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Forum Review

Reactive Oxygen Species and Endothelial Activation

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ABSTRACT

Endothelial activation refers to a specific change in endothelial phenotype, characterized most notably by an increase in endothelial–leukocyte interactions and permeability, which is pivotal to inflammatory responses in both physiologic and pathologic settings. An increasing body of evidence indicates an important role for reactive oxygen species (ROS)-mediated modulation of signal-transduction pathways in many of the processes involved in endothelial activation. ROS generated by the nicotinamide adenine dinucleotide phosphate (NADPH) oxidase family of enzymes may be especially important in this regard. We discuss the evidence implicating redox signaling pathways in the molecular and cellular processes underlying endothelial activation and the role in cardiovascular diseases, and also provide a detailed description of NADPH oxidase regulation in endothelial cells, in view of its likely importance in this context. *Antioxid. Redox Signal.* 10, 1089–1100.

INTRODUCTION

The single layer of endothelial cells that lines the lumen of all blood vessels and the heart is recognized to be not just a barrier between circulating blood and the vessel wall but critical for the maintenance of vascular function and homeostasis (9). The endothelium has many important functions, including (a) the maintenance of a selective permeability barrier between circulating blood and the underlying tissues; (b) the maintenance of vessel integrity on the one hand and blood fluidity on the other, through tight spatial and temporal interplay between prothrombotic, anticoagulant, antiplatelet and fibrinolytic activities; (c) the regulation of blood cell–vessel wall interactions; (d) involvement in innate and adaptive immune responses; (e) the modulation of vascular smooth muscle tone and thus blood-flow distribution; and (f) a contribution to the maintenance of a quiescent, differentiated vascular smooth muscle phenotype. These actions are achieved through the coordinated release of numerous paracrine factors [such as nitric oxide (NO), prostacyclin, endothelin, endothelium-derived hyperpolarizing factors, and growth factors], the expression of specific enzyme activities [*e.g.*, angiotensin-converting enzyme (ACE), tissue plasminogen factor (tPA)], the surface expression of proteins such as cell adhesion molecules, and above all, by the ability to alter cellular phenotype in response to appropriate stimuli.

In keeping with the importance of the endothelium in maintaining vascular homeostasis, dysfunction of the endothelium is implicated in the pathophysiology of several cardiovascular diseases, including atherosclerosis, diabetic vasculopathy, heart failure, and hypertension (9). Although the term endothelial dysfunction potentially encompasses all aspects of the functions of the endothelium, it is widely used specifically to refer to a disturbed balance between vasodilator and vasoconstrictor activity that is usually due to reduced NO bioavailability. This is often, but not necessarily, associated with other abnormalities of endothelial function. The importance of endothelial dysfunction defined in this manner has been confirmed by clinical studies that have demonstrated that the presence of impaired endothelium-dependent relaxation is an independent predictor of future cardiovascular morbidity and mortality (24, 85).

Endothelial activation is a term used to denote a specific and complex change in endothelial phenotype, characterized most notably by an increase in endothelium–leukocyte interactions, which is pivotal to inflammatory responses both in physiologic and pathologic settings (Table 1) (11, 38). Activation of the en-

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Table 1. Pathophysiologic Settings in Which Endothelial Activation May Be Involved

Inflammation	
Immune responses	Physiologic
Sepsis	
Atherosclerosis	
Hypertension	
ARDS	Pathologic
Sickle cell disease	
Autoimmune disease	
Interstitial fibrosis	

dothelium may occur in response to diverse stimuli, including inflammatory cytokines, lipopolysaccharide (LPS), activation of the renin–angiotensin system, hypercholesterolemia, CD40/CD40 ligand interactions, ischemia–reperfusion, physical trauma, and diabetes. The process of activation involves upregulation of the expression of chemotactic factors [such as monocyte chemoattractant protein-1 (MCP-1)] and cell surface adhesion molecules [such as intercellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1), platelet–endothelial cell adhesion molecule-1 (PECAM-1), and selectins], which enhance leukocyte adhesion and transmigration across the endothelial barrier into the underlying tissue (Fig. 1). The process is further enhanced by the release of proinflammatory cytokines and other factors by activated leukocytes.

The proinflammatory, procoagulant state of activated endothelium is implicated in many pathologic conditions, including the early stages of atherosclerosis, sepsis, hypertension, development of interstitial fibrosis, autoimmune diseases, acute respiratory distress syndrome (ARDS), sickle cell disease, and others (9). An increasing body of evidence indicates an important role for reactive oxygen species (ROS)-mediated modulation of signal-transduction pathways in many of the processes involved in endothelial activation. Of interest, ROS generated by the nicotinamide adenine dinucleotide phosphate (NADPH) oxidase family of enzymes may be especially important in this regard. In this article, we discuss the evidence implicating redox signaling pathways in the molecular and cellular processes underlying endothelial activation and its role in cardiovascular diseases, and also provide a detailed description of NADPH oxidase regulation in endothelial cells in view of its likely importance in this context. The involvement of ROS in endothelium-dependent vasodilator dysfunction has been covered by excellent recent reviews and is not dealt with in this article.

SOURCES OF ROS IN ENDOTHELIAL CELLS

The major sources of ROS generation in endothelial cells include the mitochondrial electron-transport chain, NADPH oxidases, xanthine oxidase, cytochrome P450, and uncoupled endothelial NO synthase (eNOS) (9, 57). Depending on the balance between ROS production and antioxidant status, these sources are implicated in ROS-mediated damage to macromolecules, membranes, and DNA, and in superoxide-mediated inactivation of NO and generation of peroxynitrite ($ONOO^-$) (32). Because NO is able to inhibit leukocyte adhesion and prevent platelet adhesion, at least a component of ROS-dependent endothelial activation could be secondary to a reduction in NO bioavailability. However, the main mechanisms through which ROS are involved in endothelial activation are *via* the direct modulation of redox-sensitive signaling pathways (*e.g.*, the activation of specific kinases and transcription factors) (83). NADPH oxidases are of particular importance in this regard as they appear to be specifically designed for involvement in redox signaling (10, 30, 52).

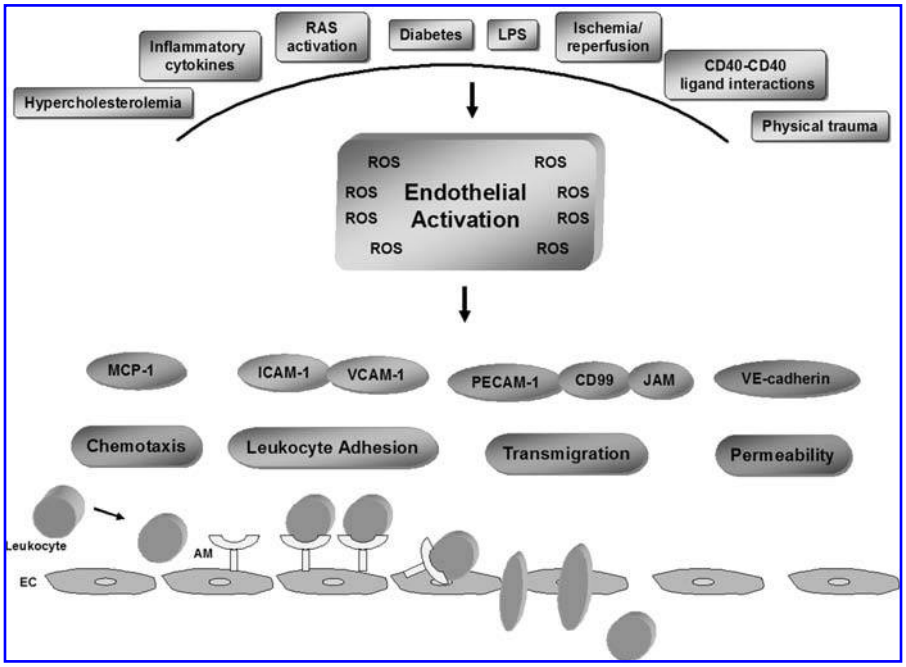

FIG. 1. Schematic diagram showing effect of endothelial activation on interactions with leukocytes. Reactive oxygen species (ROS) may play an important role in many of the processes underlying endothelial activation. Stimuli that induce endothelial activation generally increase ROS production in the endothelial cell. ROS-dependent signal transduction may be involved in the upregulation of chemotactic factors (*e.g.*, MCP-1) and cell-surface adhesion molecules (ICAM-1, VCAM-1, PECAM-1) and alterations in VE-cadherin that lead to enhanced leukocyte–endothelial cell interactions, transendothelial migration of leukocytes, and increased endothelial permeability. AM, adhesion molecule; EC, endothelial cell; JAM, junctional adhesion molecule; RAS, renin–angiotensin system; LPS, lipopolysaccharide.

THE NADPH OXIDASE FAMILY OF ENZYMES

The NADPH oxidase was first characterized in phagocytes, where it plays an essential role in nonspecific host defense against microbes (4). It catalyzes the process of electron transfer from NADPH through the enzyme and onto molecular oxygen, thereby resulting in the generation of superoxide and a proton. The classic phagocytic NADPH oxidase is a multi-subunit enzyme comprising a membrane-bound heterodimeric flavocytochrome containing 1 gp91^{phox} catalytic subunit (now also known as Nox2) and 1 p22^{phox} subunit, which associates with four regulatory cytosolic subunits during the process of enzyme activation (52). These subunits are p47^{phox}, p67^{phox}, p40^{phox}, and Rac1 or Rac2, each of which appears to have distinct roles during the activation process. Thus, the p47^{phox} subunit is thought to be important for organization of complex assembly after its phosphorylation, the p67^{phox} subunit for activation of electron transfer on binding to gp91^{phox}, and Rac1 for optimal oxidase activation. The role of the p40^{phox} subunit remains incompletely understood, but a recent study in p40^{phox}-knockout mice provides quite convincing evidence that it also has an essential role in oxidase activation (22).

Biochemical and functional evidence for the presence of NADPH oxidase–like activities in numerous nonphagocytic cells eventually led to the discovery of a whole family of NADPH oxidase enzymes over the last 7- to 8-year period, each based on a distinct gp91^{phox} or Nox isoform. Five Nox isoforms (*i.e.*, Nox1-5), each encoded for by separate genes have now been identified, of which the Nox5 isoform appears not to be expressed in rodents (52). The Nox2-based oxidase is the classic phagocyte enzyme in this terminology. In addition, two other proteins, Duox1 and 2, were identified in the thyroid and stomach; these share significant homology with Nox2 but contain additional peroxidase homology domains in their N-termini (18). Nox1 through 4 each appear to associate with p22^{phox} but exhibit tissue-specific distribution and significant differences in their regulation (15). For instance, the Nox1 oxidase is preferentially activated by homologues of p47^{phox} and p67^{phox} termed NoxO1 and NoxA1, respectively (for Nox organizer and Nox activator, respectively), whereas activation of the Nox4 oxidase appears not to require the conventional regulatory subunits at all (2, 3, 68). In the cardiovascular system, Nox1 is reported to be expressed in vascular smooth muscle cells; Nox2, in the endothelium, cardiomyocytes, fibroblasts, and some vascular smooth muscle; Nox4, in all cell types; and Nox5, probably in some human endothelial and vascular smooth muscle cells.

NADPH OXIDASES IN ENDOTHELIAL CELLS

Both the Nox2 and Nox4 oxidase isoforms are expressed in endothelial cells, with a few reports also suggesting the expression of Nox1, at least in some cultured endothelial cells (30, 57). The molecular and protein expression of the key components of a Nox2-based oxidase in endothelial cells was confirmed several years ago (6, 7, 29, 44, 59, 98). Subsequently, the Nox4-based oxidase was also identified in endothelial cells (2, 88); indeed, the mRNA expression level of Nox4 is reported to be at least 20-fold greater than that of Nox2 (88). It appears likely that the Nox2 and Nox4 isoforms could subserve distinct functions when coexpressed in endothelial cells, because it is increasingly clear that they may be expressed in different subcellular locations and are subject to different regulation (Fig. 2) (10).

The regulation of the Nox2-based oxidase in endothelial cells has now been investigated in many studies. The oxidase subunits p22^{phox} and gp91^{phox} were described to have a perinuclear localization, based on confocal microscopic studies (6), which was subsequently confirmed by cell subfractionation studies that also suggested an association with the cytoskeleton (59). The latter study also found evidence for the presence of fully assembled, functional oxidase complexes in unstimulated endothelial cells, which could account for the findings that low-

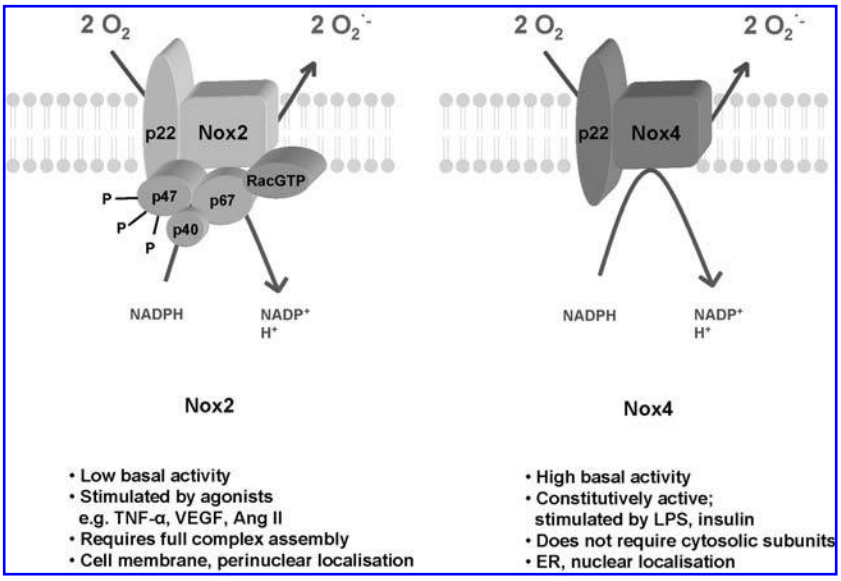

FIG. 2. Schematic diagram of the NADPH oxidases Nox2 and Nox4.

level NADPH-dependent superoxide generation could readily be detected in unstimulated cells (in marked contrast to phagocytic cells, where the oxidase is quiescent unless cells are activated). However, despite the presence of low-level ROS generation, the endothelial Nox2-based oxidase can also be acutely activated by several agonists, including angiotensin II (8, 58, 73), TNF-α (19, 26, 31, 56), vascular endothelial growth factor (VEGF) (97, 101), chronic oscillatory shear (40), hypoxia–reoxygenation (12, 49, 63), nutrient deprivation (64, 67), atrial natriuretic peptide (27), glucose (37, 42), and endothelin (21). In some cases, the evidence that these agonists are activating the Nox2-based oxidase is indirect, in that either $p47^{phox}$ or Rac1 or both have been found to be involved, neither of which is thought to be required for Nox4 oxidase activation. The acute activation of the Nox2 oxidase involves the formation of new oxidase complexes, with evidence that this may occur in distinct parts of the cell (*e.g.*, the cell membrane) (58). Consistent with this, it has been reported that $p47^{phox}$ resides associated with the intracellular cytoskeleton in resting cells, but translocates to membrane ruffles on stimulation by TNF-α (28, 31, 55, 98) or VEGF (101) .Van Buul and colleagues (98) also found that TNF-α stimulation of human umbilical vein endothelial cells (HUVECs) grown at low density resulted in translocation of $p67^{phox}$ and Rac1 (as well as $p47^{phox}$) to the tips of membrane ruffles (98). Wu *et al*. (102) reported that $p47^{phox}$ was found tethered to nascent focal complexes in the lamellae of motile endothelial cells (102). Petry *et al*. (80) reported that whereas most Nox2 was found in the perinuclear region in unstimulated EaHy926 hybrid endothelial cells, Nox2 was also detected at the cell surface where it partially colocalized with the actin cytoskeleton. Another recent study reported good evidence that after TNF-α stimulation of endothelial cells, active Nox2-containing oxidase complexes were found in the caveolae (105). Interestingly, the latter study found that Nox2 was in close proximity to eNOS, suggesting the potential for local peroxynitrite formation in this setting. Taken together, these studies clearly demonstrate that the Nox2-containing oxidase can become specifically assembled and activated in discrete compartments in endothelial cells, often in distinct cell-surface membrane locations.

In contrast to Nox2, the regulation of the Nox4 oxidase remains poorly understood. Several studies involving the transfection of Nox4 into various cell lines (*e.g.*, HEK293 cells) suggest that the Nox4-based oxidase is constitutively active (3, 53, 68), and it has been suggested that Nox4 may account for the basal ROS production usually detected in unstimulated endothelial cells (2). This is consistent with the data that Nox4 activity is not affected by known cytosolic regulatory subunits (3, 68). Nox4 was reported to colocalize with an endoplasmic reticulum marker in HUVECs (98), human microvascular endothelial cells (HMEC-1), and EaHy926 cells (80). Other studies found Nox4 to localize to the nucleus of human vascular endothelial cells, where the authors suggest it may play a role in gene expression (51). In vascular smooth muscle cells, Nox4 was reported to colocalize with vinculin in focal adhesions (36). Despite the apparent constitutive activity of the Nox4-containing oxidase, studies in other cell types suggest that its activity can also be acutely augmented.

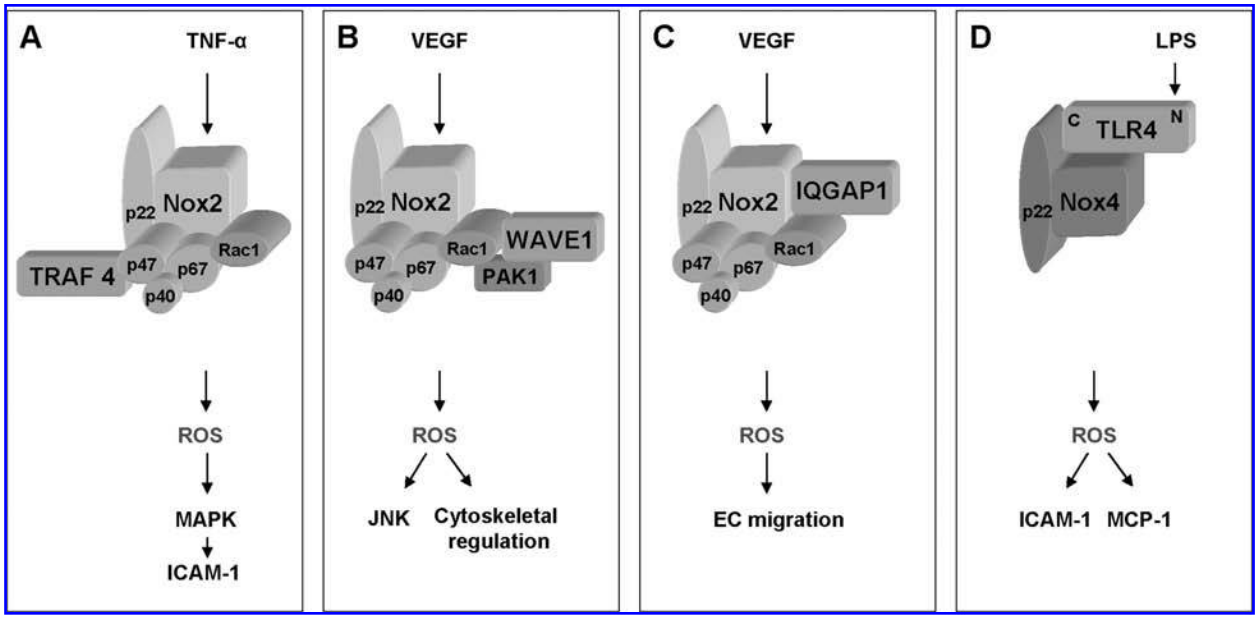

FIG. 3. Putative mechanisms underlying specific Nox-dependent redox signaling. Protein–protein interactions between oxidase components and various signaling or scaffolding molecules may play a key role in redox signaling. (**A**) Potential role of binding between TRAF4 and $p47^{phox}$ in TNF-α–induced downstream MAPK activation and ICAM-1 upregulation. (**B**) Interactions between WAVE1, Rac1, and PAK1 in VEGF-induced cytoskeletal regulation. (**C**) Binding between Nox2, IQGAP1, and Rac1 during VEGF-induced migration. (**D**) Toll-like receptor 4 (TLR4) as a binding partner for Nox4 during LPS-induced endothelial activation. EC, endothelial cell; TRAF4, TNF-α receptor–associated factor-4; WAVE1, WASP-family verprolin homologous protein-1.

For example, insulin was reported to increase Nox4-dependent ROS production in adipocytes (66), whereas LPSs appeared to activate Nox4 in HEK293 cells (78). Recently, it was also reported by the same group that LPS activates Nox4 in human aortic endothelial cells (77). This study suggested that a direct interaction occurs between the cytosolic region of the Toll-like receptor 4 (TLR4) activated by LPS and the C-terminal of Nox4.

SPECIFICITY OF NOX-DEPENDENT REDOX SIGNALING

An important question in the field is how ROS generated by individual NADPH oxidase isoforms could modulate specific signaling pathways in a spatial- and temporal-specific manner, given that the ROS that is most likely to be involved (*i.e.*, H_2O_2) is thought to be freely diffusible. Several recent studies indicate different ways in which such specificity of signaling could be imparted, all of which involve the concept of compartmentalized ROS-dependent signaling. The distinct subcellular localization of individual Nox isoforms (including the possibility of distinct pools of the same isoform), as well as their differential activation by different stimuli, provides at least part of the requirements for specific compartmentalized signaling. For example, trafficking and assembly of NADPH oxidase-containing signaling complexes within caveolae has been reported (100). With regard to the highly diffusible nature of H_2O_2, Rhee and colleagues (82) convincingly demonstrated that the spatial targeting of H_2O_2-dependent signaling involves a critical role for peroxiredoxin, which degrades H_2O_2 highly effectively. In the model proposed by Rhee, H_2O_2 generated within the cell briefly inactivates peroxiredoxin in the vicinity of its production site, thereby allowing local H_2O_2-dependent redox signaling, which is terminated on the reactivation of peroxiredoxin.

In addition, an emerging paradigm is that components of the NADPH oxidase may undergo direct protein–protein interactions with a variety of non–oxidase-binding partners, which could serve to localize ROS generation to the close vicinity of signaling targets (Fig. 3). In a yeast two-hybrid screen, Xu *et al*. (103) found that p47^{phox} could potentially interact with many different proteins apart from the other oxidase subunits (55, 103). The binding of one of these, the TNF-α receptor–associated factor-4 (TRAF4), with phospho-p47^{phox} was subsequently shown by Li *et al*. (55) to be essential in TNF-α–induced acute activation of ERK1/2 in human microvascular endothelial cells. TRAF4, TRAF4-p47^{phox} binding, and p47^{phox}-dependent ROS generation were all essential for ERK1/2 activation in this study. In another study, the interaction of p47^{phox} with WASP-family verprolin homologous protein-1 (WAVE1), an important regulator of the cytoskeleton, was found to be involved in VEGF-induced JNK activation and membrane ruffle formation in endothelial cells; the WAVE1-dependent complex also contained Rac1 and the kinase PAK1 (101). Also in endothelial cells, the interaction of Nox2 and Rac1 with the molecule IQGAP1, an actin-binding scaffolding protein, was found to be critical during VEGF-induced migration (41, 104). Recently, Engelhardt and colleagues (60) reported that Nox2 oxidase–containing signaling complexes may be assembled in endosomes after agonist stimulation. In this study, the authors found that stimulation of MCF-7 epithelial cells with interleukin-1β induced internalization of Nox2 and the interleukin receptor into endosomes in a Rac1-dependent manner; the consequent Nox2-derived H_2O_2 generation within the endosomes played a critical role in activation of an I-κB kinase complex and NF-κB in these cells (Fig. 4). Taken together, these studies demonstrate ways in which Nox2 oxidase–dependent compartmentalized redox signaling may be effected.

In contrast to Nox2, possible mechanisms involved in Nox4 oxidase–dependent redox signaling remain poorly defined, apart from the interaction between the TLR4 receptor and Nox4 in response to LPS stimulation, referred to earlier (77) (see Fig. 3).

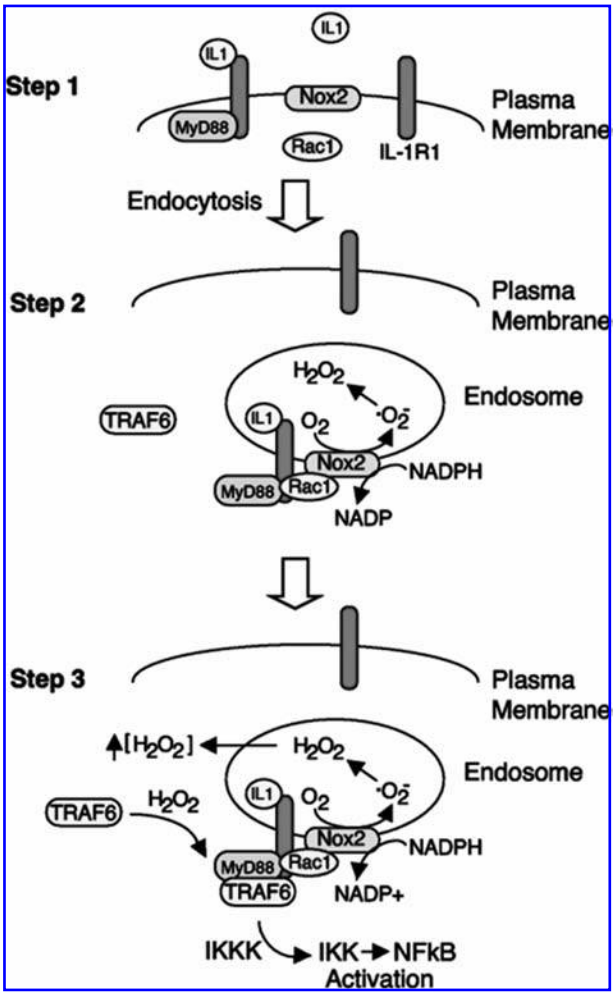

FIG. 4. Compartmentalization of Nox2 oxidase–containing signaling complexes in endosomes. Stimulation with IL-1 induced internalization of Nox2 and the interleukin receptor into endosomes in a Rac-1–dependent manner, followed by assembly of a signaling complex leading to downstream NF-κB activation. [Reprinted with permission from Li *et al.* (60)].

OVERVIEW OF CELL BIOLOGY OF ENDOTHELIAL ACTIVATION

Endothelial activation is essential for the development of the inflammatory response, a pivotal component of which is the transmigration of leukocytes across the endothelium (known as transendothelial migration or diapedesis). This involves a complex process of capture, rolling, and then firm adhesion of leukocytes onto the endothelium, followed by transmigration (11, 38). As part of the process, significant changes occur in endothelial protein expression, cytoskeletal organization, cell shape, and permeability. The initial capture and rolling of leukocytes involves the establishment of transient interactions between E- and P-selectins on endothelial cells and their ligands on leukocytes. Chemokines on the endothelial surface then induce the activation of leukocyte integrins, which mediate firm interactions with the endothelial surface adhesion molecules ICAM-1 and VCAM-1. These adhesion molecules cluster in F-actin–rich docking structures that form cuplike extensions around leukocytes. Subsequent paracellular transmigration or transcellular transmigration (through intercellular junctions) of leukocytes involves PECAM-1, members of the junctional adhesion molecule (JAM) family, CD99 and ICAM-1. Changes in vascular permeability are also an important component of the inflammatory response and involve significant changes in the location and properties of vascular endothelial cadherin (VE-cadherin), an adhesion molecule essential for the stability of endothelial cell contacts and specifically enriched at intercellular adherens junctions. The phosphorylation of VE-cadherin is involved in increased intercellular gap formation and increased endothelial permeability. It should be noted that the specific details of this process may vary significantly, depending on the vascular bed, type of leukocyte, and the inflammatory stimulus.

Unactivated endothelial cells express low levels of ICAM-1 on the cell surface but no VCAM-1. During activation (*e.g.*, in response to proinflammatory stimuli such as TNF-α, interferon γ (IFN-γ), thrombin, and shear stress), a substantial increase occurs in the cell surface expression of ICAM-1 and VCAM-1. This may occur either very acutely through translocation to the cell surface (*i.e.*, without requiring gene transcription or *de novo* protein synthesis) or over a slower time course through the induction of protein transcription in a Rho GTPase and NF-κB–dependent manner (71). In addition, matrix metalloproteases (MMPs) are activated, which can regulate adhesion molecules by proteolytic cleavage of their extracellular domains; similar cleavage is also induced by leukocyte elastase. During activation, an increased gene and protein expression of chemokines such as MCP-1 occurs, which involves NF-κB and other transcription factors such as AP-1. The engagement of adhesion molecules by their ligands not only facilitates adhesion but also induces intracellular signaling, which contributes to the amplification and prolongation of the overall process. For example, signaling *via* the intracellular tail of ICAM-1 is implicated in transcellular diapedesis; the tail interacts with the cytoskeleton-associated proteins ezrin and α actinin and with F-actin, and can activate a variety of signaling molecules, including Src kinase, focal adhesion kinase, RhoA, and ERK1/2 (11). ICAM-1 cross-linking may also activate AP-1, increase VCAM-1 expression, and increase the production of IL-8.

ROLE OF ROS IN ENDOTHELIAL ACTIVATION

A significant body of evidence now exists to suggest that ROS are involved in many of the processes underlying (or associated with) endothelial activation (*e.g.*, the upregulation of adhesion molecules and chemokines, increased activity of MMP2 and MMP9, cytoskeletal reorganization, formation of intercellular gaps, and an increase in endothelial permeability and leukocyte transmigration). Indeed, many of the key signal-transduction molecules involved in endothelial activation, such as various MAPKs and the transcription factors AP-1 and NF-κB, are known to be redox sensitive. Whereas a wealth of evidence implicates ROS-mediated signaling (and in particular, ROS derived from NADPH oxidases) in endothelial activation in various *in vitro* settings, still only limited data demonstrate the involvement of such pathways *in vivo*.

TNF-α

TNF-α and other proinflammatory cytokines increase the expression of ICAM-1, VCAM-1, and MCP-1 through an NF-κB–dependent redox-sensitive mechanism, which can be inhibited by antioxidants or the NADPH oxidase inhibitor

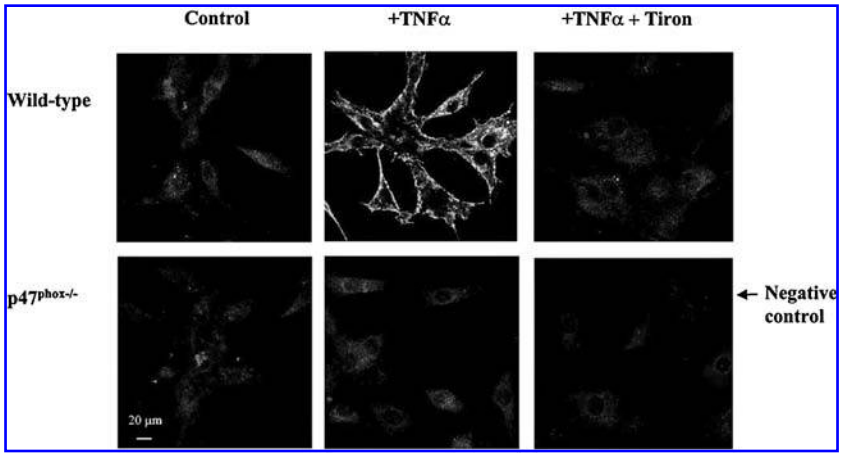

FIG. 5. TNF-α induces endothelial-surface expression of ICAM-1 through a p47^{phox}-dependent mechanism. ICAM-1 upregulation was inhibited in coronary microvascular endothelial cells from p47^{phox}-knockout mice. [Reprinted with permission from Li *et al.* (55)].

apocynin (94). The rapid TNF-α–induced upregulation of endothelial P-selectin expression was reported to depend on $O_2^{\cdot-}$ generation from both NADPH oxidase and xanthine oxidase (92). In another study, suppression of TNF-α–induced ICAM-1, VCAM-1, and E-selectin expression after adenoviral-mediated overexpression of dominant-negative Rac1 or of superoxide dismutase, through inhibition of NF-κB activation, suggested a role for Nox2 oxidase (13). Recently, it was found that the rapid surface expression of ICAM-1 induced by TNF-α in murine coronary microvascular endothelial cells was dependent on $p47^{phox}$ (*i.e.*, most likely Nox2 oxidase), because it was inhibited in cells isolated from $p47^{phox}$-knockout mice (Fig. 5) (55). In this study, $p47^{phox}$-dependent ROS generation also induced acute activation of ERK1/2. TNF-α–induced stimulation of NADPH oxidase was also implicated in the activation of JNK and p38MAPK, leading to AP-1 and NF-κB activation and finally an increase in ICAM-1 expression (61). TNF-α–induced increases in the permeability of HUVEC monolayers (through the formation of gaps) were found to involve NADPH oxidase activation, JNK activation, the tyrosine phosphorylation of VE-cadherin, and decreased endothelial cell junctional integrity (75). It should be noted that *in vivo*, at least some of the ROS released in response to TNF-α may also be derived from neutrophil NADPH oxidase, which then induces ICAM-1 expression in microvascular endothelial cells (23).

LPS

LPS is well recognized to induce TLR4-dependent endothelial cell activation (107), and it has been shown that this involves the ROS-dependent activation of NF-κB (84). Recently, it was reported that Nox4 oxidase–derived ROS generation plays a key role in this process (77). This group demonstrated that LPS-induced ROS-dependent expression of ICAM-1, IL-8, and MCP-1 in human aortic endothelial cells was inhibited by siRNA-mediated downregulation of Nox4. Furthermore, Nox4 siRNA also inhibited the adhesion of monocytes to endothelial cells and their transmigration after stimulation by LPS (Fig. 6). Didion *et al.* (20) showed that SOD overexpression in transgenic mice protects against LPS-induced NADPH oxidase–dependent ROS production and vascular-dependent relaxation. However, endothelial activation was not studied by these authors.

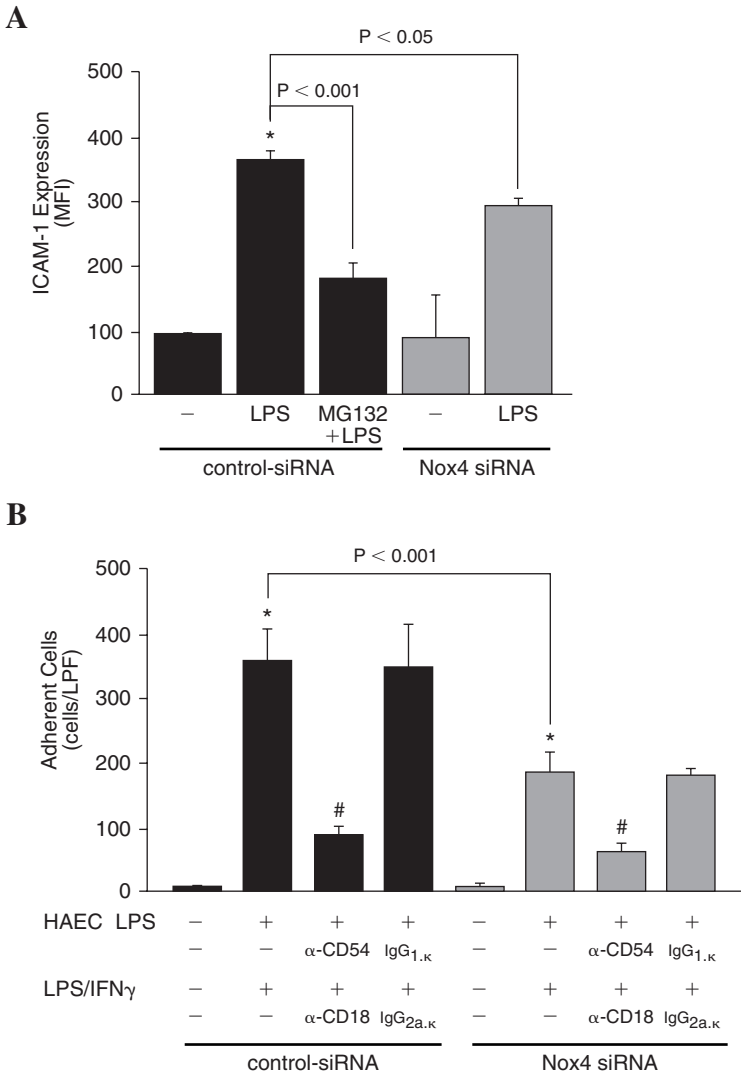

FIG. 6. Role of Nox4 in LPS-induced endothelial activation. (**A**) LPS-induced expression of ICAM-1 in human aortic endothelial cells was inhibited by the antioxidant MG132 or by siRNA against Nox4. (**B**) LPS-induced adhesion of monocytic cells to endothelial cells was inhibited by CD54 antibodies or by siRNA against Nox4. [Reprinted with permission from Park *et al.* (77)].

Angiotensin II

Angiotensin II acting *via* angiotensin 1 (AT_1) receptors is a potent stimulus for intracellular ROS generation in endothelial cells, especially from Nox2 oxidase (53, 58, 93). Many studies have reported an angiotensin II–mediated expression of ICAM-1 by endothelial cells *in vitro* and *in vivo*, which in several cases has been shown to involve ROS (14, 16, 62, 74, 79, 87). Antioxidant-inhibitable activation of p38MAPK and NF-κB was implicated in this effect (16). Angiotensin II–induced ROS production has also been reported to augment the expression of VCAM-1 and MCP-1 in endothelial cells (74, 81, 106).

Aldosterone

The mineralocorticoid-receptor agonist, aldosterone, also induces endothelial cell activation and the expression of VCAM-1, ICAM-1, MCP-1, and E-selectin mRNA. Interestingly, a recent study suggested that aldosterone increased the expression of Nox4 NADPH oxidase in HUVECs, although no direct evidence for its involvement in endothelial activation was reported (33).

Soluble CD40 ligand

CD40 ligand is a member of the TNF family that binds to CD40, a member of the TNF-receptor family; CD40 and CD40 ligand are coexpressed on many vascular and blood cells, including endothelial cells, vascular smooth muscle cells, and macrophages. Soluble CD40 ligand is derived mainly from activated platelets (35), whereas macrophages express CD40 ligand after exposure to oxidized LDL (86). Engagement of CD40 on endothelial cells stimulates ROS production (96) and induces the expression of E-selectin, ICAM-1, VCAM-1, MCP-1, and the secretion of other chemokines and cytokines that promote leukocyte recruitment (35). Endothelial CD40 signaling also increases MMP expression (65). However, the precise source of ROS activated by CD40 ligand signaling remains to be elucidated.

Oscillatory shear stress

Oscillatory shear stress is well known to cause endothelial activation, with this being a likely mechanism underlying the proatherogenic effects of disturbed flow in arteries *in vivo*. Oscillatory shear causes an increase in endothelial cell ROS production derived from NADPH oxidase (40), at least in part through the activation of Rac1 (95). Recently, an important role for bone morphogenic protein 4 (BMP4) in the process of shear-induced endothelial activation was discovered, based on DNA microarray analyses of cells subjected to chronic shear (90). Subsequently, the same group reported that BMP4 stimulated ICAM-1 expression and monocyte adhesion through the stimulation of ROS production by a $p47^{phox}$-dependent oxidase (89). Interestingly, it was suggested that this was a Nox1-containing (rather than a Nox2-containing) oxidase, based on the use of siRNA, which is one of the very few reports suggesting functional effects of Nox1 in endothelial cells. In another recent study, BMP2 (which may also be expressed by endothelial cells) was reported to induce endothelial activation and increase monocyte adhesion through a ROS- and PKC-dependent mechanism (17).

ROS production triggered by adhesion molecules

In addition to redox signaling after the agonist activation of NADPH oxidases, ROS-dependent signaling may also be involved downstream of adhesion molecule activation. For example, the clustering of VCAM-1 or its experimental cross-linking has been shown to trigger the formation of gaps between endothelial cell–cell contacts in a process that is both Rho and Rac1 dependent and involves downstream ROS release (99). Given the Rac1 dependence of the process, it seems likely that activation of Nox2 oxidase is involved. At least part of the mechanism for gap formation involves the activation of endothelial MMPs, which may induce the release of adhesion molecules in cell–cell junctions. ROS also influence the process of actin reorganization triggered by VCAM-1 cross-linking. Similar mechanisms may also be evoked after ICAM-1 clustering or cross-linking. In HUVECs, ICAM cross-linking has been shown to result in AP-1 activation and an increase in VCAM expression independent of NF-κB (54), although the authors did not look into whether these processes were ROS dependent.

Atrial natriuretic peptide (ANP)

ANP is known to antagonize TNF-α–induced activation of endothelial cells through the induction of mitogen-activated protein kinase phosphatase-1 (MKP-1), which acts by inhibiting TNF-α–induced activation of p38MAPK (48). Thus, it abrogates increases in MCP-1 expression, endothelial stress fiber formation, and endothelial permeability. Recently, Furst *et al.* (27) reported that this antiinflammatory effect of atrial natriuretic peptide involved Nox2 NADPH oxidase–dependent induction of MKP-1 *via* a JNK/AP-1–mediated pathway. This report therefore raises the intriguing possibility that Nox2 oxidase can both promote endothelial activation (*e.g.*, when activated by TNF-α or angiotensin II) and inhibit activation (when activated by atrial natriuretic peptide). The precise mechanisms underlying these dual effects (*e.g.*, whether different pools of Nox2 are involved) remain unknown.

Cigarette smoke

Endothelial adhesion molecules such as ICAM-1 are reported to be upregulated in sera obtained from cigarette smokers (1) and in cultured HUVECs exposed to cigarette-smoke extract (45). In animals exposed to prolonged cigarette smoke, it was found that vascular mRNA expression of IL-1β, IL-6, TNF-α and ICAM-1 was significantly increased in association with enhancement of NADPH oxidase activity and H_2O_2 generation (76). Similar effects in isolated arteries exposed to cigarette-smoke extract could be abrogated by the oxidase inhibitors apocynin or diphenyleneiodonium, whereas cigarette-smoke extract also augmented levels of NF-κB activation and monocyte adhesion in endothelial cells. However, the precise Nox isoform involved was not defined.

Ischemia–reperfusion

During microvascular dysfunction induced by ischemia–reperfusion, the increase in endothelial permeability is thought to involve an increase in xanthine oxidase–derived ROS generation (70). However, the activation of xanthine oxidase has

not been found directly to increase ICAM-1 expression (25, 72). Studies using induction of heme oxygenase-1 (HO-1), which acts as an antioxidant through the generation of bilirubin, support an involvement of ROS in endothelial activation during ischemia–reperfusion *in vivo*. Hayashi *et al*. (34) studied rats treated with either an HO-1 inducer or control, and then subjected to ischemia–reperfusion or exposure to H_2O_2, and found that leukocyte adhesion in microvessels was inhibited in the former group (34). Another study by Kawamura *et al*. (46) showed that an HO-1 inducer decreased the expression of VCAM-1 and MCP-1 in endothelial cultures exposed to oxidized low-density lipoprotein or TNF-α, although this was not studied *in vivo*. Intraperitoneal injection of bilirubin was also found to inhibit leukocyte migration into lung alveoli in a murine asthma model characterized by VCAM-dependent airway inflammation (47).

Hypercholesterolemia

Endothelial activation induced by hypercholesterolemia *in vivo* may be of relevance to atherogenesis. Grainger and colleagues (91) reported compelling evidence implicating Nox2 oxidase–dependent signaling in this setting. By using intravital microscopy studies in hypercholesterolemic p47^{phox}-knockout mice, this group found that both leukocyte-endothelial cell adhesion and leukocyte emigration into tissues required NADPH oxidase (91). In further studies using bone marrow chimeras, they could show that both endothelial and leukocyte NADPH oxidase (most likely Nox2 oxidases) were important in these effects. In a follow-up study, the same investigators also showed that hypercholesterolemia-induced P-selectin–dependent adhesion of platelets and leukocytes in the cerebral microcirculation was blunted in Nox2-deficient mice (43).

Atherosclerosis

It was reported that after crossing p47^{phox} knockout mice into an ApoE$^{-/-}$ background, the double-knockout mice did not demonstrate differences in atherosclerotic lesion size compared with single-knockout ApoE$^{-/-}$ mice, although no assessment of endothelial activation was made in this study (39). Similarly, Nox2-knockout mice fed a high-fat diet for 20 weeks developed the same degree of atherosclerosis as the wild-type mice, even after crossing into an ApoE$^{-/-}$ background (50). Interestingly, however, Barry-Lane *et al*. (5) found that lesions in the abdominal and thoracic aorta were reduced in p47^{phox} knockout mice crossed into an ApoE$^{-/-}$ background compared with single knockouts. Another study in which ApoE$^{-/-}$ mice were crossed with mice deficient in glucose 6-phosphate dehydrogenase and therefore in NADPH production showed that the double-deficient mice had lower expression of VCAM-1, lower superoxide anion release, and reduced aortic lesion area, suggesting that a reduction in NADPH oxidase activity attenuated cell activation and aortic lesion growth (69).

CONCLUSIONS

ROS-dependent modulation of signal transduction appears to play a key role in the process of endothelial activation in response to diverse stimuli, which in turn is implicated in disease states such as atherosclerosis, sepsis, autoimmune disease, and hypertension. Both the Nox2 and Nox4 isoforms of NADPH oxidase are implicated as key sources of these ROS through the specific modulation of different processes involved in endothelial activation. Because NADPH oxidase activity can be inhibited by drugs such as statins and angiotensin-receptor blockers, the targeting of these enzymes may be a useful therapeutic approach to inhibiting endothelial activation.

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ABBREVIATIONS

ACE, angiotensin-converting enzyme; ARDS, acute respiratory distress syndrome; AT_1, angiotensin 1 receptor; eNOS, endothelial NO synthase; JAM, junctional adhesion molecule; HMEC-1, human microvascular endothelial cell; HUVEC, human umbilical vein endothelial cell; ICAM-1, intercellular adhesion molecule-1; IFN-γ,_interferon γ;_LPS, lipopolysaccharide; MCP-1, monocyte chemoattractant protein-1; MKP-1, mitogen-activated protein kinase phosphatase-1; MMPs, matrix metalloproteases; NADPH, nicotinamide adenine dinucleotide phosphate; NO, nitric oxide; $ONOO^-$,peroxynitrite; PECAM-1, platelet–endothelial cell adhesion molecule-1; ROS, reactive oxygen species; TLR4, Toll-like receptor 4; tPA, tissue plasminogen activator; TRAF4, TNF-α receptor–associated factor-4; VCAM-1, vascular cell adhesion molecule-1; VE-cadherin, vascular endothelial cadherin; VEGF, vascular endothelial growth factor; WAVE1, WASP-family verprolin homologous protein-1.

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