Superoxide Production and Expression of Nox Family Proteins in Human Atherosclerosis

Dan Sorescu, MD; Daiana Weiss, MD; Bernard Lassègue, PhD; Roza E. Clempus, MD; Katalin Szöcs, MD; George P. Sorescu, MD; Liisa Valppu, BS; Mark T. Quinn, PhD; J. David Lambeth, MD; J. David Vega, MD; W. Robert Taylor, MD, PhD; Kathy K. Griendling, PhD

Background—NAD(P)H oxidases are important sources of superoxide in the vasculature, the activity of which is associated with risk factors for human atherosclerosis. This study was designed to investigate the localization of superoxide production and the expression of the Nox family of NAD(P)H oxidase proteins (gp91phox, Nox1, and Nox4) in nonatherosclerotic and atherosclerotic human coronary arteries.

Methods and Results—In coronary artery segments from explanted human hearts, we examined intracellular superoxide production with dihydroethidium. In nonatherosclerotic coronary arteries, superoxide was present homogenously throughout the intima, media, and adventitia. In atherosclerotic arteries, there was an additional intense area of superoxide in the plaque shoulder, which is rich in macrophages and α-actin-positive cells. p22phox colocalized with gp91phox mainly in macrophages, whereas Nox4 was found only in nonphagocytic vascular cells. Expression of gp91phox and p22phox mRNA was associated with the severity of atherosclerosis. gp91phox correlated with the plaque macrophage content, whereas Nox4 correlated with the content of α-actin-positive cells. Nox1 expression was low both in human coronary arteries and isolated vascular cells.

Conclusions—Several Nox proteins, including gp91phox and Nox4, may contribute to increased intracellular oxidative stress in human coronary atherosclerosis in a cell-specific manner and thus may be involved in the genesis and progression of human coronary atherosclerotic disease. (Circulation. 2002;105:1429-1435.)

Key Words: enzymes ▪ coronary disease ▪ arteries ▪ atherosclerosis

The oxidative stress hypothesis of atherosclerosis posits that it is an inflammatory disease triggered by subendothelial accumulation of LDL particles modified by reactive oxygen species (ROS). Recent work, however, showed that ROS mediate many additional pathological processes in the vessel wall, including endothelial dysfunction as well as smooth muscle cell (SMC) migration, growth, and apoptosis.1

In vitro studies demonstrated that proinflammatory stimuli activate proteins that generate both intracellular and extracellular ROS in virtually all vascular cells. Work in cultured cells and animal models led to the identification of the NAD(P)H oxidase family of enzymes as major sources of these ROS.1 NAD(P)H oxidases are present in endothelial cells, SMCs, fibroblasts, and phagocytic mononuclear cells and provide a mechanism for localized release of ROS, which can influence discrete redox-sensitive signaling pathways with fundamental effects on atherogenesis.

Vascular NAD(P)H oxidases have structures similar to, but distinct from, the neutrophil respiratory burst enzyme. The neutrophil NAD(P)H oxidase is an electron transport system that includes the membrane-bound flavocytochrome b_{558} (formed by gp91phox and p22phox) and three cytosolic proteins, p47phox, p67phox, and Rac.2 gp91phox contains the putative binding sites for NADPH, heme, and FAD, and together with p22phox, supports the flow of electrons from NADPH to oxygen. Recently, novel gp91phox homologues, termed Nox1, Nox3, Nox4, and Nox5, were identified in nonphagocytic cells, including Nox1 and Nox4 in the vasculature.3-8 These homologues share with gp91phox (Nox2) putative NAPDH and flavin binding sites and a 30% to 60% mRNA identity.4 Expression of Nox1 and Nox4 in fibroblasts increases O_{2}^{-} and H_{2}O_{2} production,3,7 indicating that these proteins represent functional oxidases.

There are few human studies examining the relation between NAD(P)H oxidase expression and activity and atherosclerosis. Guzik et al8 provided the first evidence that NAD(P)H oxidases are a major source of O_{2}^{-} in human vessels and showed an association between enzymatic activ-
Superoxide Detection

Superoxide was detected in frozen 30-μm-thick arterial sections, with dihydroethidium (DHE) (10 μmol/L) as described previously.6

Cell Culture

Human coronary artery SMCs and human cardiac fibroblast cells were obtained from Cell Systems and grown in SmGM-2 media (Biowhittaker). Human coronary artery endothelial cells were purchased from BioWhittaker and grown in EGM-2 media (Biowhittaker). All cells were harvested at passage 3 and used at confluence.

Immunofluorescent Histochemistry

Single- and double-label fluorescent immunohistochemistry was performed on frozen 7-μm OCT-embedded tissue sections as described previously.6 The antibodies used were rabbit polyclonal anti-p22phox R317911 (1:100 dilution), rabbit polyclonal anti-Nox46 (1:100 dilution), mouse monoclonal anti-gp91phox (clone 54.1,12 1:50 dilution), mouse monoclonal anti-smooth muscle actin (clone A4, 1:400 dilution, Sigma), mouse monoclonal anti-CD68 antibody (1:50 dilution, Dako), and mouse monoclonal anti–von Willebrand factor (1:50, Dako). Serial sections treated with secondary antibodies alone did not show specific staining.

Quantitative Real-Time Polymerase Chain Reaction

Quantification of human gp91phox, p22phox, nox1, nox4, monocyte colony-stimulating factor receptor-1 (c-fms or MCSFR-1), human smooth muscle-specific α-actin, and 18S rRNA was performed by amplification of artery cDNA with the LightCycler real-time thermocycler (Roche), as described previously.6 Optimized amplification conditions were 100-nmol/L primers for gp91phox, p22phox, nox1, nox4, MCSFR-1, and α-actin, and 18S rRNA primers, 4 mmol/L MgCl2, annealing at 68°C; for 18S, 50 mmol/L universal 18S rRNA primers, 4 mmol/L MgCl2, and annealing at 62°C; extension at 70°C. Copy numbers were calculated by the instrument software from standard curves generated from human gp91phox, p22phox, nox1, nox4, MCSFR-1, α–actin, and 18S templates.

Statistical Analysis

All data are expressed as mean±SEM. Statistical significance (P<0.05) was assessed by Student’s t test on untransformed data and linear regression analysis with SPSS 7.5 for Windows (SPSS).

Results

In Vivo Detection of Superoxide in Human Coronary Arteries

To localize O2·− production in coronary arteries from explanted human hearts, we used DHE staining. DHE is a fluorescent dye that specifically reacts with intracellular O2·− and is converted to the red fluorescent compound ethidium, which then binds irreversibly to double-stranded DNA and appears as punctate nuclear staining.13 O2·− was detected throughout the vessel wall (Figure 1A). DHE fluorescence was abolished by preincubation with liposomal-SOD, demonstrating the specificity of the assay for O2·−.14

Figure 1B (top) shows that the O2·− signal in nonatherosclerotic arteries (defined as stage I or II per AHA classification of lesions)10) is present homogeneously in the intimal, medial, and adventitial layers, suggesting that all vascular cells produce intracellular O2·− in vivo. This is consistent with previous observations showing significant O2·− production in normal internal mammary arteries.13 In atherosclerotic vessels (stages III to VI), although O2·− staining is evident in all layers, an intense area of O2·− production occurs in the plaque shoulder (Figure 1B, bottom; typical of 9 of 11
arteries). This region is an area of severe inflammation and has been suggested to be prone to rupture.16

Expression of NAD(P)H Oxidase mRNAs in Isolated Human Vascular Cells

The electron transfer component of the phagocytic NADPH oxidase resides in the catalytic subunit gp91phox, the association of which with p22phox is essential for flavocytochrome b function.2 SMCs express only low levels of gp91phox but exhibit higher levels of the novel gp91phox homologues Nox1 and Nox4.3 However, the expression of these components in each type of cell present in the vessel wall is unclear. To gain insight into the expression pattern of these subunits, we compared mRNA levels in freshly isolated human monocytes, cultured coronary artery endothelial and SMCs, and cardiac fibroblasts with the use of quantitative real-time polymerase chain reaction (PCR) (Table 3). p22phox is expressed at high levels in all cell types, gp91phox is most abundant in monocytes, which express 465-fold more gp91phox mRNA than do endothelial cells. SMCs and fibroblasts have much lower levels of gp91phox (1050- and 32 000-fold lower, respectively), near the limit of detection of the assay. In contrast, nox4 mRNA is undetectable in monocytes but is abundantly expressed in nonphagocytic cells (endothelial cells/fibroblasts/SMCs, 125:3:1). nox1 levels are low in all cell types. These data indicate that monocytes express almost exclusively gp91phox, whereas the most abundant gp91phox homologue in endothelial cells, SMCs, and fibroblasts is Nox4. However, because these measurements reflect mRNA and not protein expression, one cannot make a firm conclusion concerning the stoichiometry of NAD(P)H oxidase complexes.

Localization of p22phox, gp91phox, and Nox4 in Human Coronary Arteries

To examine the distribution of NAD(P)H oxidase proteins in intact arteries, we used fluorescent immunohistochemistry. In nonatherosclerotic coronary arteries, p22phox is expressed in a pattern similar to $O_2^{-}$, with staining apparent in intimal, medial, and adventitial cells (Figure 2, left). The distribution of gp91phox is more restricted, with staining readily observable in the adventitia, to a lesser extent in intimal cells and notably almost absent from the medial layer. This is in striking contrast to the distribution of Nox4, which is expressed intensely in the media and weakly in adventitial fibroblasts and intimal cells. In atherosclerotic arteries, the pattern of p22phox expression is again similar to $O_2^{-}$ (Figure 2, right). In these vessels, both $O_2^{-}$ production and p22phox expression are intense in the plaque shoulder. gp91phox is also localized to the central region of the plaque shoulder and coincides with the most intense area of p22phox staining. Nox4 expression is evident in the media and in the intima surrounding the central core of the plaque.

On the basis of the distinct distribution of NAD(P)H oxidase components and the apparent ubiquitous expression of p22phox, we hypothesized that gp91phox colocalizes with p22phox primarily in macrophages. To test this premise, we used double-label fluorescent immunohistochemistry of oxidase subunits and cell type-specific markers. In both nonatherosclerotic and atherosclerotic arteries, as expected, p22phox colocalizes with endothelial, smooth muscle, and macrophage markers (Figure 3). In nonatherosclerotic arteries, p22phox and gp91phox colocalization exhibits a pattern similar to the distribution of macrophages and endothelial cells (Figure 3, left), whereas in atherosclerotic arteries it is quite intense in the area of the plaque rich in macrophages (Figure 3, right). p22phox staining is, however, also detected in other areas of the vessel wall, suggesting that in these cells p22phox might associate with another gp91phox homologue.

Correlation of NAD(P)H Oxidase Subunit mRNA Expression With Severity of Atherosclerotic Lesions

To approach the relation between oxidase subunits and the severity of atherosclerotic lesions in a more quantitative way, we measured mRNA expression in segments of human coronary arteries that were also evaluated for lesion severity according to AHA classification guidelines.10 As shown in

---

**TABLE 2. PCR Primers**

<table>
<thead>
<tr>
<th>Human Gene</th>
<th>Primer sequence 5’ to 3’</th>
<th>Genebank Identification Number</th>
</tr>
</thead>
<tbody>
<tr>
<td>gp91phox</td>
<td>+1: GTCACACCTTCCGATCCATTGCTGTCAGTCTAGT</td>
<td>Gl: 6996020</td>
</tr>
<tr>
<td></td>
<td>−2: CGTACACCTTCCGATCCATTGCTGTCAGTCTAGT</td>
<td></td>
</tr>
<tr>
<td>p22phox</td>
<td>+1: AACGACGGAGCGTGGCTGTCGCCAAGCAGAG</td>
<td>Gl: 4557504</td>
</tr>
<tr>
<td></td>
<td>−2: GCTGGGCTGATGGGCTGTCACCTACT</td>
<td></td>
</tr>
<tr>
<td>nox4</td>
<td>+1: CTGAGGAGCTGCTGCGCCTGGCAACAGAG</td>
<td>Gl: 7738703</td>
</tr>
<tr>
<td></td>
<td>−2: TGAATCATGAGGATTAGCAGCCACACCATGAGCAG</td>
<td></td>
</tr>
<tr>
<td>nox1</td>
<td>+1: TTCACCAATTCCAGGATGAACTGGATGTC</td>
<td>Gl: 6138993</td>
</tr>
<tr>
<td></td>
<td>−2: CAGCTGACAGATGCACTGGCGTCAATGA</td>
<td></td>
</tr>
<tr>
<td>M-CSF-1 (c-fms)</td>
<td>+1: CATTCGACAGTGGCATTGCCCCACACATC</td>
<td>Gl: 29899</td>
</tr>
<tr>
<td></td>
<td>−2: AAACAGCCAAGCAGAGACCAAACGAGG</td>
<td></td>
</tr>
<tr>
<td>Smooth muscle α-actin</td>
<td>+1: GCAGCCAGCCAAGCAGACTGTCAGAAT</td>
<td>Gl: 4501882</td>
</tr>
<tr>
<td></td>
<td>−2: AGGCAGAGCCATTGCTCACACACCAAGG</td>
<td></td>
</tr>
</tbody>
</table>
expression of both gp91phox and p22phox increases with increasing severity of atherosclerosis. Between stages I and IV, gp91phox mRNA increases 3.9±0.5-fold, and by stage VI, levels are 8.6±0.3-fold higher than in stage I. p22phox mRNA increases by 2.7±0.7-fold at stage IV and 4.5±1.3-fold by stage VI. We hypothesized that this increase in gp91phox was due to increased infiltration of macrophages in more severe lesions. To estimate macrophage content in these samples, we measured MCSFR-1 mRNA. As shown in Figure 4C, there was a strong linear correlation between gp91phox and MCSFR-1 mRNA (r²=0.61, P<0.0005), supporting the concept that the increase in gp91phox is largely due to the increased macrophage content of the arteries. Figure 5A shows the relation of nox4 mRNA to lesion severity. nox4 is highest in stage IV atherosclerosis (2.3±0.9-fold increase), and, as opposed to gp91phox, is dramatically decreased in the most complicated plaques (12.8% of stage IV levels). We hypothesized that this expression pattern might be due to the relative proportion of SMCs at different TABLE 3. Expression of Nox Isoforms in Cell Types

<table>
<thead>
<tr>
<th>Cell Type</th>
<th>gp91phox (×10⁵/10⁹ copies 18S)</th>
<th>nox4 (×10⁵/10⁹ copies 18S)</th>
<th>nox1 (×10⁵/10⁹ copies 18S)</th>
<th>p22phox (×10⁵/10⁹ copies 18S)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Monocyte</td>
<td>6140.0</td>
<td>undetectable</td>
<td>0.10</td>
<td>447.0</td>
</tr>
<tr>
<td>Endothelial cells</td>
<td>13.2</td>
<td>270.0</td>
<td>0.87</td>
<td>45.8</td>
</tr>
<tr>
<td>Smooth muscle cells</td>
<td>0.19</td>
<td>2.15</td>
<td>0.22</td>
<td>81.6</td>
</tr>
<tr>
<td>Fibroblasts</td>
<td>0.58</td>
<td>6.25</td>
<td>0.45</td>
<td>10.9</td>
</tr>
</tbody>
</table>

RNA was harvested from the indicated cultured human cells, reverse-transcribed, and quantified by real-time PCR. Values are expressed as copy numbers per 10⁹ copies of 18S measured in the same sample and are representative of 3 experiments.
stages of atherogenesis. Thus, we examined the relation between smooth muscle–specific α-actin content and nox4 mRNA. Indeed, α-actin content varied with lesion severity in a manner similar to nox4 (Figure 5B). Furthermore, there was a significant correlation between nox4 and α-actin mRNAs in these vessels (r²=0.59, P<0.0005, Figure 5C).

Discussion

In the present study, we investigated the spatial localization of O₂⁻ production and NAD(P)H oxidase expression in both nonatherosclerotic and atherosclerotic human coronary arteries. We provide strong evidence that cell type–specific expression of gp91phox, p22phox, and the novel Nox proteins in human coronary arteries correlates with the severity of atherosclerosis. The enhanced expression of gp91phox and p22phox in atherosclerotic arteries is associated with macrophage infiltration in complicated lesions. In contrast, Nox4 expression correlates with the proportion of smooth muscle–specific α-actin–positive cells in the lesions. These results clearly demonstrate a link between human coronary disease, superoxide, and NAD(P)H oxidase expression and provide insight into the cellular and molecular basis for oxidative stress in atherosclerosis.

Recent work showed an association between risk factors for coronary artery disease and NAD(P)H oxidase-dependent production of ROS in human internal mammary arteries and saphenous veins. These investigators used lucigenin to detect O₂⁻, which provides quantitative data but does not give information about the cellular source. The present study extends these observations to human coronary arteries and establishes that virtually all cell types in the vessel wall produce intracellular O₂⁻. Both nonatherosclerotic and atherosclerotic arteries show clear DHE staining throughout the intima, media, and adventitia, suggesting that all vascular cells continually produce O₂⁻ during normal metabolic function. The observed differences in O₂⁻ production with atherosclerosis most likely are underestimated because arterio-

![Figure 3. Colocalization of p22phox with gp91phox and cell-type–specific markers. Human coronary arteries were harvested and costained for p22phox (green) and endothelial cells (EC) with von Willebrand factor (red); SMCs and myofibroblasts with α-actin (red), and macrophages with CD68 (red) and gp91phox (red). Colocalization is indicated by yellow staining and is particularly evident in insets. Left, Sections of nonatherosclerotic coronary arteries; Right, sections of atherosclerotic coronary arteries.](http://circ.ahajournals.org/)

![Figure 4. Correlation of gp91phox and p22phox mRNA with lesion severity. Coronary artery segments were classified for lesion severity according to AHA guidelines, and RNA was extracted. gp91phox (A) and p22phox (B) mRNAs were measured by quantitative real-time PCR. Numbers of segments of each type of lesion severity are indicated in parentheses. *P<0.01. C, Correlation between gp91phox mRNA and MCSFR-1 mRNA in 40 coronary segments. MCSFR-1 is a measure of macrophage content in the lesion.](http://circ.ahajournals.org/)
ies from explanted hearts are necessarily exposed to complex mechanical and biochemical stresses that may increase basal O$_2$ formation.

In atherosclerotic arteries, however, the most intense O$_2$ staining is apparent in the central inflammatory area of the plaque core (Figure 1B), which is vulnerable to plaque rupture. We also observed that O$_2$ production is low in stable, collagen-rich, macrophage-deficient plaques (unpublished observations). Taken together, these observations support the concept that O$_2$ production in atherosclerotic coronary arteries correlates with the biological activity of the plaque.

Similar to Azumi et al, we found p22phox expression throughout the vessel wall and in the plaque. p22phox staining correlates well with O$_2$ production in terms of the overall pattern and intensity of staining. In contrast, distribution of the gp91phox homologues is more limited. In nonatherosclerotic arteries, gp91phox expression is highest in the plaque core, where macrophages are abundant and O$_2$ staining is most intense. Once again, the pattern of Nox4 staining is complementary and is found in the areas where gp91phox is absent. Correlation of gp91phox and Nox4 with specific cellular markers (MCSFR-1 for macrophages and smooth muscle α-actin for SMCs and myofibroblasts) confirms their differential expression by macrophages and SMCs, respectively (Figures 4 and 5), consistent with in vitro data. Quantitative mRNA analysis showed that gp91phox expression in monocytes is ~500-fold higher than in any of the nonphagocytic cells, whereas nox4 is not present in monocytes (Table 3). In contrast, nox4 mRNA is 10- to 20-fold higher than that of gp91phox in other vascular cells. These data raise the possibility that novel Nox homologues such as Nox4 serve as a catalytic NAD(P)H oxidase subunit in nonphagocytic cells. In cells that express more than one gp91phox homologue, it is possible that each Nox protein serves a specific biological function.

The apparent lack of gp91phox in the SMC-rich medial layer is consistent with our previous findings in cultured cells and the report of Souza et al, who showed that SMCs from gp91phox-/- mice have similar NAD(P)H oxidase activity as cells from wild-type mice. Furthermore, we have previously shown that gp91phox staining of medial SMCs in rat carotid artery is minimal but is upregulated in myofibroblast-like cells after balloon injury. Götlich et al reported that gp91phox was expressed in endothelial cells and not SMCs but were unable to detect Nox1 in the endothelium. The present data suggest that this may be because Nox1 is only expressed at very low levels in these cells. In contrast, Wang et al were able to detect gp91phox staining in the aortic media of mice infused with angiotensin II, which may be a consequence of this experimental model.

A correlation between p22phox and severity of atherosclerotic lesions was suggested by Azumi et al, who showed that p22phox is more abundant in advanced atherosclerotic plaques than in nonatherosclerotic arteries. The present study confirms and extends this observation to show differential correlation of lesion severity with the catalytic subunits gp91phox and Nox4. The strong correlation of gp91phox with the evolution of atherosclerosis (Figure 4) suggests a possible causal link between the classic NAD(P)H oxidase and the development of lesions. This is in apparent contrast to studies in apoE-/- mice that lack gp91phox, which showed no improvement in lesion area when compared with apoE-/- mice expressing gp91phox. However, in that study, only lesions in the ascending aorta were quantified, which could underestimate the differences in the extent of atherosclerosis. Two other studies used animals in which the regulatory subunit of NAD(P)H oxidases, p47phox, was genetically disrupted in apoE-/- mice. Although lesion formation in the ascending aorta was not affected in either study, Barry-Lane et al found that lesion area in the descending aorta was reduced by 75%.

In our study, the contribution of gp91phox to lesion progression is likely to be due almost entirely to macrophage...
accumulation, because expression of this subunit correlated with a macrophage marker. In contrast, the nonphagocytic homologue Nox4 is upregulated mainly during the atheroma stage of the plaque (Figure 5), which is known to contain an abundance of α-actin–positive cells, and is downregulated in more advanced stages of atherosclerosis that are characterized by fibrosis and a reduction in intimal SMCs. The functional significance of altered expression of these NAD(P)H oxidases was confirmed by the findings of Guzik et al,8 who showed a clear association between NAD(P)H oxidase activity, atherosclerotic risk factors, and endothelial dysfunction.

Perhaps the most important clinical evidence to date for the role of vascular oxidative stress in human atherosclerosis was revealed by a recent study designed to determine whether oxidative stress has prognostic significance for cardiovascular morbidity and mortality. Heitzer et al23 found that cardio-oxidative stress has prognostic significance for cardiovascular events likely to develop in those patients whose vascular oxidative stress was revealed by a recent study designed to determine whether NAD(P)H oxidase activation and human atherosclerosis were associated with histopathological correlation. Circulation. 2002;97:1494–1498.

Acknowledgments
This work was supported by National Institutes of Health grants HL-38206, HL-58863, HL-58000, and HL-66575 and an AHA Fellowship to Dr D. Sorensen. We thank Dr Margaret Talpey for a generous gift of liposomal SOD, Drs Yoshihiro Taniyama and Piwe Seshiah for critical reading of the manuscript, Dr Lula Hileński for help with confocal microscopy, and Marcie Burnham for excellent secretarial assistance.

References
Superoxide Production and Expression of Nox Family Proteins in Human Atherosclerosis

_Circulation_. 2002;105:1429-1435; originally published online March 4, 2002; doi: 10.1161/01.CIR.0000012917.74432.66
_Circulation_ is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2002 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7322. Online ISSN: 1524-4539

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circ.ahajournals.org/content/105/12/1429

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in _Circulation_ can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to _Circulation_ is online at:
http://circ.ahajournals.org//subscriptions/