Increased NADPH Oxidase Activity, gp91phox Expression, and Endothelium-Dependent Vasorelaxation During Neointima Formation in Rabbits

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Abstract—Reactive oxygen species including superoxide and hydrogen peroxide are important mediators in atherogenesis. We investigated the enzymatic source of vascular superoxide and its role in endothelium-dependent vasorelaxation during neointima formation. Silastic collars positioned around carotid arteries of rabbits for 14 days induced neointimal thickening. Using lucigenin-enhanced chemiluminescence, superoxide production was detectable in collared artery sections, but not in controls, only after inactivation of endogenous Cu2+/Zn2+-superoxide dismutase (Cu2+/Zn2+-SOD) with diethyldithiocarbamate (DETCA). Dihydroethidium staining indicated that endothelium and adventitia were the major sites of superoxide generation. Superoxide production in DETCA-treated collared arteries was enhanced further by NADPH and was inhibited by diphenyleneiodonium, suggesting NADPH oxidase was the source of the radical in collared arteries. Moreover, real-time PCR demonstrated 11-fold higher expression of the gp91phox subunit of NADPH oxidase in collared arteries than in controls. In vascular reactivity studies, endothelium-dependent vasorelaxation to acetylcholine did not differ between collared and control sections. However, treatment with DETCA reduced relaxations to acetylcholine in collared rings, but not in controls. NADPH further reduced relaxations to acetylcholine in DETCA-treated collared sections, but not in controls. In DETCA/NADPH-treated collared rings, sensitivity to nitroprusside, in contrast to acetylcholine, exceeded that of controls. Moreover, further treatment of such rings with exogenous Cu2+/Zn2+-SOD restored acetylcholine relaxations without altering nitroprusside responses. Thus, early neointimal lesions induced by perivascular collars are associated with elevated gp91phox expression and increased NAPDH-oxidase–dependent superoxide production in endothelium and adventitia. However, endothelium-dependent vasorelaxation is largely preserved due to the actions of Cu2+/Zn2+-SOD and increased smooth muscle sensitivity to nitric oxide.

Key Words: NADPH oxidase • superoxide • gp91phox • endothelium-dependent vasorelaxation • collar-induced atherosclerosis

Reactive oxygen species (ROS) are important mediators in atherogenesis. Not only is superoxide production increased in atherosclerotic arteries from humans, rabbits, and mice, but ROS have also been shown to induce many proatherogenic cellular responses in vivo. These include inactivating endothelium-derived nitric oxide, inducing endothelial cell apoptosis, upregulating endothelial cell adhesion molecule expression, stimulating the proliferation and migration of vascular smooth muscle cells, and oxidatively modifying lipoproteins.

To our knowledge, only one study has attempted to characterize the oxidase responsible for superoxide production in atherosclerotic lesions. Warnholtz et al demonstrated that superoxide production in aortas from Watanabe heritable hyperlipidemic and cholesterol-fed rabbits was markedly higher than that in controls. The enzyme responsible for superoxide production in these atherosclerotic arteries was membrane-associated, stimulated by NADH and NADPH, and inhibited by the flavoprotein inhibitor, diphenyleneiodonium (DPI). Collectively, these observations provided evidence for the involvement of an NADPH oxidase, at least in these hypercholesterolemic models of atherosclerosis. NADPH oxidases are a family of enzymes that generate superoxide by transferring electrons from NADPH and/or NADH to molecular oxygen via flavins within their structure. NADPH oxidases are made up of at least 3 cytosolic protein subunits, p47phox, p67phox, and the G-protein, Rac, as well as a membrane-bound cytochrome reductase domain.

The cytochrome domain is a heterodimeric protein comprising a 22-kDa α-subunit called p22phox as well as a larger, flavin-containing β-subunit that is required for substrate binding and electron transfer. The nature of the β-subunit appears to depend on cell type. Thus, although gp91phox is associated with p22phox in neutrophils, endo-
thelial cells and adventitial fibroblasts, homologues of this subunit such as Nox1 or Nox4 may be important in vascular smooth muscle cells.\textsuperscript{11} It was recently found that expression of p22phox and gp91phox are elevated in human atherosclerotic lesions.\textsuperscript{12,13} Moreover, polymorphisms in the gene that encodes p22phox appear to be independently associated with increased superoxide production\textsuperscript{14} and elevated risk factors for atherosclerosis in some populations.\textsuperscript{15} Neointimal lesions displaying many characteristics of early stage human atherosclerosis can be rapidly induced in rabbits by periarterial collars.\textsuperscript{16–18} Such characteristics include infiltration of macrophages, accumulation of cholesterol esters, and deposition of collagen and fibronectin within the intima.\textsuperscript{16,17} In addition, the neointima contains smooth muscle cells that replicate in the media before migrating across the internal elastic lamina.\textsuperscript{17} Collar-induced lesions also display functional changes similar to those that occur in early human atherosclerosis including hypersensitivity to the vasoconstrictor action of 5-hydroxytryptamine (5-HT).\textsuperscript{17,18} Importantly, the endothelial layer remains morphologically intact, although functionally impaired.\textsuperscript{18,19} Thus, although relaxations to directly acting nitrovasodilators are preserved in collared arteries, some authors have reported impaired endothelium-dependent vasorelaxations to acetylcholine,\textsuperscript{17,20,21} whereas others found acetylcholine-induced relaxations were unaltered.\textsuperscript{22,23}

We set out to determine the enzymatic source of superoxide in arterial lesions induced by periarterial collars and to examine its effect on endothelium-dependent vasorelaxations. Our results provide the first evidence for a role for NADPH oxidase in superoxide production and the ensuing endothelial dysfunction in a non-hyperlipidemic model of atherosclerosis.

## Materials and Methods

### Animals

Male New Zealand White rabbits (3 to 4 kg) were maintained on a normal chow diet. All procedures were approved by the Institute’s Animal Ethics Committee (No. 99056).

### Collar Implantation

Animals were anesthetized with propofol (5 mg/kg, IV) followed by ketamine/xylazine (50/10 mg/kg, IM). Left and right common carotid arteries were exposed surgically and cleared of connective tissue along a 30-mm length. Hollow, nonocclusive silastic collars (length 20 mm; internal diameter along bore 4 mm; internal diameter at ends 1 mm) were then placed around each artery and held in place with a nylon sleeve. The space inside the collar was filled with sterile saline (0.9%). Muscle, fat, and skin layers were sutured, the wound dressed with antibiotic, and animals allowed to recover for 14 days.

### Tissue Harvesting

Rabbits were heparinized (1500 U, IV) before euthanasia with sodium pentobarbitone (90 mg/kg, IV). Carotid arteries were excised and placed in ice-cold Krebs-HEPES buffer (pH 7.4). Collars were removed and arteries cleaned of fat and connective tissue. Three ring segments (~3 mm) were cut from the area enclosed by the collar and from the sham-operated region of carotid artery lying upstream from the collar for use as within-animal controls. An additional ring segment (~1 mm) was taken from each region for morphological analysis.

### Measurement of Neointima Formation

Ring segments (1 mm) were fixed in 4% paraformaldehyde for 4 hours at 4°C, dehydrated in ethanol, and embedded in paraffin. Transverse sections (4 μm) were stained with hematoxylin and eosin. Using a Microcomputer Imaging Device (version 3.0, Imaging Research Inc), the cross-sectional areas of the intimal and medial regions were measured in micrometers squared and expressed as an intima-media ratio (IMR) to quantify neointima formation.

### Detection of Superoxide by Lucigenin-Enhanced Chemiluminescence

Superoxide production by control and collared carotid artery ring segments was measured using 5 μmol/L lucigenin-enhanced chemiluminescence as previously described.\textsuperscript{3}

### Localization of Superoxide by Dihydroethidium

Dihydroethidium (2 μmol/L) was used as previously described to detect superoxide in frozen sections (20 μm) of control and collared artery.\textsuperscript{2}

### RNA Preparation

Carotid artery ring segments were placed in a mortar, overlaid with liquid nitrogen, and pulverized. The resulting powder was transferred to a microcentrifuge tube containing 500 μL of RNAWiz (Ambion), and total RNA was extracted according to the manufacturer’s instructions. RNA concentrations were determined spectrophotometrically by measuring absorbance at 260 nm.

### Reverse Transcription (RT) Reaction

RNA (2 μg) was used for RT in a final reaction volume of 200 μL. The reaction mixture consisted of 1X Taqman RT buffer, 5.5 mmol/L MgCl\textsubscript{2}, 2.5 μmol/L random hexamers, 2 mmol/L deoxynucleoside triphosphates, 0.4 U/μL RNase inhibitor, and 1.25 U/μL MultiScribe Reverse Transcriptase (PE Biosystems). As a control for genomic DNA contamination in subsequent real-time PCR, 500 ng of each RNA sample was also added to parallel RT reaction mixtures that contained all of the above reagents except the Reverse Transcriptase. Reverse transcription was performed in a GeneAmp 9600 thermocycler (Perkin Elmer) using the following parameters: 10 minutes at 25°C, 30 minutes at 48°C, and 5 minutes at 95°C. Samples were stored at ~20°C.

### Real-Time PCR

Real-time PCR and the ∆∆Ct method were used as previously described to examine mRNA expression of the flavin-containing NADPH oxidase subunits gp91phox and Nox1. For Nox4, where a rabbit sequence has not been described, a region of high homology between the mouse and rat homologues of the gene was identified and used to design optimal primers, again with Primer Express software (PE Biosystems) and the published sequences for the rabbit homologues of each gene (Table). For Nox4, where a rabbit sequence has not been described, a region of high homology between the mouse and rat homologues of the gene was identified and used to design optimal primers, again with Primer Express (Table). Because probe binding in real-time PCR does not tolerate a single base mismatch, SYBR Green (PE Biosystems) was used in the Nox4 PCR mixture in place of a labeled probe. Ribosomal RNA (18s) was used as the internal standard for each reaction and was detected with commercially available rodent 18s primers and 5'-VIC–labeled probes (PE Biosystems; Table).

### Assessment of Endothelial Nitric Oxide Function

Carotid artery rings were suspended between 2 stainless steel wire hooks, one connected to an isometric force transducer (FT-03, Grass Instruments) and the other to an adjustable support. Preparations were immersed in water-jacketed organ baths containing Krebs-bicarbonate buffer (pH 7.4) bubbled with 95% O\textsubscript{2} and 5% CO\textsubscript{2} and maintained at 37°C. Circumferential force was measured with a Powerlab running Chart software (version 3.6.3; AD Instruments).

Tissues were equilibrated for 20 minutes under zero tension and a
Table 1: Optimized primer and probe sequences and concentrations used to detect gp91phox, Nox1, and Nox4 by real-time PCR. For gp91phox and Nox1, primer and probe sequences were designed with Primer Express software (PE Biosystems). For Nox4, primers were also designed with Primer Express; however, SYBR Green was used in place of a fluorescent probe.

<table>
<thead>
<tr>
<th>Gene (NCBI Acc.)</th>
<th>Primer Sequence (concentration in nmol/L)</th>
<th>Probe Sequence (concentration in nmol/L)</th>
<th>cDNA Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>gp91phox (AF323788)</td>
<td>Fwd: 5'-AGCGTCGCTAAGTCATTCAAGCT-3' (80)</td>
<td>Rev: 5'-CTGGACCCCTGAGAAGAGGAGA-3' (150)</td>
<td>5 ng</td>
</tr>
<tr>
<td>Nox1 (AJ271882)</td>
<td>Fwd: 5'-CACCTGTGTTAATCTGACGCTACA-3' (300)</td>
<td>Rev: 5'-AAGATTCCAGGGATATCA-3' (150)</td>
<td>50 ng</td>
</tr>
<tr>
<td>Nox4</td>
<td>Fwd: 5'-TGTTGGCCTAGTATGTGTTG-3' (300)</td>
<td>Rev: 5'-AAAAGGATGAGGCTGCAGTTG-3' (300)</td>
<td>Not applicable</td>
</tr>
<tr>
<td>18s</td>
<td>Fwd: 5'-CGGCATTACCATCAAGGAA-3' (80)</td>
<td>Rev: 5'-GCTGAATTACCGGCCGCT-3' (80)</td>
<td>10 ng</td>
</tr>
</tbody>
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Further 20 minutes under 2 g resting tension. Bath contents were then replaced with an isotonic, 125 mmol/L physiological salt solution (KPSS) to induce a maximum contraction (KPSSmax). After washing the tissues with fresh Krebs-bicarbonate buffer, cumulative concentration-response curves to 5-HT (0.1 to 10 μmol/L) were constructed to confirm that collared vessels had undergone functional changes characteristic of atherosclerosis. Tissues were again washed with fresh Krebs-bicarbonate buffer and treated with one or more of the following drugs: paratox (10 mmol/L); diethylidithiocarbamate (DETC, 3 mmol/L); NADPH (100 μmol/L); and Cu2+/Zn2+-superoxide dismutase (Cu2+/Zn2+-SOD, 600 U/mL). Note, when DETCA was used, the bathing solution was replaced with fresh Krebs-bicarbonate buffer and the appropriate drug treatment(s) replaced in the organ bath. Rings were then precontracted to 50% to 60% KPSSmax with titrated concentrations of 5-HT and relaxed with cumulatively increasing concentrations of acetylcholine (0.001 to 30 μmol/L). In some experiments, rings were again washed with fresh Krebs-bicarbonate buffer and the appropriate drug treatment(s) replaced in the organ bath. Rings were then precontracted with 5-HT and relaxed with cumulatively increasing concentrations of sodium nitroprusside (0.001 to 30 μmol/L). Contractile responses were expressed as change in force (g), and relaxations expressed as percent relaxation of 5-HT–induced precontraction. Nonlinear regression (Prism, version 3.0) was used to determine pEC50 and maximum responses (Rmax) to 5-HT, acetylcholine, and nitroprusside in each ring.

Data Analysis

All results are expressed as mean±standard error of the mean (SEM). Statistical comparisons were made by paired t tests or by 1- or 2-way repeated measures ANOVA with Bonferroni corrections where appropriate. A value of P<0.05 was considered significant.

An expanded Materials and Methods section can be found in the online data supplement available at http://www.circresaha.org.

Results

Induction of Atheroma-Like Lesions by Periarterial Collars

Carotid artery segments that had been collared for 14 days developed a substantial neointima (IMR 0.22±0.06 versus 0.01±0.01 in control arteries; n=39, P<0.05) while maintaining a morphologically intact endothelium. In contrast, paired control segments did not display any neointimal thickening (Figure 1).

Production of Superoxide in Collared Sections

Superoxide production could not be detected in untreated control or collared artery ring segments using lucigenin-enhanced chemiluminescence (n=4). However, after inactivation of endogenous Cu2+/Zn2+-SOD with DETCA, superoxide was detected in collared ring segments (27±9 counts/s per milligram; n=6) but not in control rings (Figure 2). Superoxide production was increased further in DETCA-treated collared rings by NADPH (156±32 counts/s per milligram; P<0.05 versus DETCA-treated alone; n=6; Figure 2). In contrast, although NADH increased superoxide in some DETCA-treated collared rings (70±24 counts/s per milligram; n=6), this effect did not reach statistical significance overall (Figure 2). Neither NADPH nor NADH had any significant effect on superoxide production in DETCA-treated control segments (n=6; Figure 2).

Cellular Localization of Superoxide in Collared Sections

Dihydroethidium staining of DETCA-treated control and collared segments of carotid artery revealed that superoxide production was primarily localized in the endothelial layer (n=4; Figure 3). Staining was also visible in the adventitial layer, particularly in collared arteries in a thin outer rim of cells, which were presumably invading inflammatory cells (n=4; Figure 3). Only low levels of superoxide were detected in the medial smooth muscle layer and this did not differ between control and collared sections (n=4; Figure 3). In adjacent sections of artery treated with the superoxide scavenger, tiron, fluorescence intensity, particularly in the endothelial and adventitial layers, was markedly reduced, confirming specificity for superoxide (n=4; Figure 3).

Characterization of Enzymatic Source of Superoxide

Superoxide production in DETCA-treated collared sections (83±33 counts/s per milligram) was reduced by the flavin antagonist and NADPH oxidase inhibitor, DPI (26±8 counts/s per milligram; n=5, P<0.05). 6-Aminonicotinamide, an inhibitor of pentose phosphate-dependent NADPH production, also appeared to attenuate superoxide production in unstimulated collared rings (27±29 counts/s per milligram; n=5), but this effect did not reach statistical significance (Figure 4).

Similar to the above, DPI inhibited NADPH-stimulated superoxide production in DETCA-treated collared rings (134±29 versus 45±23 counts/s per milligram in DPI-treated
rings; \( n=4, P<0.05 \). NADPH-stimulated superoxide production was also blocked by the SOD-mimetic, tiron (14±10 counts/s per milligram; \( n=4, P<0.05 \)), but not by L-NAME, an inhibitor of nitric oxide synthase, or allopurinol, an inhibitor of xanthine oxidase (Figure 4). None of the above treatments affected superoxide production in control segments (Figure 4).

**NADPH Oxidase Expression**

To determine whether increased NADPH oxidase activity in collared arteries was associated with upregulation of one or more NADPH oxidase isoforms, real-time PCR was used to measure mRNA expression of gp91phox, Nox1, and Nox4. In all cases, 18s was used as the internal standard. Gp91phox was detected \( \Delta C_t, 15.2 \pm 0.3 \) than in control samples (\( \Delta C_t, 18.5 \pm 0.1 \)), indicating that gp91phox is \( \sim 11 \)-fold more highly expressed in collared arteries (\( n=3, P<0.05 \); Figure 5). In contrast, Nox4 expression did not differ between control (\( \Delta C_t, 19.8 \pm 0.0 \)) and collared (\( \Delta C_t, 19.7 \pm 0.2 \)) arteries (Figure 5). We were unable to detect Nox1 message in either collared or control samples, even when 50 ng of cDNA template was used.

**Contractile Responses in Collared Sections**

In all experiments, rings were initially contracted with KPSS followed by cumulatively increasing concentrations of 5-HT to confirm that the collar had induced functional changes as previously observed.\(^{17,18}\) Maximal contractions to KPSS were slightly lower in collared segments (3.5±0.2 g; \( n=24 \) from 10 animals) than in controls (4.3±0.1 g; \( n=24 \) from 10 animals; \( P<0.05 \)). In contrast, both the sensitivity and maximum contractions to 5-HT were enhanced in collared rings (\( pEC_{50}, 7.3 \pm 0.1; R_{\text{max}}, 5.0 \pm 0.3 \) g) compared with controls (\( pEC_{50}, 6.4 \pm 0.1; R_{\text{max}}, 3.2 \pm 0.1 \) g; \( n=24 \) from 10 animals; \( P<0.05 \)).

**Endothelium-Dependent Vasorelaxations Associated With Increased Superoxide Levels**

Acetylcholine caused concentration-dependent vasorelaxations in precontracted control rings (\( pEC_{50}, 7.6 \pm 0.1; R_{\text{max}}, 95 \pm 1\% ; n=5 \); Figure 6) that were not different, in these studies, from those in collared rings (\( pEC_{50}, 7.4 \pm 0.2; R_{\text{max}}, 102 \pm 1\% ; n=5 \); Figure 6). However, although DETCA treatment had no effect on responses to acetylcholine in control segments, it caused a 6-fold reduction in sensitivity (\( pEC_{50}, 14 \pm 1.0; R_{\text{max}}, 66 \pm 2.8 \) g; \( n=20 \) segments; \( P<0.05 \); Figure 6).

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**Figure 1.** Transverse sections of rabbit carotid arteries showing a thickened neointima after 14 days with a periarterial collar. Control (A) and collared (B) segments of artery (4-μm sections) were stained with hematoxylin and eosin. Scale bar=100 μm. Arrows indicate the endothelium (E), internal elastic lamina (IEL), and external elastic lamina (EEL). Images are representative of sections from 39 rabbits.

**Figure 2.** Effect of NADH and NADPH on superoxide levels in control (open bars) and collared (filled bars) carotid artery rings. All rings were incubated with DETCA (3 mmol/L) and the appropriate drug treatment for 45 minutes before assay for superoxide with lucigenin. Values (mean±SEM from 6 experiments) are expressed as counts/s per milligram dry tissue weight. *\( P<0.05 \) vs DETCA-treated control rings; †\( P<0.05 \) vs DETCA-treated collared rings; ‡\( P<0.05 \) vs DETCA/NADH-treated collared rings.
6.8±0.2; n=5, P<0.05) without altering maximum relaxations (Rmax, 94±3%) to acetylcholine in collared rings (Figure 6). Additional treatment with NADPH attenuated further the maximum relaxations to acetylcholine in collared rings (Rmax, 83±7%; n=5, P<0.05) but still had no effect in control rings (Figure 6).

Like DETCA and NADPH, an exogenous superoxide-generating compound, paraquat (10 mmol/L), significantly reduced both sensitivity (pEC50, 6.0±0.1 versus 7.2±0.2 in controls; n=7, P<0.001) and maximum relaxations (Rmax, 73±9% versus 94±2% in controls; n=7, P<0.05) to acetylcholine in noncollared ring segments (Figure 6).

To confirm that the DETCA/NADPH-induced attenuation of acetylcholine responses in collared rings was due to increased superoxide, in a separate series of experiments, we examined the effects of exogenous Cu2+/Zn2+-SOD. In col-

Figure 3. Cellular localization of superoxide production in transverse sections of rabbit carotid artery. Control and collared sections of artery (20 μm) were treated with dihydroethidium (2 μmol/L) and DETCA (3 mmol/L) in the absence (Top) and presence (Bottom) of tiron (10 mmol/L) for 45 minutes before visualization under a confocal microscope (100× magnification). Scale bar=100 μm. Arrows indicate the endothelium (E), internal elastic lamina (IEL), and external elastic lamina (EEL). Each image is representative of 2 to 3 sections from 4 rabbits.

Figure 4. Effect of the NADPH oxidase inhibitor, DPI (5 μmol/L), an inhibitor of NADPH regeneration via the pentose phosphate pathway, 6-amino-nicotinamide (6-AN, 300 μmol/L) and the SOD mimetic, tiron (10 mmol/L), on basal (A) and NADPH-stimulated (B) superoxide generation in control (open bars) and collared (filled bars) carotid artery rings. C, Lack of effect of the nitric oxide synthase inhibitor, L-NAME (100 μmol/L), and the xanthine oxidase inhibitor, allopurinol (100 μmol/L), on NADPH-stimulated superoxide production in control (open bars) and collared (filled bars) ring segments. Note that all rings were incubated with DETCA (3 mmol/L) and the appropriate vehicle or drug treatment for 45 minutes before assay for superoxide with lucigenin. Values (mean±SEM from 4 to 6 experiments) are expressed as counts/s per milligram dry tissue weight. *P<0.05 vs DETCA-treated collared rings; †P<0.05 vs DETCA/NADPH-treated collared rings.
lared rings cotreated with DETCA and NADPH, acetylcholine caused concentration-dependent relaxations (pEC50, 6.7±0.1; Rmax, 77±10%) that were of lower sensitivity and Rmax than those obtained in similarly treated control rings (pEC50, 7.4±0.1; Rmax, 92±2%; n=6, P<0.05 for both values; Figure 7). However, additional treatment of DETCA/NADPH-treated collared rings with native Cu2+/Zn2+-SOD significantly increased both the pEC50 and Rmax to acetylcholine (pEC50, 7.3±0.1; Rmax, 95±4%) such that the response was not significantly different from that in control rings (Figure 7). Cu2+/Zn2+-SOD had no effect on relaxations to acetylcholine in DETCA/NADPH-treated control rings (Figure 7).

In marked contrast to the impaired responses to acetylcholine, relaxations to the nitric oxide donor, sodium nitroprusside, in DETCA/NADPH-treated collared rings (pEC50, 7.8±0.1; n=5) were located to the left of similarly treated control rings (pEC50, 7.4±0.1; n=5, P<0.05), although there was no difference in maxima. Unlike responses to acetylcholine, relaxations to nitroprusside in control and collared rings were not altered by exogenous Cu2+/Zn2+-SOD (Figure 7).

**Discussion**

The major finding in this study is that neointimal thickening induced by periartrial collars in rabbits is accompanied by increased superoxide production, most likely derived from upregulation of a gp91phox-containing NADPH oxidase in the endothelial and adventitial layers of the artery wall. Our conclusion that superoxide is elevated in collared arteries, and that this has functional consequences in terms of reduced bioavailability of nitric oxide, is supported by functional studies performed after inactivation of endogenous Cu2+/Zn2+-SOD by DETCA. This study thus exposes 2 mechanisms that compensate for increased superoxide production in the artery wall: endogenous Cu2+/Zn2+-SOD, which disposes of excess superoxide production, as well as a compensatory increase in the sensitivity of the underlying smooth muscle to nitric oxide. This is the first study to implicate a role for NADPH oxidase in elevated superoxide production in a non-hyperlipidemic model of early stage atherosclerosis.

To measure superoxide production in carotid artery segments, we utilized the chemiluminescent probe, lucigenin. Superoxide was undetectable in control arteries even after inactivating endogenous Cu2+/Zn2+-SOD with DETCA. In contrast, DETCA markedly increased superoxide production in collared arteries, suggesting that Cu2+/Zn2+-containing isoform(s) of SOD may play an important protective role in atherosclerotic arteries. This is despite the fact that total SOD activity in rabbit carotid arteries was reduced by 50% after 14 days with periartrial collars and activity of extracellular SOD (the most abundant Cu2+/Zn2+-containing isoform of SOD in vascular tissue) is known to be suppressed in arteries from patients with coronary artery disease. In the presence of SOD (albeit reduced activity) in atherosclerotic arteries, elevated superoxide may lead to increased generation of hydrogen peroxide. Hydrogen peroxide and its myeloperoxidase and Fenton-derived products, hypochlorite and hydroxyl radicals, could themselves promote atherogenesis via several mechanisms, including upregulation of endothelial cell adhesion molecule expression, vascular smooth muscle cell proliferation and migration, and oxidative modification of lipoproteins. However, the status of hydrogen peroxide–metabolizing pathways such as catalase and glutathione peroxidase in periartrial collar–induced neointima formation, or indeed in other models of atherosclerosis, remains unclear.

In addition to the quantitative chemiluminescence data, we also sought to localize superoxide production in DETCA-treated collared arteries. Staining of arterial sections with dihydroethidium clearly demonstrated that the endothelium was the primary source of superoxide in collared arteries with lower levels of staining evident in the outer layers of the adventitia. Because the endothelium is the major site for production of vasoprotective nitric oxide, generation of su-
peroxide in this location in the early stages of atherosclerosis has important implications for subsequent lesion development. Not only would superoxide react with and reduce the bioavailability and antiatherosclerotic actions of nitric oxide, but the product of this reaction, peroxynitrite, may itself directly contribute to atherogenesis. In addition to oxidizing lipoproteins, modifying protein function and inducing endothelial cell apoptosis, peroxynitrite may further compromise endothelial function in atherosclerosis by oxidizing tetrahydrobiopterin, an essential cofactor for nitric oxide synthase.3

To investigate the enzymatic source of superoxide in collared arteries we first excluded nitric oxide synthase and xanthine oxidase using the inhibitors L-NAME and allopurinol, respectively. We also demonstrated that the oxidase utilized exogenous NADPH in preference to NADH as an electron donor substrate and that oxidase activity was inhibited by the flavin antagonist and NADPH oxidase inhibitor, diphenyleneiodonium. Taken together, these results suggest that an NADPH oxidase is the most likely source of superoxide in these neointimal lesions. To determine the isoform(s) of NADPH oxidase involved, we examined expression of gp91phox, Nox1, and Nox4. Within the vascular wall, gp91phox is thought to be associated with endothelial isoforms of NADPH oxidase,27 whereas Nox1 appears to be involved in superoxide production by vascular smooth muscle cells.10,11 Nox4 is highly expressed in all vascular cell types but its role in superoxide production in these cells remains to be determined.11,13 Using real-time PCR, we have now demonstrated that expression of gp91phox is markedly increased in collared arteries compared with controls. In contrast, Nox1 expression was not detectable in either tissue and, although Nox4 was detectable in carotid arteries, its expression was not altered by the collar. These findings are in agreement with those from a recent study on human atherosclerotic arteries where it was shown that gp91phox expression is strongly correlated with lesion severity.13 In contrast, no such correlation was evident between Nox4 expression and disease stage.13

Sequence analysis of gp91phox family members across a number of species reveals the presence of a highly conserved canonical nucleotide binding sequence Gly-Xaa-Gly-Xaa-Xaa-Pro.28 This is followed by Phe or Tyr, which is characteristic of flavoproteins that bind NADPH in preference to NADH28 and is thus consistent with our findings that exog-

Figure 6. Effect of increased superoxide levels on endothelium-dependent relaxations to acetylcholine (ACh) in tissues precontracted to ∼50% KPSSmax with 5-HT. Segments of control (A) and collared (B) arteries were either left untreated (open circles) or were preincubated with DETCA alone (3 mmol/L; filled circles) or DETCA and NADPH (100 μmol/L; closed triangles) for 20 minutes before precontraction. In a separate set of experiments, control arteries from rabbits that had not undergone surgery (C) remained untreated (open circles) or were treated with paraquat (10 mmol/L; filled circles) for 20 minutes before precontraction. Values (mean±SEM from 5 to 7 experiments) are expressed as percent relaxation of the 5-HT-induced precontraction. *P<0.05 vs untreated collared rings; †P<0.05 vs DETCA-treated collared rings; ‡P<0.05 vs DETCA/NADPH-treated paired control rings; §P<0.05 vs untreated rings.
enous NADPH is more efficacious than NADH at driving superoxide production in collared arteries. To examine the role of endogenous NADPH in superoxide production, we used the pentose phosphate pathway inhibitor, 6-amino-nicotinamide. The pentose phosphate pathway is one of two major metabolic pathways responsible for maintaining NADP(H) in its reduced form in mammalian cells.29 Although 6-amino-nicotinamide appeared to reduce basal superoxide production in collared arteries (ie, '50% reduction), this effect just failed to reach statistical significance. This may be because even after inhibition of the pentose phosphate pathway, the intracellular concentration of NADPH is preserved by the other major pathway of NADPH regeneration, namely the malic enzyme-catalyzed conversion of malate to pyruvate.30 In support of this, Gupte et al31 recently showed that 6-amino-nicotamide only reduced intracellular NADPH levels by 30% to 35% in isolated bovine pulmonary arteries.

Increased production of oxyradicals has been shown to compromise acetylcholine-induced relaxations in many isolated blood vessels.4,32 Indeed, in the present study, we confirmed that increased superoxide production is capable of attenuating endothelial function in rabbit carotid arteries by demonstrating that paracut, a known generator of intracellular superoxide, reduced relaxations to acetylcholine. Surprisingly, in contrast to our earlier studies with a shorter period of neointima formation,18 relaxations to acetylcholine in collared arteries were not different from those in control arteries, despite the increase in NADPH oxidase activity in these arteries. Only after inactivation of endogenous Cu2+/Zn2+-containing isoforms of SOD that “dispose” of the increased superoxide produced by NADPH oxidase, thereby maintaining normal endothelium-dependent vasodilator function. It is also important to note that at the level of the contractile vascular smooth muscle, the sensitivity (presumably of the soluble guanylate cyclase) to nitric oxide appears to be increased in collared sections, because vasorelaxations to the nitric oxide donor nitroprusside are enhanced. Therefore, at this stage of pathology in this model, two mechanisms appear to compensate for the increase in NADPH oxidase activity, such that the vasodilator function of nitric oxide is preserved. It is less likely, however, given that endothelial dysfunction is clearly evident in atherogenesis in human patients and many animal models, that these mechanisms are always able to compensate adequately for the compromised bioavailability of nitric oxide. Also, although vasodilator function may be partially protected, other antiatherogenic actions of endothelium-derived nitric oxide may not be as well preserved throughout all stages of atherogenesis.

Supplementation with exogenous Cu2+/Zn2+-SOD completely restored responses to acetylcholine after DETCA/NADPH-treatment in collared arteries, confirming that the effects of DETCA and NADPH were attributable to increased superoxide generation. In contrast, the increased sensitivity to nitroprusside in collared arteries was not reversed by exogenous Cu2+/Zn2+-SOD, indicating that this compensation of the vascular smooth muscle for reduced nitric oxide bioavailability was not an event that could be rapidly reversed by restoring the nitric oxide/superoxide balance.

In conclusion, we have now demonstrated increased gp91phox expression associated with elevated endothelial and adventitial NADPH oxidase–dependent superoxide production in a normocholesterolemic model of vascular remod-

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**Figure 7.** Effect of exogenous Cu2+/Zn2+-SOD on endothelium-dependent relaxations to acetylcholine (ACh; A) and sodium nitroprusside (SNP; B) in tissues precontracted to ~50% KPSSmax with 5-HT. All segments of control (open symbols) and collared (filled symbols) arteries were preincubated with DETCA (3 mmol/L) and NADPH (100 μmol/L) for 20 minutes before precontraction. Artery segments were then either left without further treatment (circles) or were incubated with Cu2+/Zn2+-SOD (600U/mL; triangles) together with DETCA and NADPH. Values (mean±SEM from 6 experiments) are expressed as percent relaxation of the 5-HT–induced precontraction. *P<0.05 vs DETCA/NADPH-treated control rings.
eling. Increased superoxide caused impairment of endotheli-
um-dependent vasorelaxation that was revealed after
inhibition of endogenous Cu^{2+}/Zn^{2+}-SOD. Whether increased
dismutation of superoxide might protect against atherogene-
sis awaits further studies into the status of hydrogen peroxide
metabolizing pathways in atherosclerosis. Whatever the out-
come of these studies, targeting superoxide production at its
enzymatic source may be a more effective approach than
conventional antioxidants for preventing arterial damage by
reactive oxygen species.

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