Cytochrome $b_{558}$–Dependent NAD(P)H Oxidase–Phox Units in Smooth Muscle and Macrophages of Atherosclerotic Lesions

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**Objective**—Despite studies implicating superoxide anion–producing oxidases in atherosclerosis, their characteristics, expression, and regulation in cells of lesions are poorly understood. We examined the following: (1) whether cytochrome $b_{558}$–dependent NAD(P)H oxidase–phox peptides are expressed by intimal smooth muscle cells (iSMCs) and macrophages of human aortic atherosclerotic lesions and their regulation and (2) whether cytochrome $b_{558}$–dependent NAD(P)H oxidase represents a major NAD(P)H oxidase in iSMCs.

**Methods and Results**—Using a combination of immunochemical and reverse transcription–polymerase chain reaction procedures, we demonstrate that p22 phox and gp91 phox (cytochrome $b_{558}$) expression in normal intima was restricted to a quarter of the iSMCs. In fatty streaks, a similar fraction of iSMCs expressed cytochrome $b_{558}$, whereas macrophages also expressed low levels of p47 phox and p67 phox. In fibrofatty lesions, the majority of iSMCs expressed the cytochrome $b_{558}$ subunits; p67 phox was also detected. Macrophages and macrophage-derived foam cells expressed the 4 phox subunits that constitute superoxide-producing cytochrome $b_{558}$–dependent NAD(P)H oxidase. These were upregulated by transforming growth factor-β1 and interferon-γ. Aortic lesions also expressed Thox1 and Nox4, and although their expression also increases with lesion severity, their expression is less frequent than that of gp91 phox.

**Conclusions**—In human aortic fibrofatty lesions, a cytochrome $b_{558}$–dependent NAD(P)H oxidase appears to be a major iSMC and macrophage oxidase whose expression is upregulated by cytokines. (Arterioscler Thromb Vasc Biol. 2002;22:2037-2043.)

**Key Words:** atherosclerosis • gp91 phox • Thox1 • Nox4 • cytokines

**Reactive oxygen species (ROS),** such as superoxide anions and hydrogen peroxide (H$_2$O$_2$), have been implicated in the development and progression of human atherosclerotic lesions.1 Their effects range from reducing vascular smooth muscle cell (SMC) survival2 to oxidizing LDLs.3 Oxidized LDLs can stimulate macrophages to secrete metalloproteases, which together with SMC apoptosis can promote lesion instability.2,4 Intracellular ROS modulate gene transcription via nuclear factor-kB and redox-sensitive pathways, increasing monocyte and neutrophil adhesion via elevations in adhesion receptor expression.5

Despite substantial knowledge of the potential in vitro consequences of high ROS production, little is known about the characteristics or regulation of the oxidases responsible for their generation in human atherosclerotic lesions. Pharmacological studies in human internal mammary arteries indicate that yet-to-be-defined NAD(P)H oxidases are an important source of superoxide anion.6 Azumi et al7 have demonstrated large elevations in the expression of p22 phox within severe human coronary lesions. This phox peptide is known to complex with gp91 phox to form cytochrome $b_{558}$, a key component of phagocytic NAD(P)H oxidase; p22 phox was highly expressed by endothelial cells, vascular SMCs, and macrophages associated with complex coronary atherosclerotic lesions, with little or no expression by SMCs of normal coronary arteries.7 However, although gp91 phox can be highly expressed by macrophages8 and endothelial cells,9 studies in cultured vascular SMCs from arteries of experimental animals indicate that homologues of gp91 phox are mostly likely to be expressed by vascular SMCs.10 Nox4 and Nox1 are the major gp91 phox homologues expressed by cultured rat aortic SMCs, with gp91 phox (Nox2) mRNA expression barely detectable.10 This led to the proposal that gp91 phox expression in vascular SMCs may be restricted to a very minor phenotype.10

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In the present study, we investigated the extent to which intimal SMCs (iSMCs) and macrophages in human aortic fibrofatty lesions express cytochrome b$_{558}$-dependent NAD(P)H oxidases, characterized by the presence of phox peptides, including gp91phox, and we also investigated the ability of interferon (IFN)-γ and transforming growth factor (TGF)-β1, 2 cytokines present in atherosclerotic lesions, to regulate their expression. The present study indicates that populations of iSMCs within atherosclerotic lesions express ≥1 NAD(P)H oxidase that is dependent on either gp91phox, Nox4, or Thox1 (the latter is a novel multidomain oxidase/peroxidase not previously reported in SMCs). Nox4 and Thox1 expression by iSMCs within fibrofatty lesions is more restricted than that of gp91phox. Macrophages and the majority of iSMCs in human aortic fibrofatty lesions express gp91phox and other key phox components that constitute superoxide-producing cytochrome b$_{558}$-dependent NAD(P)H oxidase. The phox components of this oxidase are upregulated by IFN-γ and TGF-β1 in both cell types.

**Methods**

Please refer to http://atvb.ahajournals.org for online Methods supplement.

**Aortic Tissues**

Thoracic and abdominal aortas were collected during autopsy, not later than 6 hours after death, from 37 male and female individuals, aged 16 to 67 years, at the Russian Cardiology Research and Industrial Complex, Moscow. The causes of death included skull-brain traumas, traffic accidents, acute hemorrhages, and (nonhemorhagic) strokes but not infections. The collection of tissues for the study was approved by the Human Ethics Experimentation Committee of the Russian Cardiology Research and Industrial Complex. Aortic segments were either frozen in OCT (Miles Inc) and stored at −80°C or dissected to remove the intima from the endothelial cells. The intimal segments were used for iSMC isolation (see below). Atherosclerotic lesions were classified as previously described by us. Nine aortas (from subjects aged 23 to 38 years) appeared normal, possessing an SMC-rich intima and extracellular matrix; 14 aortas (from subjects aged 29 to 42 years) contained fatty streaks, and 13 (from subjects aged 43 to 67 years) contained fibrofatty lesions.

**Immunohistochemistry**

**Antibodies**

Antibodies to p22phox and gp91phox were monoclonal IgGs. The antibodies to p47phox (No. P33720) and p67phox (No. P69820) were from Transduction Laboratories; CD-68 monoclonal and smooth muscle α-actin antibodies were from DAKO Corp. Secondary biotinylated horse anti-mouse antibodies, streptavidin-peroxidase, streptavidin-alkaline phosphatase conjugates, and the visualization kit for alkaline phosphatase (AP) were from Vector Laboratories. 3,3’-Diaminobenzidine tetrachloride (DAB) was from Sigma Chemical Co.

**Immunohistochemistry and Western Blotting**

The expressions of p22phox, gp91phox, p47phox, and p67phox were examined in aortic cross sections, essentially as previously described. The sections were incubated (1 hour) with p22phox (1:200), gp91phox (1:300), p47phox (1:75), or p67phox (1:75) antibodies in 10% normal horse serum in PBS (NHS/PBS). Antigens were visualized by using DAB solution containing 0.5% NiCl$_2$. To simultaneously detect macrophages, the sections were incubated with anti-CD-68 (1:800 NHS/PBS, 1 hour). Immunoreactive peptides were visualized as a red color by using the AP ABC red visualization kit. Nuclei were counterstained with hematoxylin (∼15 seconds). The percentage of CD68$^+$ and CD68$^-$ cells expressing the phox peptides was determined after the counting of these and the total number of cells within the intimal/lesion of each cross section by 2 independent histologists.

**Monocyte/Macrophage and Aortic iSMC Cultures**

The promonocyte (THP-1) human cell line (American Cell Type Collection) was cultured in RPMI-1640 medium containing 10% FCS and β-mercaptoethanol. Differentiation to macrophages was induced by 4-day exposure to either TGF-β1 (6 ng/mL), IFN-γ (150 U/mL), or phorbol myristate acetate (PMA, 1 μmol/L). Human aortic iSMCs were isolated by 0.25% collagenase (Sigma, C6885) digestion and cultured in 15% FCS/DMEM for 1 week before culturing for 48 hours in serum-free DMEM. They were then exposed to either TGF-β1 (6 ng/mL) or IFN-γ (150 U/mL) for 24 hours.

**mRNA Expression in Cultured Cells and Aortic Intima**

mRNAs encoding different phox peptides and homologues of gp91phox in cultured monocytes/macrophages and aortic iSMCs were assessed by using reverse transcription (RT)-polymerase chain reaction (PCR) and DNase-treated RNA. Oligonucleotide primer pairs for RT-PCR were designed by using previous defined criteria and purchased from Generwoks. In situ RT-PCR was performed on frozen sections as previously described elsewhere with the use of a GeneAmp EZTth RNA PCR kit (Roche). Conditions of RT and PCR cycles were the same as for RT-PCR in solution. Amplified PCR products were visualized by using a digoxigenin detection kit (Roche) and developed a dark purple-black color. Negative controls included sections taken through the digestion reactions without oligonucleotide primer pairs. Nuclei were weakly counterstained with hematoxylin (10 minutes).

Amplifications of mRNA extracted from cultured cells were optimized so that the amount of PCR product reflected mRNA levels. RT-PCR was performed by using a 1-step procedure (SuperScript RT-PCR System, Life Technologies). PCR products were electrophoresed on 2% agarose gels at 120 mV (Bio-Rad), together with HaeIII-digested dX174 DNA size markers (Promega). Cycle sequencing of gel-purified PCR products confirmed the specificity of the amplifications.

**Statistical Analyses**

Results are expressed as mean±SEM. Differences between lesions and/or phox peptides were assessed by using either unpaired t tests or, for multiple comparisons, 1-way ANOVA, followed by a post hoc t test. Results were considered statistically significant if P<0.05.

**Results**

**Expression of p22phox**

In nonatherosclerotic aortic intima, the expression of p22phox by iSMCs was low, with 24.7±1.1% expressing immunoreactive p22phox (Figure 1A). SMCs within the media did not express detectable levels of p22phox (Figure 1A). Aortic fatty streaks contained higher levels of p22phox, mostly localized in macrophages (Figure 1B). Up to 80% of the CD-68$^+$ positive macrophages expressed p22phox.
quency of p22phox expression by iSMCs was similar to that in normal intima (25.3 ± 1.5%, P > 0.05; Figure 1A). The highest p22phox expression was in fibrofatty lesions, where 66.1 ± 2.1% of the iSMCs expressed the peptide (P < 0.05 from fatty streaks) as well as all macrophages, including macrophage-derived foam cells (Figure 1C and 1D); medial SMCs associated with this lesion did not express p22phox (Figure 1E).

Expression of gp91phox, Thox1, and Nox
In nonatherosclerotic aortic intima, gp91phox expression was restricted to 28.4 ± 1.4% of the iSMCs (Figure 2A), similar to p22phox expression (Figure 1); medial SMCs did not express gp91phox (Figure 2A). In fatty streaks, macrophages and macrophage-derived foam cells expressed high amounts of gp91phox (Figure 2B), as did 46.6 ± 2.2% of the iSMCs. In fibrofatty lesions, nearly all macrophages and macrophage-derived foam cells and 67.6 ± 1.9% of the iSMCs expressed gp91phox (P < 0.05 from fatty streaks), similar to the frequency of p22phox expression (P for difference > 0.05, Figure 2C and 2D). Medial SMCs underlying the fibrofatty lesions did not express gp91phox (Figure 2E).

In contrast to gp91phox, Nox4 and Thox1 are expressed in aortic media and either not at all or rarely in unaffected intima (please see online Figure I, available at http://www.ahajournals.org). When expressed by the SMCs, mRNAs encoding Nox4 and Thox1 exhibit a cytoplasmic and perinuclear distribution, similar to endothelin mRNA. Nox4 mRNA is also expressed in fatty streaks, fibrofatty lesions, and underlying media. In these regions, Thox1 mRNA is expressed in a somewhat similar pattern. In both instances, expression of these gp91phox homologues is more restricted than that of gp91phox. As with gp91phox, expression of Thox1 and Nox4 mRNA by cells was greatest in fibrofatty lesions. Solution RT-PCR confirmed Thox1 and Nox4 expression in the fibrofatty lesions and Thox1 expression by iSMCs (see online Figure I). Monocytes (THP-1) did not express detectable Thox1 mRNA (not shown). Thus, within aortic fibrofatty lesions, gp91phox compared with its homologues (Thox1, Nox4, and Nox1) is the most abundant and is expressed by the majority of iSMCs and all macrophages.

Cell Type–Specific p47phox and p67phox Expression in Fibrofatty Lesions
Macrophages and macrophage-derived foam cells within the fibrofatty lesions expressed high levels of p47phox and p67phox (Figure 3A, 3B, 3D, and 3E; yellow arrows), contrasting with low expression by macrophages in fatty streaks (Figure 3C and 3F). In the fibrofatty lesions, some iSMCs express low levels of p67phox (P < 0.05 from fatty streaks), similar to the frequency of p22phox expression (P for difference > 0.05, Figure 2C and 2D). Medial SMCs underlying the fibrofatty lesions did not express p47phox (Figure 2E).

Regulation of Phox Subunits in Monocytes/Macrophages by TGF-β1 and IFN-γ
Because TGF-β1 and IFN-γ, which are associated with cells within vessels, have been implicated in the pathogenesis of atherosclerosis,11,12 because they can induce monocyte/macrophage differentiation,21,22 and because they can possibly alter the expression of NAD(P)H oxidase subunits,23 we next examined, by using the human THP-1 monocytes, the possi-
bility that these cytokines might elevate the expression of the phox peptides.

Four-day exposure of the monocytes to TGF-β1 or IFN-γ resulted in large elevations in all 4 phox peptides (Figure 4). PMA, a stimulator of monocyte/macrophage differentiation, also elevated these peptides, but to a lesser extent. TGF-β1, IFN-γ, and PMA also elevated the levels of mRNAs encoding gp91 phox and p67 phox (please see online Figure II, available at http://www.ahajournals.org), implicating transcriptional activation in their regulation. The inability of these agents to increase mRNAs encoding p22 phox and p47 phox suggests that posttranscriptional mechanisms increase their peptide levels. There were, at most, only small changes in thymosin-β10 mRNA levels, but L7 mRNA levels were reduced, consistent with monocyte to macrophage differentiation.

Regulation of Phox Subunits in iSMCs

Because TGF-β1 and IFN-γ elevated the 4 key phox peptides critical for cytochrome b558-dependent NAD(P)H oxidase activity in macrophages, we investigated whether these 2 cytokines might also influence the expression of phox peptides in the human aortic iSMCs, particularly gp91 phox and p67 phox. TGF-β1 has been reported to elevate p22 phox and superoxide anion generation in human aortic SMCs, whereas p47 phox is upregulated by thrombin. TGF-β1 and IFN-γ increased the expression of gp91 phox, the 91-kDa form that is known to be extensively glycosylated: the increase with IFN-γ was ~2-fold (Figure 5). The expression of p67 phox peptide was elevated to a similar extent by IFN-γ; TGF-β1 was less effective (please see online Figure III, available at http://www.ahajournals.org).

Discussion

Our findings indicate for the first time that iSMCs and macrophages express the phox peptides that constitute cytochrome b558-dependent NAD(P)H oxidase in human aortic atherosclerotic lesions, with expression increasing with lesion severity. The high expression of gp91 phox relative to its homologues in fibrofatty lesions (Thox1, Nox4, and Nox1) indicates that cytochrome b558-dependent NAD(P)H oxidase is a major contributor to ROS generation. TGF-β1 and IFN-γ upregulate its expression in iSMCs and macrophages. The expression in aortic lesions of Thox1, a novel multidomain oxidase with an ectoperoxidase domain, which cannot substitute for gp91 phox in phagocytic NAD(P)H oxidase, and the expression of Nox4 provide evidence for the presence of additional unique NAD(P)H oxidases in iSMCs that can also contribute to ROS generation.

It has been proposed that Nox4 and not gp91 phox is the major gp91 phox homologue expressed by SMCs in atherosclerotic lesions. These investigators used cultured coronary artery SMC lines, colocalization with p22 phox, and correlations with SMC and macrophage markers to assess the expression of gp91 phox homologues by SMCs within lesions. Although the present study also indicates that Nox4 is expressed by SMCs within aortic media and in atherosclerotic lesions, gp91 phox appears to be the major homologue expressed by the iSMCs in aortic lesions. The expression of gp91 phox by iSMCs is apparent only in regions containing fatty streaks and fibrofatty lesions, suggesting that specific cytokines/growth factors regulate its expression. Our findings suggest that TGF-β1 and IFN-γ contribute to this elevated expression.
Why iSMCs within atherosclerotic lesions express multiple gp91 phox homologues is not immediately apparent. Predictions of their locations within cells, based on the amino acids that encode protein-sorting signals and localization sites (PSORT Program, available at http://psort.ims.u-tokyo.ac.jp/), localize Nox4 predominantly to the endoplasmic reticulum and Thox1 primarily to the plasma membrane. On the basis of these predicted locations, it is tempting to speculate that ROS produced by a Nox4-dependent NAD(P)H oxidase may primarily modulate intracellular cell signaling processes, whereas Thox1 contributes more to ROS in the extracellular environment. In Caenorhabditis elegans, Thox1 catalyzes the cross-linking of tyrosine residues that stabilize its cuticular extracellular matrix. It is probable that the Thox1-dependent NAD(P)H oxidase within human atherosclerotic lesions also catalyzes the cross-linking of tyrosine to dityrosines during LDL oxidation with tyrosyl radicals. Macrophage cytochrome b558-dependent NAD(P)H oxidase also participates in LDL oxidation.

The differentiation of monocytes to macrophages is a key inflammatory response in atherosclerotic lesions and is a potential mechanism that could contribute to the coordinate increase in phox peptides observed in aortic lesions. By use of THP-1 cells (a homogenous and well-characterized monocyte/human cell adapted to long-term culture), we have demonstrated that TGF-β, IFN-γ, or PMA, both potent stimulators/promoters of monocyte/macrophage differentiation, elevate all 4 NAD(P)H oxidase–phox peptides. We not only confirm that IFN-γ elevates gp91 phox and p67 phox during the differentiation of THP-1 cells to macrophages, but also determine the expression of p47 phox and p67 phox in human aortic fibrofatty lesions and fatty streaks. A and B, High frequency of p47 phox expression in macrophage/macrophage-derived foam cells (red stain, CD-68–expressing cells; yellow arrows) within fibrofatty lesions. C, Expression of p47 phox in fatty streaks (yellow arrows point to macrophages expressing CD68). D and E, Expression of p67 phox by macrophage/macrophage-derived foam cells (red stain, yellow arrows) and some iSMCs (green arrow) in fibrofatty lesions. F, p47 phox in fatty streaks (yellow arrows point to macrophages). Original magnification ×160 (A and D) and ×400 (B, C, E, and F).

Figure 3. Expression of p47 phox and p67 phox (black stain) in human aortic fibrofatty lesions and fatty streaks. A and B, High frequency of p47 phox expression in macrophage/macrophage-derived foam cells (red stain, CD-68–expressing cells; yellow arrows) within fibrofatty lesions. C, Expression of p47 phox in fatty streaks (yellow arrows point to macrophages expressing CD68). D and E, Expression of p67 phox by macrophage/macrophage-derived foam cells (red stain, yellow arrows) and some iSMCs (green arrow) in fibrofatty lesions. F, p47 phox in fatty streaks (yellow arrows point to macrophages). Original magnification ×160 (A and D) and ×400 (B, C, E, and F).

Figure 4. Regulation of NAD(P)H-phox peptide components in human THP-1 monocytes by cytokines. NAD(P)H oxidase-phox peptides (brown stains) are shown before (control) and after differentiation to macrophages stimulated by 4-day exposure to either TGF-β, IFN-γ, or PMA. Nuclei are counterstained with hematoxylin. Original magnification ×400.

Figure 5. Effects of TGF-β and IFN-γ on gp91 phox expression in cultured human aortic iSMCs. Cells were exposed to either TGF-β, or IFN-γ for 48 hours, and gp91 phox levels were determined by using Western blots. The minor band (≈65 kDa) and a major band (≈91 kDa) represent unglycosylated and glycosylated forms of gp91 phox. In THP-1 cells, gp91 phox is seen as a minor band of ≈91 kDa and also as higher molecular weight glycosylated forms.
monocyte differentiation via increased transcription, but we also demonstrate the importance of posttranscriptional mechanisms in elevating p22phox and p47phox peptides. Whether regional differences in macrophage p47phox and p67phox expression in fibrofatty lesions are due to the presence of additional “inhibitory” cytokines, such as interleukin-10, requires further study. Interleukin-10 is known to inhibit ROS production in IFN-γ-stimulated monocytes by reducing mRNA transcripts encoding p47phox and gp91phox. In cultured SMCs, TGF-β doubles p22phox mRNA expression and increases p22phox-dependent NAD(P)H oxidase ROS production. Our demonstration that TGF-β also induces increases in gp91phox and p67phox peptides suggests that the upregulation of multiple phox peptides contributes TGF-β-mediated increases in ROS production. IFN-γ also consistently elevated gp91phox and p67phox peptides in cultured iSMCs. Thus, both cytokines can contribute to the observed elevations in phox peptides in aortic atherosclerotic lesions via transcriptional and posttranscriptional mechanisms.

Although they are not specifically investigated, there is also the possibility that risk factors for atherosclerosis, such as hypertension, diabetes, and hyperlipidemia, might also contribute to the upregulation of NAD(P)H oxidase–phox peptides and/or the gp91phox homologues Thox1 and Nox4. Because developing and advanced fibrofatty lesions contain a rich milieu of cytokines capable of elevating NAD(P)H oxidase activities, any influence of risk factors might be most apparent during the early stages of lesion development in fatty streaks and more pronounced in endothelium than in iSMCs or macrophages. Increased superoxide production by diabetic arteries and veins has been attributed to NAD(P)H oxidase in endothelial cells and has been associated with increases in p22phox, gp91phox, and p67phox. Similarly, the increases in vascular ROS levels in experimental animals made hypertensive by the infusion of angiotensin II have been attributed to NAD(P)H oxidase in endothelial cells and adventitial fibroblasts. Experimental hypercholesterolemia also elevates superoxide production by endothelial cells. Further studies are required to determine whether these risk factors can contribute to the upregulation of NAD(P)H oxidases in iSMCs and macrophages of human aortic lesions.

A potential limitation of using postmortem material is the possibility of significant protein and mRNA degradation. We collected postmortem material within 6 hours of death, times during which protein and mRNA degradation are known to be minimal. However, we cannot exclude the possibility that some degradation limited our detection ability to detect p47phox in iSMCs, although its mRNA was readily detected. Despite the potential disadvantage of using even early postmortem material, the vessels collected from subjects provide novel insights into the differential expression of phox peptides as well as Nox and Thox1 mRNAs during human lesion progression. This procedure led to the identification in aortic iSMCs of a cytokine-regulated cytochrome b55–dependent NAD(P)H oxidase, whose expression increases with lesion severity, as well as the expected oxidase in macrophages, and the findings further support the hypothesis that p22phox/gp91phox-dependent NAD(P)H oxidases contribute to the progression of atherosclerosis in humans. Our ability to identify novel potential NAD(P)H oxidases in iSMCs of atherosclerotic lesions, based on gp91phox, Thox1, and Nox4 expression, suggests that their ROS products may be influencing specific signaling processes within iSMCs and LDL oxidation in the extracellular environment.

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