A gp91phox Containing NADPH Oxidase Selectively Expressed in Endothelial Cells Is a Major Source of Oxygen Radical Generation in the Arterial Wall

A. Görlach,* R.P. Brandes,* K. Nguyen, M. Amidi, F. Dehghani, R. Busse

Abstract—Reactive oxygen species (ROS) play an important role in regulating vascular tone and intracellular signaling; the enzymes producing ROS in the vascular wall are, however, poorly characterized. We investigated whether a functionally active NADPH oxidase similar to the leukocyte enzyme, ie, containing the subunits p22phox and gp91phox, is expressed in endothelial cells (ECs) and smooth muscle cells (SMCs). Phorbol 12-myristate 13-acetate (PMA), a stimulus for leukocyte NADPH oxidase, increased ROS generation in cultured ECs and endothelium-intact rat aortic segments, but not in SMCs or endothelium-denuded arteries. NADPH enhanced chemiluminescence in all preparations. p22phox mRNA and protein was detected in ECs and SMCs, whereas the expression of gp91phox was confined to ECs. Endothelial gp91phox was identical to the leukocyte form as determined by sequence analysis. In contrast, mitogenic oxidase-1 (mox1) was expressed in SMCs, but not in ECs. To determine the functional relevance of gp91phox expression, experiments were performed in aortic segments from wild-type, gp91phox2/2, and endothelial NO synthase (eNOS)2/2 mice. PMA-induced ROS generation was comparable in aortae from wild-type and eNOS2/2 mice, but was attenuated in segments from gp91phox2/2 mice. Endothelium-dependent relaxation was greater in aortae from gp91phox2/2 than from wild-type mice. The ROS scavenger tiron increased endothelium-dependent relaxation in segments from wild-type, but not from gp91phox2/2 mice. These data demonstrate that ECs, in contrast to SMCs, express a gp91phox-containing leukocyte-type NADPH oxidase. This enzyme is a major source for arterial ROS generation and affects the bioavailability of endothelium-derived NO. (Circ Res. 2000;87:26-32.)

Key Words: oxygen radicals ■ endothelial function ■ smooth muscle cells ■ p22phox

Reactive oxygen species (ROS) play an important role in vascular homeostasis by affecting signal transduction, proliferation, apoptosis, aging, gene expression, and the biologically effective concentration of NO.1–4 The enzymatic mechanisms leading to vascular ROS generation, however, are still incompletely understood. Several reports have suggested that endothelial cells (ECs) and vascular smooth muscle cells (SMCs) express a ROS-generating, NADH/NADPH-dependent oxidase containing p22phox,5–9 which is a membrane-bound component of the leukocyte NADPH oxidase.10 In leukocytes, this complex enzyme is responsible for the production of superoxide anions during the respiratory burst and, in addition to p22phox, contains the membrane-bound protein gp91phox.11 On activation of the enzyme by phorbol-12-myristate-13-acetate (PMA), for example, several cytosolic proteins, including rac, p47phox, p67phox, and p40phox, translocate to the membrane and associate with the membrane-bound subunits. The assembled enzyme complex facilitates electron transfer from NADPH to molecular oxygen, leading to the generation of superoxide anions, which subsequently react to form further ROS such as hydrogen peroxide and hydroxyl radicals.12

Whereas p22phox is widely expressed in mammalian tissues,10 the other subunits, and in particular gp91phox, are thought to be specifically expressed in myeloid cells.11 The identification of p22phox in vascular cells and the observation that addition of NADPH/NADH to intact cells elicits ROS generation led to the suggestion that a NAD(P)H oxidase resembling the leukocyte enzyme is present in vascular cells.13 Because p22phox and gp91phox, which together make up cytochrome b558, are essential for NADPH oxidase activity in leukocytes, we compared the expression of these subunits in leukocytes, ECs and SMCs. Moreover, we determined the functional importance of these subunits for ROS generation in vascular cells.

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Materials and Methods

Measurements of ROS

Measurement of ROS in vascular segments and in cultured cells seeded on 9×9-mm glass coverslips was performed by chemiluminescence using the chemiluminescence enhancer lucigenin (220 μmol/L) or the recently described improved Cypridina bilgen-dorffii luciferin analog (CLA) derivative 6-(4-methoxy-phenylethynyl)-2-methyl-7H-imidazo[1,2-a]pyrazin-3-one (compound 5) (3 μmol/L) (provided by Dr O. Shimomura, Woods Hole, Mass.). Dichlorofluorescein (DCF) fluorescence was recorded in human umbilical vein ECs (HUVECs) loaded with 2′,7′-dichlorodihydrofluorescein-diacetate (H₂DCFDA, 5 μmol/L) or the recently described improved lucigenin, 329 μmol/L) to obtain identical preconstriction levels (lucigenin, 329 μmol/L). The phenylephrine concentration was adjusted to 3 mol/L to obtain identical preconstriction levels (~80% of the initial KCl constriction). Relaxations to acetylcholine (ACh) in the presence or absence of the ROS scavenger tiron (5 mmol/L) and relaxations to sodium nitroprusside (SNP) were recorded.

Statistical Analysis

All values are mean±SEM. Statistical significance was tested using ANOVA for repeated measures followed by Newman-Keuls test.

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Results

PMA Stimulates ROS Formation in ECs but Not in SMCs

Although basal ROS formation was close to the detection limit in both cell types, HUVECs generated significantly more ROS than SMCs as assessed by chemiluminescence. PMA (0.01 to 1 μmol/L) induced a sustained increase in ROS formation in HUVECs, but not in SMCs, whereas NADPH increased chemiluminescence in both cell types (Figures 1A and 1B). Chemiluminescence elicited by PMA as well as by NADPH was sensitive to the flavin inhibitor diphenylene iodonium (DPI, 10 μmol/L) (Figure 1C, compound 5 chemiluminescence). To exclude the possibility that the lack of PMA response in SMCs was due to repetitive passaging, experiments were repeated in primary cultures from porcine coronary SMCs and porcine aortic ECs. The data obtained were comparable with those obtained with the passaged human cells. PMA induced a chemiluminescence response in porcine aortic ECs, which was similar to that in HUVECs (lucigenin, 329 μmol/L), whereas no increase in ROS generation was detected in porcine coronary SMCs. Basal and PMA-induced chemiluminescence in SMCs (lucigenin, −1±0.9, and compound 5, −2×10⁶ CPM/coverslip, P<0.05, n=5).

To exclude the possibility that the contamination of cell cultures with leukocytes contributed to PMA-induced ROS generation in HUVECs, measurements were repeated using different cell passages. Basal and PMA-induced chemiluminescence was not affected by passage (P) number (for basal

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Immunoblotting

Western blot analysis was performed as described previously. p22phox SDS-PAGE was performed using a membrane preparation of HUVECs and SMCs. For detection of gp91phox, purified glycosylated proteins were isolated by wheat germ agglutinin agarose affinity purification. Antibodies were provided by Dr J. Kreutzer (Heidelberg, Germany; p22phox) and Dr D. Roos (Amsterdam, Netherlands; gp91phox).

Reverse Transcriptase–Polymerase Chain Reaction (RT-PCR) and Sequence Analysis

RT-PCR was performed with total RNA. PCR primers were selected on the bases of the published sequences (Table). PCR products were gel purified and subjected to cycle sequencing (Applied Biosystems, Perkin-Elmer).

Screening of a HUVEC cDNA Library

A HUVEC cDNA library (gift of Dr R. Bowditch, Oklahoma City, OK) was screened with full-length human p22phox or gp91phox cDNA probes (provided by Dr M. Dinauer, Indianapolis, IN). Positive clones were amplified by PCR and sequenced.

Organ Chamber Experiments

Organ chamber experiments were performed as described using phenylephrine-precontracted mouse aortic segments from gp91phox−/− and wild-type mice. The phenylephrine concentration was adjusted (0.03 to 0.3 μmol/L) to obtain identical preconstriction levels (~80% of the initial KCl constriction). Relaxations to acetylcholine (ACh) in the presence or absence of the ROS scavenger tiron (5 mmol/L) and relaxations to sodium nitroprusside (SNP) were recorded.

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lucigenin signal, \( P_0 \), 327±129; \( P_1 \), 234±94; and \( P_2 \), 231±131 CPM/cover slip; for PMA-induced lucigenin signal, \( P_0 \), 1082±373; \( P_1 \), 1147±144; and \( P_2 \), 1275±445 CPM/cover slip; \( n=3, \ P=NS \). Using the ROS-sensitive fluorophore \( \text{H}_2\text{DCFDA} \), a 2.5-fold increase in fluorescence in response to stimulation with PMA was observed in all ECs studied (basal, 1.1±0.3, and PMA, 2.6±0.6 light units/min, \( P<0.02, n=7 \) each group; Figure 2).

PMA-Stimulated Oxygen Radical Formation in Intact Segments Requires the Presence of Endothelium

In intact rat aortic segments, PMA as well as NADPH increased ROS generation (Figure 3). Mechanical denudation of the endothelium abolished the response to PMA, whereas the response to NADPH remained detectable. Pretreatment with the flavin inhibitor DPI attenuated the responses to both PMA (Figure 3C) and NADPH (data not shown).

Expression of \( p22\text{phox} \) and \( gp91\text{phox} \) in Vascular Cells

\( p22\text{phox} \) was detected by RT-PCR in the monocytic cell line THP1, which is known to express the leukocyte NADPH oxidase, and in HUVECs, the EC line EA.Hy926, HSMCs, and the human colon carcinoma cell line CaCo-1 (Figure 4, top). Screening of a HUVEC cDNA library with a human \( p22\text{phox} \) probe revealed 5 positive clones. The HUVEC library was positive for endothelial NO synthase (eNOS) as a marker enzyme for ECs, but negative for tumor necrosis factor-\( \alpha \) (TNF-\( \alpha \)), which is expressed in leukocytes, demonstrating that the HUVEC cDNA library was free of contaminating leukocytes (Figure 4, bottom). Sequence analysis of 84% of the \( p22\text{phox} \) coding sequence revealed that \( p22\text{phox} \) in HUVECs and HSMCs is identical to the human leukocyte form. The \( p22\text{phox} \) sequence was also identical in rat SMCs and rat leukocytes (data not shown).

RT-PCR performed with human leukocyte \( gp91\text{phox} \) primers led to PCR fragments of expected sizes in THP1 cells as well as in HUVECs and EA.Hy926 cells. No \( gp91\text{phox} \) PCR product could be obtained using HSMC or CaCo-1 cell cDNA (Figure 4, top). Screening of the HUVEC cDNA library with a human \( gp91\text{phox} \) probe revealed 1 positive clone. Sequence analysis of cloned and PCR-amplified cDNA

**Figure 1.** ROS generation in response to PMA and NADPH in cultured ECs and SMCs. A, ROS formation in HUVECs (EC) or HSMCs (SMC) measured using lucigenin-enhanced chemiluminescence. Cells were stimulated with 1 \( \mu \text{mol/L} \) PMA or 100 \( \mu \text{mol/L} \) NADPH. B, Statistical analysis of the effects of PMA and NADPH on HUVECs (open bars) and HSMCs (solid bars) on lucigenin-enhanced chemiluminescence (\( n=12; \ ^*P<0.05 \)). C, PMA-induced ROS formation as measured by compound 5-enhanced chemiluminescence in HUVECs (EC) and HSMCs (SMC) before and after the addition of DPI (10 \( \mu \text{mol/L} \)). A representative experiment of 5 independent experiments is shown.

**Figure 2.** PMA-induced DCF fluorescence in primary cultures of HUVECs. Cells were exposed to solvent (A) or PMA (B), and fluorescence was recorded using a confocal microscope. False color images show fluorescence 5 minutes after stimulation.
composing the complete gp91phox coding sequence as well as 148 bp of the 5′ flanking sequence and 208 bp of the 3′ flanking region demonstrated that gp91phox is identical in HUVECs and human leukocytes. To exclude the possibility of gp91phox expression in HSMCs being lost during culture, mRNA from the media of human umbilical arteries was isolated and subjected to gp91phox RT-PCR. In accordance with the results obtained in HSMCs, gp91phox was not detectable (data not shown).

An isoform of gp91phox termed mitogenic oxidase-1 (mox1) was recently identified in SMCs and CaCo-1 cells. RT-PCR with primers derived from the human mox1 sequence (Table) resulted in PCR products of the predicted size in HSMCs and CaCo-1 cells (Figure 4, top). However, no mox1 PCR product was obtained from HUVECs, EA.Hy926 cells, or THP1 cells.

Western blot analysis demonstrated the presence of p22phox protein in membrane but not cytoplasmic fractions prepared from HUVECs and HSMCs (Figure 5A). Because gp91phox is extensively glycosylated and the protein appears as a smear rather than a distinct band in SDS-PAGE, it is difficult to detect small amounts of the protein in whole-cell lysates. To circumvent this problem, Western blotting was performed using glycoproteins isolated from polymorphonu-

clear neutrophils (PMNs), HUVECs, and HSMCs. gp91phox protein was detected in PMNs and HUVECs, but not in HSMCs (Figure 5B).

**gp91phox Is Involved in Vascular ROS Generation**

To investigate whether gp91phox is involved in vascular ROS formation, lucigenin-enhanced chemiluminescence was assessed in aortic segments from gp91phox−/−, eNOS−/− and wild-type mice (Figure 6). PMA-stimulated ROS production was detectable in aortae from wild-type and eNOS−/− mice and was abolished by endothelial denudation. In contrast,
PMA did not elicit ROS generation in endothelium-intact or endothelium-denuded aortic segments from gp91phox−/− mice. Lucigenin chemiluminescence in unstimulated aortic preparations or after the addition of NADPH was similar in the 3 strains, suggesting that the PMA-induced, but not the NADPH-mediated ROS formation requires a functional gp91phox.

**ACh-Mediated Relaxation Is Increased in gp91phox Knockout Mice**

Compared with responses obtained using aortic segments from wild-type mice, the ACh-induced relaxation of segments from gp91phox−/− mice was more pronounced. The ROS scavenger tiron (5 mmol/L) enhanced the ACh-induced relaxation of aortic segments from wild-type, but not from gp91phox−/− mice, so that there was no longer a difference in the ACh-induced relaxation of segments from the 2 strains. Endothelium-independent relaxations to SNP were similar in aortic segments from both groups (Figure 7).

**Discussion**

In the present study, we have demonstrated that gp91phox is expressed in ECs, but not in SMCs, and that the endothelial gp91phox is identical to that isolated from leukocytes.

The initial concept that gp91phox is myeloid-specific was strengthened by the demonstration of binding sites for several “myeloid-specific” transcription factors such as Pu.1 and Elf-1 in its promoter region. However, Elf-1 protein is constitutively expressed in ECs and has been proposed to play a role in the control of gene expression of eNOS. Thus, the presence of “myeloid-specific” transcription factors may account for the expression of gp91phox in ECs, but not in SMCs. The recent identification of mox1, a gp91phox homologue, in colon carcinoma cells and SMCs suggests that the composition of the NADPH oxidase is fundamentally different in ECs and SMCs. Indeed, although we found evidence for the presence of mox1 in HSMCs, we could not detect mox1 cDNA in the myeloid cell line THP1 or in ECs. It is therefore tempting to speculate that in SMCs mox1, instead of gp91phox, associates with p22phox to constitute an NADPH oxidase.

Although the expression of gp91phox mRNA in ECs has been reported previously, the expression of functionally relevant amounts of protein has been questioned. In addition to demonstrating the presence of gp91phox protein in ECs, our results indicate that this protein is functionally active. Indeed, gp91phox appears to be crucial for the burstlike ROS generation observed in response to PMA, as knockout of the gp91phox gene abolished ROS generation in endothelium-intact aortic segments.

The exact mechanism by which PMA activates the endothelial NADPH oxidase remains to be elucidated. However, in leukocytes, PMA directly activates protein kinase C, which leads to the phosphorylation of p47phox, possibly to the activation of rac, and to the subsequent activation of the oxidase. Although p47phox, p67phox, and rac are present in
ECs (A.G. and R.P.B., unpublished observation, 2000), the role played by these proteins in the specific signaling pathways leading to the activation of the oxidase in ECs has not been established.

To measure ROS formation, we mainly used lucigenin-enhanced chemiluminescence, although the validity of this method has recently been questioned.23 We obtained similar results using compound 5, one of the imidazopyrazinone-type chemiluminescence enhancers that do not undergo redox cycling.24 Moreover, PMA-stimulated ROS formation was also observed by using DCF fluorescence and by using cells derived from different passages. Such experiments indicate that PMA-induced ROS formation in ECs was not due to an artifact associated with the use of lucigenin and did not reflect contamination of EC cultures with leukocytes.

PMA increases [Ca\(^{2+}\)], and activates eNOS, which is another important source of ROS under certain conditions.25 Because PMA stimulated ROS generation in wild-type and in eNOS\(^{-/-}\) mice to a similar extent, we could exclude a substantial contribution of eNOS-derived ROS to the PMA response. The specificity of this effect was further demonstrated by the finding that the addition of NADPH increased chemiluminescence in ECs and SMCs as well as in aortic segments from gp91phox\(^{-/-}\) mice. A number of studies have demonstrated increased ROS formation in vascular segments in response to NADPH and, to an even greater extent, in response to NADH.13,26 Our finding that NADPH, but not PMA, stimulated ROS formation in aortae from gp91phox\(^{-/-}\) mice clearly indicates that NADPH does not activate the gp91phox-containing leukocyte/EC NADPH oxidase. Whether the NADPH-elicted chemiluminescence originates from ROS generated by other oxidases or by auto-oxidation of endogenous redox cyclers such as ubiquinones, or can be attributed to redox cycling of the chemiluminescence enhancers on the outer surface of the cell membrane, remains to be determined.

The amount of ROS generated by ECs after stimulation with PMA was significantly lower than that measured in leukocytes. This is not unexpected, because ROS production is determined by the amount of oxidase protein in the cell27 and ECs express only 1/100 of p22phox and gp91phox protein detected in leukocytes.

ECs are able to generate low levels of ROS under basal conditions. However, basal chemiluminescence was not significantly different in aortic segments from wild-type and gp91phox\(^{-/-}\) mice, suggesting that the gp91phox-containing NADPH oxidase might not contribute significantly to basal ROS generation. However, it cannot be excluded that the chemiluminescence assay used was not sensitive enough to detect small differences in basal aortic ROS production between wild-type and gp91phox\(^{-/-}\) mice.

Aortic segments from gp91phox\(^{-/-}\) mice exhibited a more pronounced endothelium-dependent relaxation to ACh than that observed in aortae from wild-type mice. An antioxidant, tiron, selectively enhanced endothelium-dependent relaxation in wild-type mice, indicating that the reduced scavenging of endothelial NO by O\(_2^-\) in the aorta from gp91phox\(^{-/-}\) mice could account for the enhanced relaxation. ACh is known to increase [Ca\(^{2+}\)], in ECs, release arachidonic acid, and facilitate the activation of protein kinase C, all mechanisms known to activate the leukocyte NADPH oxidase.11 It is, however, unclear whether ACh, phenylephrine, or both are required to stimulate gp91phox-dependent ROS generation in ECs.

In conclusion, our data demonstrate that the endothelium is a major generator of vascular ROS and that the formation of endothelial ROS can be acutely modulated by activation of protein kinase C. One prominent source of endothelial ROS is the gp91phox-containing NADPH oxidase, which is similar to that found in leukocytes but distinct from the enzyme in vascular SMCs. However, this endothelial NADPH oxidase generates ROS in a concentration sufficient to affect the bioavailability of endothelium-derived NO.

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