, 182, 215, 361), but the possible role of NADPH oxidase-dependent MAPK activation in the heart remains speculative.

**XVII. CARDIAC REMODELING AND FIBROSIS**

Interstitial fibrosis contributes significantly to the pathophysiology of cardiac dysfunction associated with LVH, myocardial ischemia, senescence, inflammatory processes, and diabetes. Under these conditions, interstitial fibroblasts transform into myofibroblasts that express α-smooth muscle actin, angiotensin converting enzyme, high densities of Ang II receptors, and various MMPs and tissue inhibitors of MMP (TIMPs) (322). Following significant myocardial infarction, the heart typically dilates and becomes more spherical over a period of weeks and months in a process known as adverse remodeling, which is associated with alterations in contractile function and the development of CHF. Like fibrosis, adverse cardiac remodeling involves profound alterations in the composition of the extracellular matrix. Both fibrosis and remodeling are therefore markedly influenced by the balance between collagen deposition and matrix degradation, the latter being modulated largely by the activity of MMPs and TIMPs (16).

Persuasive evidence implicates intracellular redox balance as a key regulator of fibrosis and remodeling. Oxidative stress is profibrotic in the liver, lungs, kidney, and vasculature (276), and ROS modulate fibroblast proliferation and their transformation into matrix-producing myofibroblasts (13, 159). Profibrotic stimuli such as Ang II (216, 320), aldosterone (320), and cyclic load (181) all stimulate intracellular ROS production as discussed earlier. In addition, many signaling pathways and transcription factors implicated in fibrogenesis are redox-sensitive (32, 181, 253). Notably, MMP expression and activation are exquisitely redox-sensitive (13, 93, 248, 308). In the context of remodeling post-MI, local activation of the renin–angiotensin system may be important in increasing ROS production (41, 319).

Emerging evidence supports a role for NADPH oxidase in interstitial cardiac fibrosis and remodeling. Although a profibrotic role of Ang II is well recognized (29, 132, 165, 287), the involvement of NADPH oxidase in this process has been unclear. We addressed this question in Nox2−/− mice infused with Ang II and found that Ang II-induced increases in interstitial cardiac fibrosis were completely abolished independent of the hypertrophic and pressor effects of Ang II (29, 165). Furthermore, mRNA expression of procollagen I and III and connective tissue growth factor (CTGF) as well as MMP-2 activation were suppressed in Ang II-treated Nox2−/− mice compared to wild type (165). Aldosterone is also a potent profibrotic agent and has recently been reported to activate vascular p38MAPK and NADPH oxidase (40). In line with this, we found in an experimental model of aldosterone-driven interstitial cardiac fibrosis that this was inhibited in Nox2−/− mice (165). Sun et al. (320) also reported evidence of increased myocardial oxidative stress together with increased Nox2 expression in a similar model although a cause–effect relationship between these observations was not established. Nox2 also appears to be profibrotic in pressure-overload LVH since we found that Nox2-deficient mice subjected to aortic banding had reduced interstitial fibrosis compared to banded wild-type controls (120). Thus, these studies strongly support a specific role of Nox2 in the development of cardiac fibrosis (Fig. 15). However, the role of different cell types in this response remains unclear. Both Nox2 (263) and Nox4 (44) are expressed in aortic adventitial fibroblasts of rabbit and mouse, but in human cardiac fibroblasts it was recently reported that the main isoforms expressed at mRNA level were Nox4 and Nox5, whereas Nox1 and Nox2 were barely detectable (58). In the latter study, TGFβ-1 potently upregulated Nox4 mRNA expression and oxidase activity which led to increased expression of the myofibroblast marker smooth muscle α-actin (58). However, the role of Nox4 in mediating in vivo fibrosis was not addressed. Taken together, these results could be consistent with a role for both Nox2 (in nonfibroblasts) and Nox4 (in fibroblasts) in vivo fibrosis or they could indicate significant species-specific differences.

NADPH oxidase could have a similarly important role in adverse cardiac remodeling but this remains an area under continuing investigation. An increase in ROS production and oxidative stress is well recognized to occur post-MI (140, 176, 307), and antioxidant treatment (e.g., with dimethylthiourea or probucol) reportedly attenuates LV remodeling following MI by attenuating increases in collagen volume fraction and MMP activity (176, 306). An increased myocardial expression of the NADPH oxidase subunits, Nox2 and p22phox, has been reported after MI both in animal models (97, 220) and human myocardium (180). In recent preliminary studies in Nox2−/− mice subjected to coronary ligation, we have found that cardiac remodeling is significantly reduced compared to wild type, supporting an important role for Nox2 in this process (unpublished data).

**XVIII. MYOCARDIAL ISCHEMIA–REPERFUSION AND CARDIOPROTECTION**

Oxidative stress is increased in cellular and experimental models of ischemia–reperfusion injury, with reperfusion thought to be the more potent stimulus for ROS production
(26, 173). However, as yet, no convincing evidence for an involvement of NADPH oxidases in this process has been reported. Indeed, myocardial infarction following 30 min ischemia and 24 h reperfusion in p47phox−/− mice was not found to be significantly different from wild-type mice (146).

In contrast, a recent study suggests that NADPH oxidase-derived ROS may play a significant role in the signaling of early ischemic preconditioning (i.e., may have beneficial effects). Bell et al. (27) showed that ischemic preconditioning-induced reductions in infarct size were abolished in Nox2−/− mice although these animals could be preconditioned by an adenosine analogue, suggesting a significant role for Nox2 in the adaptive response to brief ischemia.

**XIX. SEPSIS**

The systemic sepsis syndrome (e.g., in response to gram negative bacterial infection) is characterized by hypotension, vascular hyporeactivity, intrinsic cardiac depression, and multiorgan dysfunction, and has a high mortality despite treatment (123, 270). Significant oxidative stress is a well-recognized feature of the syndrome (33, 51), at least in part the result of inflammatory cytokine-induced production of ROS (51, 92, 291). The increased ROS production may contribute to cardiac contractile depression and reversible injury (101, 226) and endotoxemia-induced dysfunction is significantly decreased in transgenic mice overexpressing either human extracellular glutathione peroxidase or the intracellular isoform (242). A few studies suggest that NADPH oxidase-derived ROS may contribute to the oxidative stress. DeLeo et al. (67) demonstrated that LPS rendered neutrophils more responsive to other stimuli as a result of increased translocation of Rac2, p47phox, and p67phox (i.e., “priming”). Sanlioglu et al. (294) also reported that LPS induced Rac1-dependent ROS production and TNFα secretion in macrophages. Ben Shaul et al. (28) found that NADPH oxidase activity increased in rat hearts subjected to LPS injection in vivo, while pretreatment with the oxidase inhibitor apocynin significantly reduced mortality. Similarly, LPS treatment of
rats enhanced vascular expression of p22phox, p67phox, Nox2, and xanthine oxidase, and increased O$_2^-$ and ONOO$^-$ formation (33). ROS formation was partially sensitive to both DPI and the xanthine oxidase inhibitor oxypurinol but scavenging of O$_2^-$ did not restore endothelial dysfunction (33). More recently, a study in isolated cultured mouse neonatal cardiomyocytes showed that LPS-induced TNF$\alpha$ expression and myocardial depression involved a Nox2 oxidase (273).

XX. CONCLUSIONS

The knowledge that increased oxidative stress plays important roles in the pathophysiology of many cardiovascular disorders has naturally led to consideration of the potential therapeutic benefit of antioxidant agents. Although treatment with antioxidants or with SOD has been found to be effective in reducing markers of oxidative stress and improving functional parameters such as endothelium-dependent relaxation in many settings, the results of large antioxidant trials in patients at risk of cardiovascular morbidity and mortality have been singularly disappointing (163). However, recent advances in our understanding of the complexity of oxidative stress and redox signaling, as well as the concept that ROS production may be specifically regulated by distinct stimuli and pathways, has led to renewed enthusiasm regarding therapeutic possibilities. The NADPH oxidases provide perhaps the best example of an enzyme system that appears to be specifically designed for redox signaling. Furthermore, the complexity of their regulation may in itself provide the possibility of targeted therapeutic manipulation in cell-, tissue- and pathway-specific manners at appropriate points in the disease process. Instead of biologically inefficient and nonspecific antioxidants (e.g., vitamins) that have been disappointing, targeted drugs may be more promising. Indeed, several successful existing drugs are now appreciated to exert at least part of their effects in this manner (e.g., statins, ACE inhibitors, and AT$\_1$ antagonists). Further understanding of the detailed mechanisms and roles of ROS sources such as the NADPH oxidase family in cardiovascular disorders should provide the basis for devising novel therapies for some of these conditions.

ACKNOWLEDGMENTS

The authors’ work is supported by the British Heart Foundation (BHF). AMS holds the BHF Chair of Cardiology at King’s College London. RR is supported by a BHF Scholar award.

ABBREVIATIONS

AA, arachidonic acid; AGE, advanced glycation end products; Ang II, angiotensin II; CGD, chronic granulomatous disease; CHF, chronic heart failure; CTGF, connective tissue growth factor (CTGF); DOCA, deoxycorticosterone acetate; DPI, diphenylene iodonium; EC, endothelial cells; EDHF, endothelium-derived hyperpolarizing factor; GEF, guanine nucleotide exchange factor; HIF-1, hypoxia-inducible factor-1; HUVEC, human umbilical vein endothelial cells; ICAM-1, intercellular adhesion molecule-1; IFNy, interferon-$\gamma$; IL-1, interleukin 1; LPS, lipopolysaccharide; LVH, left ventricular hypertrophy; MMP, matrix metalloproteinases; NO, nitric oxide; NOS, nitric oxide synthase; Nox, NADPH oxidase; Nox01, Nox organizer 1; NoxA1, Nox activator 1; oxLDL, oxidized low density lipoprotein; PAK, p21 activated kinase; PDGF, platelet derived growth factor; PKC, protein kinase C; PMA, phorbol myristate; ROS, reactive oxygen species; RNS, reactive nitrogen species; SHR, spontaneously hypertensive rat; SOD, superoxide dismutase; TGF$\beta$, transforming growth factor $\beta$; TIMPs, tissue inhibitors of MMP; TNF$\alpha$, tumor necrosis factor $\alpha$; TRAF4, TNF receptor-associated factor 4; VEGF, vascular endothelial growth factor; VCAM-1, vascular cell adhesion molecule-1; VSMC, vascular smooth muscle cells.

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