Superoxide Production in Vascular Smooth Muscle Contributes to Oxidative Stress and Impaired Relaxation in Atherosclerosis

Francis J. Miller, Jr, David D. Gutterman, C. David Rios, Donald D. Heistad, Beverly L. Davidson

Abstract—The endothelium is a source of reactive oxygen species in short-term models of hypercholesterolemia and atherosclerosis. We examined a chronic model of atherosclerosis for increased vascular production of superoxide (\(O_2^\cdot\)) and determined whether endothelial overexpression of superoxide dismutase (SOD) would improve endotheliumpdependent relaxation. Superoxide generation was 3 times higher in isolated aortas from Watanabe heritable hyperlipidemic (WHHL) rabbits (2 to 4 years old) compared with aortas from New Zealand White (NZ) rabbits (43±10 versus 14±2 relative light units \(\cdot\) min\(^{-1}\) \(\cdot\) mm\(^{-2}\), n=9, \(P<0.05\)). After in vitro transduction with adenovirus containing the gene for CuZn-SOD (AdCMVCuZn-SOD) or extracellular SOD (AdCMVEC-SOD), endothelial \(O_2^\cdot\) levels in WHHL aortas were significantly reduced. Gene transfer of SOD to WHHL aortas, however, failed to improve the impaired relaxation to acetylcholine or calcium ionophore. By use of the oxidative fluorescent dye hydroethidine, an in situ assay indicated markedly increased generation of \(O_2^\cdot\) throughout the wall of WHHL aorta, especially within layers of smooth muscle. This finding was confirmed by demonstrating increased \(O_2^\cdot\) levels in smooth muscle cells cultured from WHHL aorta. We conclude that elevated \(O_2^\cdot\) levels in atherosclerotic vessels are not confined to the endothelium but occur throughout the vascular wall, including smooth muscle cells. Reduction in endothelial \(O_2^\cdot\) levels is not sufficient to improve endotheliumpdependent relaxation. Generation of reactive oxygen species within the media may contribute to vasomotor dysfunction in atherosclerosis. (Circ Res. 1998;82:1298-1305.)

Key Words: superoxide dismutase • reactive oxygen species • gene transfer • adenovirus • acetylcholine • atherosclerosis

Endothelial production of ROS, especially superoxide \((O_2^\cdot)\), with the subsequent reaction with nitric oxide \((\cdot\)NO) is an important mechanism of vascular dysfunction in atherosclerosis. \(O_2^\cdot\) and \(\cdot\)NO rapidly interact to generate peroxynitrite \((ONOO^-)\), a potent oxidant and mediator of vascular tissue injury.\(^1\)\(^-\)\(^4\) Excess generation of ROS has been demonstrated in atherosclerosis,\(^5\)\(^-\)\(^6\) and peroxynitrite formation has been shown to occur in atherosclerotic human vessels.\(^7\)

In animal models of atherosclerosis, endotheliumpdependent relaxation is impaired.\(^8\)\(^,\)\(^9\)\(^,\)\(^5\)\(^,\)\(^6\) Treatment of hypercholesterolemic rabbits with liposomal or PEG-SOD, but not native SOD, improves vascular responses.\(^9\)\(^,\)\(^10\) This confirms a role for \(O_2^\cdot\) in vascular dysfunction and suggests the necessity to deliver the enzyme intracellularly. However, these studies do not address whether enhanced oxidative products responsible for vasomotor dysfunction originate from endothelial or other vascular cells.

Mechanisms of vascular dysfunction may differ in models of long-term atherosclerosis versus short periods of hypercholesterolemia. A chronic animal model of atherosclerosis may more closely resemble the severity of atherosclerosis in patients. We designed experiments to examine the role of ROS in impaired vascular function of long-term atherosclerosis. We hypothesized that in chronic atherosclerosis, increased generation of \(O_2^\cdot\) would cause impaired endothelium-dependent relaxation. First, we confirmed previous findings of increased ROS in the endothelium of atherosclerotic aortas.\(^5\)\(^,\)\(^6\) However, further experiments showed that the media also significantly contributes to vascular dysfunction. These findings have important implications in the development of therapies for atherosclerotic disease.

Materials and Methods

Animals and Tissue Preparation

The atherosclerotic group consisted of WHHL rabbits of either sex that received high protein rabbit chow for 6 months and then a high fiber rabbit chow until they were studied at 2 to 4 years of age. For a control group, NZ rabbits were fed high protein rabbit chow. In some animals, blood was obtained for measurement of plasma cholesterol levels. Rabbits were killed with intravenous sodium pentobarbital, and the thoracic aorta was removed. The aorta was placed into oxygenated Krebs buffer (4°C), disected free of loosely adhering tissue, and cut into 3- to 4-mm ring segments. Some vessels...
were denuded by gently rubbing the endothelial surface with forceps as the ring was rolled over wet filter paper. Krebs buffer consisted of (mmol/L) NaCl 118.0, KCl 4.7, CaCl_2 2.5, MgSO_4 1.2, NaHCO_3 23.0, KH_2PO_4 1.2, and glucose 11.0.

Segments to be examined for oxidative fluorescent microtopography were immediately frozen in Tissue-Tek O.C.T. embedding medium and placed at –80°C. Rings to be assayed for superoxide content were transferred to MEM supplemented with penicillin (100 U/mL) and streptomycin (0.1 mg/mL) and placed in an incubator at 37°C for 2 to 4 hours until performing lucigenin-enhanced chemiluminescence.

Gene Transfer

Replication-defective recombinant adenoviruses (serotype 5, produced in 293 cells) used in these experiments were obtained from the Gene Transfer Vector Core at the University of Iowa and included the following: AdCMVlacZ containing the histochemical marker β-galactosidase, AdCMVβ-gal containing no transgene, AdCMVβ-galZn-SOD, and AdCMVEC-SOD. AdCMVCuZn-SOD was kindly provided by John Engelhardt, University of Pennsylvania, Philadelphia. AdCMVEC-SOD was constructed using routine methods and an EC-SOD containing plasmid (gift from Dr. James C. Crapo, Duke University) or 1:20 sheep paraffin for sectioning (5% paraformaldehyde for 1 hour, washed, and then embedded in paraffin) was used to detect the specific SOD isoforms. Tissue was placed in X-Gal for 4 hours at 37°C. Tissue was fixed in 2% paraformaldehyde for 1 hour, washed, and then embedded in paraffin for sectioning (5 μm thick) for histological analysis.

Immunohistochemical staining with a rabbit polyclonal antibody was used to detect the specific SOD isoforms. Tissue was placed in 2% paraformaldehyde for 1 hour, washed, and then embedded in paraffin for sectioning (5 μm thickness). Sections were incubated with primary antibody (1:500 rabbit anti-human EC-SOD, kindly provided by Dr. James Crapo, Duke University) or 1:20 sheep anti-human CuZn-SOD (Biodesign International) for 60 minutes.

After washing with PBS, sections were developed with Vectastain ABC Kit alkaline phosphatase (Vector Laboratories Inc.).

**Isometric Force Measurement**

Twenty-four hours after gene transduction, ring segments were mounted horizontally in organ baths containing 25 mL of Krebs buffer at 37°C and continuously gassed with a 95% O_2/5% CO_2 (pH 7.40). Rings were progressively stretched to 6 g of resting tension. This was determined to be the optimal tension for both control and atherosclerotic vessels by repeated stimulation with KCl (75 mmol/L). Vessels were allowed to equilibrate for 30 minutes and were then constricted twice with KCl (75 mmol/L). After the vessels were rinsed, the contraction response to cumulative concentrations of PE (10^{-9} to 10^{-5} mol/L) was determined. Vessels were washed and contracted with PE to between 50% and 90% of their maximal KC response. Responses were then obtained to cumulative concentrations of the endothelium-dependent dilator ACh (10^{-9} to 10^{-5} mol/L), the endothelium-independent dilator SNP (10^{-9} to 10^{-3} mol/L), or calcium ionophore A23187 (10^{-9} to 10^{-6} mol/L), which is an endothelium-dependent receptor-independent dilator. In some vessels, after SNP administration, the endothelium-independent cAMP-mediated dilator papaverine (10^{-4} mol/L) was given, and then the segments were washed in calcium-free Krebs buffer.

**Cell Culture**

Vascular SMCs were isolated from rabbit thoracic aortas by modification of methods described by Yuan et al. The aorta was cleaned of adventitia, opened longitudinally, and incubated in DMEM supplemented with fungizone (2.5 μg/mL), penicillin G (10 000 U/dL), and streptomycin (10 000 U/dL). After 4 hours, the tissue was transferred to PBS containing type I collagenase (2 mg/mL) for 20 minutes. The vessel segment was then scraped to remove remaining endothelium and plaque. The resulting smooth muscle tissue was incubated in high glucose DMEM supplemented with 10% heat-inactivated FCS, penicillin G (10 000 U/dL), streptomycin (10 000 U/dL), l-glutamine (2 mmol/L), basal minimal essential vitamins (1×), MEM nonessential amino acids (1×), and HEPES (10 mmol/L) for 24 hours in a 5% CO_2 incubator at 37°C. Overnight incubation before the second enzymatic digestion improves cell yield. Tissue was cut into 1-mm strips and incubated in collagenase (2 mg/mL), elastase (0.5 mg/mL), and bovine serum albumin (1 mg/mL) for 40 minutes. After 18 minutes, the tissue was triturated 5 times with a 10-mL pipette to speed digestion. Large tissue fragments were removed with sterile forceps. Cell suspensions were centrifuged for 5 minutes at 1500 rpm, and the cell pellet was resuspended in 5 mL of 10% FCS/DMEM and plated on collagen-treated surfaces.

After 24 hours, medium was removed, and cells were rinsed with DMEM to remove debris. Cells were fed 5% FCS/DMEM twice weekly and used for experiments when reaching 80% to 100% confluence. All studies were performed on cells from the first passage. At the time of study, cells from both NZ and WHHL rabbits showed similar positive immunohistochemical staining for smooth muscle actin of >99%.

**Oxidative Fluorescent Microtopography**

The oxidative fluorescent dye HE was used to evaluate in situ production of superoxide. HE is freely permeable to cells and in the presence of O_2·− is oxidized to EtBr, where it is trapped by intercalating with the DNA. EtBr is excited at 488 nm with an emission spectrum of 610 nm. In cell-free assays, addition of hydrogen peroxide to HE does not significantly increase EtBr fluorescence.

Unfixed frozen ring segments were cut into 30-μm-thick sections and placed on a glass slide. HE (2×10^{-6} mol/L) was topically applied to each tissue section and coverslipped. Slides were incubated in a light-protected humidified chamber at 37°C for 30 minutes. Images were obtained with a Bio-Rad MRC-1024 laser scanning confocal microscope equipped with a krypton/argon laser. Atherosclerotic and control tissues were processed and imaged in
parallel. Laser settings were identical for acquisition of images from atherosclerotic and control specimens. Fluorescence was detected with a 585-nm long-pass filter. Sections were subsequently stained with nuclear fast red. Cultured SMCs were rinsed in PBS and incubated in PBS containing HE (2 × 10⁻⁶ mol/L) at 37°C. After 30 minutes, cells were rinsed in PBS, and fluorescent confocal microscopic images were obtained as described above.

Detection of Superoxide

Production of superoxide was measured by lucigenin-enhanced chemiluminescence response as described previously. Ring segments were placed in a polypropylene tube containing 1 mL PBS and lucigenin (0.25 mmol/L). The tube was placed in a Monolight 2010 luminometer. The luminometer reported RLU emitted, which was integrated over 30-second intervals for 5 minutes. We found that counts did not significantly increase with longer periods of measurement. Dark current readings (photomultiplier background signal) were automatically subtracted. Background counts were determined from identically treated vessel-free preparations and subtracted from the readings obtained with vessels. Surface areas and dry weights were obtained for each vascular segment to allow normalization of activity.

For cultured SMCs, a cell suspension was created by detachment with 0.25% trypsin and 0.02% EDTA. After washing in PBS, cells were added to PBS containing lucigenin (0.25 mmol/L), and counts were obtained for a 5-minute period. Background counts (determined in cell-free preparations) were subtracted, and values were normalized to protein content (Lowry assay).

Data Analysis

Results are expressed as mean±SE. Data were obtained in duplicate and averaged such that in each set of experiments, n equals the number of animals studied. Contractions to PE are expressed as a percentage of the maximum contraction to KCl (75 mmol/L); relaxations are the percent change from the precontracted tension. For isometric ring studies, responses were compared among groups using a 2-factor repeated measures ANOVA with the Bonferroni correction for multiple comparisons (dose as factor 1, treatment as factor 2). Chemiluminescence data were analyzed with an unpaired 2-tailed t test or a 1-way ANOVA with a post hoc Bonferroni comparison. EC₅₀ was calculated as the dose of agonist achieving 50% of the maximal response. Statistical significance was accepted if the null hypothesis was rejected at P<0.05.

Chemicals

All chemicals were obtained from Sigma Chemical Co unless specified. Elastase and collagenase were from Worthington Biochemical; FCS, from Summit Biotechnology; bovine serum albumin, from Boehringer-Mannheim; and DMEM, penicillin G, streptomycin, and l-glutamine, from Gibco-BRL. HE was obtained from Molecular Probes Inc and suspended in dimethyl sulfoxide at a concentration of 10⁻² mol/L, where it was stored in aliquots at −80°C until use. Subsequent dilution was performed in PBS. Stock solutions of A23187 were prepared in ethanol with subsequent dilutions in Krebs buffer. The final concentration of ethanol in the ring bath was 0.1%. Sheep anti-human CuZn SOD was obtained from Boehringer-Mannheim, and rabbit anti-human EC-SOD was from Dr James Crapo, Duke University.

Results

In the WHHL rabbit model studied at 2 to 4 years of age (plasma cholesterol range, 400 to 600 mg/dL), the animals develop moderately severe atherosclerosis of the thoracic aorta with pronounced intimal thickening and atheromatous plaque. To test whether ROS were increased in this chronic model of atherosclerosis, we measured O₂⁻· production using the lucigenin-enhanced chemiluminescence assay. In the thoracic aorta, O₂⁻· generation was 3 times higher in WHHL than NZ rabbits (43±10 versus 14±2 RLU·min⁻¹·mm⁻², n=9, P<0.05; Figure 1). Mechanical denudation or addition of exogenous bovine erythrocyte SOD (750 U/mL) reduced O₂⁻· levels in diseased vessels to control values (Figure 1). Thus, O₂⁻· levels are elevated in chronically atherosclerotic aorta and are similar to levels in atherosclerotic models with a shorter duration of hypercholesterolemia. Effects of endothelial denudation also suggest that endothelium is a source of free radical production.

The endothelium and subendothelial space are the principal sites of NO/O₂⁻· interaction and subsequent peroxynitrite formation. Therefore, we asked whether overexpression of SOD in this region would alter O₂⁻· levels in atherosclerotic aortas. To perform these studies, we used replication-impaired adenoviral vectors because others had demonstrated their use in selectively transducing vascular endothelium. After incubation with AdCMVlacZ, NZ and WHHL aortas showed similar distribution and extent of X-Gal staining (Figure 2B and 2C). β-Galactosidase was demonstrated exclusively in the endothelium and adventitia, with no evidence of expression within the intima or media. Transduction of WHHL aortas with AdCMVCuZn-SOD or AdCMVEC-SOD reduced O₂⁻· levels (132% and 127% of NZ control, respectively) compared with vessels infected with AdCMVlacZ (221% of NZ control, P<0.05) (Figure 2A). Levels of O₂⁻· in atherosclerotic vessels after gene transfer with viruses expressing SOD were similar to those found in denuded vessels. Immunohistochemistry confirmed the presence of the human SOD enzyme in WHHL aortas after gene transfer (Figure 2D).

We hypothesized that improvements in oxidative stress after gene transfer of SOD would be manifested by a functional improvement in endothelium-dependent relaxation. In untreated WHHL aortas, relaxation to the endothelium-dependent dilator ACh and calcium ionophore A23187 was greatly impaired relative to responses in control rabbits (Figure 3). Although EC₅₀ values were similar, WHHL aortas displayed abnormal maximal contraction to PE and maximal relaxation to the endothelium-independent dilator SNP (Fig-
ure 3). Despite impaired relaxation of WHHL aortas to SNP (maximum relaxation, 75±2%), papaverine (10^{-4} \text{ mol/L}, cAMP-mediated) was capable of producing greater relaxation (90±6%), and vessels relaxed completely in calcium-free buffer (100±2%, P<0.05 versus SNP, n=3).

Vasomotor responses of WHHL vessels transduced with AdCMVCuZn-SOD or AdCMVEC-SOD were no different from untreated WHHL aortas (incubated 24 hours in MEM). Therefore, although gene transfer of AdCMVSOD reduced vascular O_2^\bullet^- levels, this treatment failed to improve vasomotor responses.

The topographical distribution of ROS in atherosclerotic vessels and, hence, the contribution of endothelium versus intima versus media in oxygen-derived free radical generation have not been described. Therefore, we developed an in situ assay to localize ROS and to confirm the ability of SOD gene transfer to reduce O_2^\bullet^- levels in the atherosclerotic vessel. The oxidative fluorescent dye HE was used to demonstrate the presence of O_2^\bullet^- in vascular tissue. HE easily diffuses across cell membranes, where it is directly oxidized by O_2^\bullet^- to EtBr, intercalates with DNA, and fluoresces red. Because EtBr is impermeable to intact cellular membranes, its fluorescence indicates that the reduction of HE by O_2^\bullet^- occurred intracellularly.

By use of confocal microscopy, tissue sections from WHHL and NZ aortas showed a marked increase in EtBr fluorescence, reflecting an increase in O_2^\bullet^- in atherosclerotic tissue (Figure 4). The increase in EtBr fluorescence was observed in endothelial cells, subendothelial space, media, and cellular atheromatous plaque. An unexpected finding was the marked elevation of EtBr fluorescence within the media of WHHL aortas, suggesting increased O_2^\bullet^- production by SMCs. After gene transfer of cytosolic or extracellular isoforms of SOD (AdCMVCuZn-SOD and AdCMVEC-SOD) to WHHL aortas, EtBr fluorescence in the endothelium and subendothelial space was reduced compared with WHHL vessels infected with control adenovirus (AdCMVBgl-II) (Figure 4). However, we noted no reduction of fluorescence in the media after gene transfer. These observations confirm the increased generation of ROS not only in the endothelium but throughout the vascular wall and the ability of adenovirus-mediated gene transfer of SOD to reduce oxidative stress in only the endothelium.

To confirm that O_2^\bullet^- levels were elevated in the media of atherosclerotic vessels and that the radical originated from SMCs, we examined cultured SMCs from WHHL aortas. In culture, WHHL aortic SMCs (>95% α-actin positive) exhibit...
edited marked EtBr fluorescence compared with SMCs cultured from NZ aortas (Figure 5A). This fluorescence was completely inhibited when the cells were incubated in PEG-SOD (500 U/mL) for 30 minutes before imaging.

As assessed by chemiluminescence, basal levels of $O_2^{-}$ were significantly greater in WHHL than NZ SMCs (42±12 versus 6±5 RLU·min$^{-1}$·mg protein$^{-1}$, n=3, P<0.05, Figure 5B). These data confirm that in this chronic model of vascular
disease, ROS extend beyond free radical generation in the endothelium and involve the SMCs.

**Discussion**

Other investigators have demonstrated ROS as mediators of vascular dysfunction in animal models in which atherosclerosis is acute or of short duration (<6 months). For example, aortas from rabbits fed a 1% cholesterol diet for 1 month showed a 2-fold increase in $O_2^-$ levels and impaired relaxation to ACh but not SNP. This impairment could be reversed by dietary supplements of the antioxidant probucol. Similarly, treatment with liposomal SOD improved relaxation in aortas from rabbits fed a 1% cholesterol diet for 6 months. Aortas from these rabbits, in which the severity of disease was less than that in our model, relaxed nearly 70% to ACh.

Thus, in animal models of atherosclerosis with a short duration of disease, generation of $O_2^-$ in endothelium may play an important role in vascular dysfunction. However, the magnitude of hypercholesterolemia in animals fed a high fat diet (>1500 mg/dL) introduces the possibility of vasomotor effects directly related to lipoproteins, since oxidized LDL directly inhibits endothelium-dependent relaxation. Therefore, the nature of vascular disease in humans and the mechanisms of vascular dysfunction in these animal models may differ. The model of chronic atherosclerosis described here may provide additional insight into the role of ROS in chronic clinical disease.

We show that reduction of vascular $O_2^-$ levels after gene transfer of SOD fails to improve agonist-stimulated vascular responses. In other studies, the administration of SOD or other antioxidants improves endothelium-dependent relaxation; however, these methods deliver the enzyme throughout the vessel wall. Adenovirus-mediated gene transfer, as used in the present study, selectively increases SOD in the endothelium and adventitia, but not the media. Our lucigenin-enhanced chemiluminescence and EtBr fluorescence data indicate successful gene transfer with reduction of endothelial $O_2^-$ levels and provide evidence that increased oxidative stress in vascular smooth muscle contributes to impaired relaxation in atherosclerosis.

A role for the media in contributing to vascular dysfunction is also supported by our finding that relaxation to the endothelium-independent dilator SNP is impaired in WHHL aortas. Nitroprusside relaxation occurs via direct delivery of $\cdot NO$ to the smooth muscle. If $O_2^-$ levels are increased in the media, its reaction with SNP/$\cdot NO$ and formation of peroxynitrite would reduce $\cdot NO$ availability and impair relaxation. Inactivation of $\cdot NO$ by $O_2^-$ has been well documented. Increasing vascular $O_2^-$ by inhibition of SOD activity impairs relaxation not only to ACh but also to nitroglycerin, supporting the ability of $O_2^-$ to impair smooth muscle.

**Figure 5.** Superoxide levels in SMCs cultured from NZ and WHHL aortas. A, Confocal fluorescent images of SMCs after incubation with HE suggest that basal $O_2^-$ levels are increased in SMCs from WHHL aorta. EtBr fluorescence is SOD inhibitable. Corresponding phase microscopic images are shown in lower panel. B, Basal $O_2^-$ levels detected by lucigenin-enhanced chemiluminescence are significantly higher in WHHL-cultured SMCs ($n=3$).
relaxation independent of effects on the endothelium.21 Weisbrod et al22 have recently shown decreased NO responsiveness of aortic SMCs from hypercholesterolemic rabbit aortas. In the present study, decreased responsiveness of the atherosclerotic media to NO is supported by the finding of impaired relaxation to SNP. This abnormal relaxation to SNP is unlikely to be primarily due to noncompliance of the vessel wall related to structural changes of atherosclerosis, since WHHL aortas were capable of additional relaxation by papaverine and complete relaxation in calcium-free buffer.

A potential alternative interpretation of our findings is that the mechanism of vasomotor dysfunction in chronic atherosclerosis may be unrelated to elevated superoxide levels. Although there is strong experimental evidence implicating a role for superoxide in vascular dysfunction of hypercholesterolemia and atherosclerosis, these data were obtained from animals with a short duration of disease.5,6,9,10 NO production by the endothelium is not reduced in hypercholesterolemic rabbits.23 Intimal thickening as a barrier to NO is unlikely to be a primary mechanism, since endothelium-dependent relaxation is restored in monkeys with cessation of high fat diet despite persistent thickened intima.24 In an advanced lesion, the accumulation of lipid peroxidation products may be important in their ability to impair endothium-dependent relaxation.25

It has recently been reported by Liochev and Fridovich26 that lucigenin can undergo univalent reduction by cellular reductases with subsequent generation of superoxide. This observation was reported in a cell-free system, and it is not known whether the concentration or availability of reductases would result in significant auto-oxidation of lucigenin in intact tissue. Using electron spin resonance, we observed superoxide production when lucigenin is added to xanthine oxidase and NADH (as described by Liochev and Fridovich) but not when added to WHHL aortic homogenate (authors’ unpublished data, 1998). These findings suggest that auto-oxidation of lucigenin by cellular reductases does not contribute to the chemiluminescence observed in the present study.

Chemiluminescence is able to detect O$_2^-$ within endothelium but is not a sensitive measurement of O$_2^-$ throughout the vessel wall. Univalent reduction of lucigenin and reaction with O$_2^-$ leads to the release of photons, which are measured with a luminometer.27 An inability of the photons emitted within the media to reach the photomultiplier tube will allow detection of O$_2^-$ generated in only the outer layer(s) of the vessel wall. The ability of the vascular wall to restrict O$_2^-$ detection is confirmed by our observation that when lucigenin and the O$_2^-$-generating system (xanthine and xanthine oxidase) are introduced into the lumen of an NZ rabbit aorta and the vessel segment ends are tied, the detectable RLU/min value is $<10\%$ of the xanthine/xanthine oxidase–generated RLU/min value when outside the vessel (data not shown). This quenching of photons by the vessel wall would be greater in the thickened atherosclerotic vessel. The apparent discrepancy between our luminescence and fluorescence data likely results from this poor sensitivity of lucigenin to detect O$_2^-$ within the vascular wall.

For this reason, we adapted an in situ assay using the fluorescent dye HE to show the presence and distribution of O$_2^-$ within atherosclerotic aortas. This compound has been used in cells and in tissues to detect ROS.14,15,28,29 HE is the sodium borohydride–reduced form of EtBr, initially developed as a vital dye.30 In contrast to EtBr, HE permeates the cell membrane easily, where it can be oxidized by O$_2^-$ to red fluorescent EtBr and trapped intracellularly by intercalation into DNA.14,15 Extracellular O$_2^-$ would not be expected to significantly contribute to the observed cellular fluorescence, since EtBr is impermeable to cell membranes. Similarly, once oxidized intracellularly, there would be minimal loss of EtBr out of the cell. Neither hydroxyl radical, NO, peroxynitrite, H$_2$O$_2$, hypochlorite, nor singlet O$_2$ significantly oxidizes HE.29 For these reasons, we interpreted EtBr fluorescence to specifically indicate O$_2^-$ generation within the fluorescing cell.

To our knowledge, it is unknown whether HE can produce superoxide via redox cycling off cellular reductases, as has been described for lucigenin.26 With electron spin resonance, we find no evidence of superoxide generation when HE is added to xanthine oxidase and NADH, or to WHHL aortic homogenate (authors’ unpublished data, 1998). These findings suggest that HE is not significantly auto-oxidized by cell reductases to produce superoxide.

All assays currently available for the detection of superoxide have potential shortcomings, limiting their sensitivity and/or specificity. In the present study, we use 2 independent methods, lucigenin-enhanced chemiluminescence and HE reduction to fluorescent EtBr, to determine superoxide content. The use of 2 methods minimizes potential errors of interpretation inherent to a particular assay. The findings of each of the 2 assays in the present study are consistent with increased superoxide in WHHL aortic rings and SMCs.

In the present study, strengths of the fluorescent dye HE over other methods of ROS detection include (1) sensitivity and selectivity for O$_2^-$, (2) detection of intracellular O$_2^-$, and, importantly, (3) the ability to indicate the cellular site of O$_2^-$ production.

In summary, the major findings of the present study are (1) in a chronic model of atherosclerosis, superoxide levels are increased throughout the vascular wall, including SMCs, and (2) despite reduction of endothelial superoxide levels after gene transfer of SOD, vasomotor responses remain abnormal. We conclude that the media is an important site of free radical production in chronic atherosclerosis, which may contribute to the inability of the vessels to relax and contract normally. Our findings suggest that reducing oxidative stress in chronic atherosclerosis with the intent of improving vascular dysfunction is dependent on the delivery of SOD to the media as well as the endothelium. Therefore, application of gene transfer techniques will require vectors capable of efficient transduction and expression in vascular smooth muscle.

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References


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