Immunolocalization of Native Antioxidant Scavenger Enzymes in Early Hypertensive and Atherosclerotic Arteries

Role of Oxygen Free Radicals

Ramesh C. Sharma, Donald W. Crawford, Dieter M. Kramsch, Alex Sevanian, and Qun Jiao

To elucidate the role of oxygen free radicals and lipid peroxidation in the pathogenesis of early hypertension and atherosclerosis, we studied the native distribution of three primary arterial antioxidant enzymes (AEs). Specific immunohistochemical localization of superoxide dismutase (Cu-Zn SOD), glutathione peroxidase (GSH-Px), and catalase (CAT) was examined in the arterial wall of New Zealand White rabbits: six sham-operated normotensive/normolipidemics (NT/NL), seven coarctation-induced hypertensive/normolipidemics (HT/NL), eight normotensive diet-induced hyperlipidemics (NT/HL), and six hypertensive/hyperlipidemics (HT/HL). All three AEs were confined primarily to the endothelium in NT/NL rabbit aortas. However, in HT and HL rabbits a greater proportion of the arterial wall, including the endothelium, inner media, and middle media, displayed immunolocalization of three AEs. Multiple linear-regression analysis revealed that more than 70% of the total variability in the depth of immunolocalization of arterial AEs could be explained by changes in blood pressure and/or total cholesterol. Also, levels of plasma and arterial cholesterol oxides were significantly different (p<0.05) in HT and HL rabbits compared with controls, with twofold increases in NT/HLs, threefold increases in HT/NLs, and fourfold increases in HT/HLs.

We conclude that intense free-radical activity in the arterial wall of HT and HL animals is one possibility and that this occurs despite the presence of abundant AEs.

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KEY WORDS • hypertension • oxygen free radicals • cholesterol oxides • antioxidant enzymes • glutathione peroxidase • superoxide dismutase • catalase • hypercholesterolemia • polyclonal antibodies

While it is known that hypercholesterolemia and hypertension are two major independent risk factors involved in the etiopathogenesis of atherosclerosis, many of the fundamental cell processes involved are yet unclear. Oxygen-derived free radicals such as superoxide (O2), hydroxyl ions (OH), and lipid peroxide (RO) and the toxic oxygen metabolite hydrogen peroxide (H2O2) have been discussed as a potential source of the deleterious effects on the vascular endothelium directly or indirectly through the oxidative modification of low density lipoprotein (LDL).

In vitro studies have indicated that monocyte-derived macrophages cannot take up unmodified LDL rapidly enough to produce lipid loading, suggesting that unmodified LDL may not play a significant role in fatty streak formation in vivo. However, other studies suggest that oxidative modification of LDL may occur in vivo, potentially converting this lipoprotein to a form that is recognized by the macrophage scavenger receptors. Both endothelial cells and arterial smooth muscle cells (SMCs) have been shown in vitro to actively oxidize LDL and could be a potential source of its oxidative modification in vivo. The cytotoxicity of oxidized LDL has been recently demonstrated in vitro with cultured fibroblasts, human umbilical vein endothelial cells, and arterial SMCs.

Direct evidence to support the existence of oxidized LDL in vivo has only recently begun to emerge. Using immunohistochemical methods and specific monoclonal antibodies, Haberland et al. demonstrated malondialdehyde-lysine residues in Watanabe heritable hyperlipidemic rabbit atherosclerotic lesions. In addition, several different oxidation-specific epitopes, including 4-hydroxynonenal, apolipoprotein B, and LDL oxidized in the presence of the promoter transition metal copper have been reported recently. Autoantibodies to malondialdehyde-lysine in human and rabbit plasma have also been described.

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well-oxygenated intima and subintima, is interesting but is scientifically unexplained. However, the oxygen distribution in the sequestered subintimal microenvironment above an area of hypoxia deeper in the media may facilitate widespread oxygen free-radical formation leading to peroxidation of lipids and thus, formation of lesions. Increased mural oxygen consumption also has been reported by others in experimental hypertension. Furthermore, evidence is mounting that in the hypertensive artery, increased metabolism as well as diffusional limitation may play a significant role in the formation of intimal thickening. Therefore, the heterogeneous mural oxygen distribution in the sequestered subintimal microenvironment may facilitate extensive oxygen free-radical generation.

On the other hand, to combat such a hostile hyperoxic stress, living cells possess an elaborate system of defense. They possess three primary antioxidant enzymes (AEs), namely superoxide dismutase (Cu-Zn SOD), glutathione peroxidase (GSH-Px), and catalase (CAT), which have the capacity to destroy the highly toxic oxidant species O$_2^\cdot$, organic hydroperoxides (ROOH), and H$_2$O$_2$, respectively. These powerful scavenger enzymes play a protective and regulatory role in the defense of cells against a variety of exogenous and endogenous oxidants.

Despite the progress made in modern technology, it has been difficult to unequivocally show the involvement of oxygen free-radical species directly in biologic tissues. However, electron spin resonance spectroscopy and spin trapping methods offer indirect evidence to confirm the presence of these short-lived labile oxygen free-radicals. Del Boccio et al. using biochemical assay methods, have studied time-related changes in aortic AEs in cholesterol-fed rabbits, but the precise localization of the AEs by immunohistochemical methods in atherosclerotic or hypertensive arteries has not been reported to our knowledge. Therefore, we determined to study the potential role of oxygen free radicals in the pathogenesis of hypertension as well as atherosclerotic intimal lesions by examining the distribution of native AEs in the states of hypertension, hyperlipidemia, or both conditions combined, using specific immunocytochemical techniques. In another series of experiments to further elucidate the role of oxygen free radicals, we analyzed the cholesterol oxide content in rabbit aortic arch sections and plasma.

Methods

Study Design

All rabbit experiments were performed according to the rules and regulations approved by the Department of Health, Education, and Welfare and the Animal Care and Use Committee of the University of Southern California, Los Angeles. Twenty-eight New Zealand White adult male rabbits weighing 2.7-3.5 kg were obtained from Irish Farms, Corona, Calif., and randomly divided into four groups. Two of these groups were kept normotensive (NT), and the other two groups were rendered hypertensive (HT) by surgical coarctation of the upper abdominal aorta as previously described. All the NT animals were sham-operated and subjected to the same surgical procedure except for actual ligation and that the suture remained loosely tied around the aorta without causing constriction. One NT group and one HT group were maintained normolipidemic (NL) by feeding the control diet (Purina Rabbit Chow, Purina Mills Inc., St. Louis, Mo.) while one NT group and one HT group were made hyperlipidemic (HL) by feeding an atherogenic diet containing 2% cholesterol and 10% peanut oil as described by Kritchevsky et al. The number of animals per group were NT/NL, six; HT/NL, seven; NT/HL, eight; and HT/HL, six. All animals were housed in steel cages with easy access to water and diet ad libitum for an average period of 6 weeks. Animals were provided with light between the hours of 6 AM and 6 PM daily in a temperature-controlled environment (76-78°F).

Blood Samples

Blood samples were collected from the central ear artery of each animal after an 18-hour fast at baseline and thereafter at biweekly intervals. Samples were drawn into glass Vacutainer tubes containing 1.5 mg EDTA/ml blood and placed on ice immediately. Plasma was separated in a centrifuge set at 4°C and spun at 3,000 rpm for 20 minutes. For the measurement of cholesterol oxides, 2-ml plasma samples were stored under argon in the dark at −70°C until analysis, as described by Sevanian and McLeod. Total plasma cholesterol measurements were done by standard enzymatic methods.

Blood Pressure Measurements

Routine weekly blood pressures were measured in a dark room by the ear capsule transillumination method of Grant and Rothschild after the animals had been warmed and were well rested. A mean of at least five successive measurements was recorded, and the average standard deviation of these groups of measurements was ±4 mm Hg. In the coarcted animals the blood pressure was recorded 2 weeks postoperatively and was followed by weekly recordings until the time the animals were killed. It has been demonstrated that pressure measured by this method is linearly correlated with mean intra-arterial pressure.

Autopsy and Tissue Preparation

All animals were anesthetized by intramuscular administration of ketamine (100 mg/ml) and xylazine (20 mg/ml) and were killed by an intravenous overdose of sodium pentobarbital. The aortic arch was dissected and cleaned in ice-cold normal saline. One 5-mm ring segment of the midaortic arch was embedded in cryomold containing OCT medium (Tissue Tek, Fisher Scientific, Tustin, Calif.) and snap frozen in liquid nitrogen. The tissues were stored at −70°C in an ultrafreezer until they were ready for cryostat sectioning. Positive control sections for the AEs were obtained from the liver of the same rabbit and processed in the same manner. Nine-micron-thick cryostat ring sections of the aortic arch were cut and placed on microscope slides that had been precoated with 10% poly L-lysine adhesive subbing solution (Sigma Diagnostics, St. Louis, Mo.) and then air dried. The sections were fixed in acetone for 10 minutes, air dried, and subjected to immunohistochemical staining. In addition, other adjacent 5-mm ring sections of aortic arch were excised and immediately immersed in Bouin's solution, postfixed in
immunohistochemical staining was performed according to the method of Atalla et al.27,28 In brief, the positive control slides and the test slides were treated with polyclonal antibodies against the three AEs at a final dilution of 1:64 for 30 minutes. The negative control slides were reacted with 1) preimmune plasma from the same animal that generated the primary antibody and 2) phosphate-buffered saline (PBS). All the slides were incubated in a temperature-controlled workstation (Stainplate, CRL, Cambridge, Mass.) concomitantly. The sections were washed with 0.1 M PBS for 10 minutes and were reacted with biotinylated goat anti-rabbit secondary antibody (Vector Labs, Burlingame, Calif.). Another negative control was done by omitting the secondary antibody and substituting it with PBS. The sections were washed for 10 minutes in PBS and reincubated with avidin-biotin-peroxidase complex (Vector Labs); this was followed by development of 3-amino-9-ethylcarbazol H2O2 substrate reaction. Some of the slides were counterstained with hematoxylin.

**Image Analysis Microscopy**

To determine the relative depth of immunolocalization of AEs as well as total intimal-medial wall thickness of the aorta of different experimental animal groups, we used a computerized image analysis/light microscopy system. Multiple immunostained arterial cross sections for each AE were evaluated through a Zeiss universal microscope with a x20 objective lens. The image was scanned by a Hitachi VK-C 2000 video camera and digitized by a data translation frame-grabber in a Micro-VAX computer, using an algorithm developed by Paul Lee at Jet Propulsion Laboratory, Pasadena, Calif., resulting in a 480 x 480 image with a resolution of 0.8 μm/pixel. The immunohistochemical staining of AEs in the arterial cross section of HT and atherosclerotic animals was not microscopically homogeneous and varied from case to case. Therefore, we determined only the maximum depth of positive AE immunostaining from the endothelium through the media by manually defining the end points with a cursor.

This was done in three consecutive readings, and the mean was calculated (Figure 5).

**Analysis of Cholesterol Oxides**

Arterial tissue. Freshly excised adjacent minced segments of the aortic arch (approximately 500 mg wet weight) were homogenized in 6 ml chloroform/methanol (2:1, vol/vol) containing 0.01% butylated hydroxytoluene, using a Tekman tissue homogenizer (Tekman, Cincinnati, Ohio). Samples were homogenized and rinsed with two successive washings of the homogenizer probe with the aforementioned solvents. All procedures were carried out in an ice-cold water bath under an atmosphere of argon. After allowing the homogenate to stand in a sealed tube for 1 hour at room temperature, the samples were centrifuged at 2,500 rpm for 15 minutes and the supernatant was recovered. The solvent was evaporated to dryness under nitrogen and the residue dissolved and reextracted according to the method of Bligh and Dyer,30 with modifications as described previously.22 The organic phase was evaporated and the residue dissolved in chloroform and associated to diol extraction columns (VWR Scientific, Cerritos, Calif.), hydrolyzed, methylated with diazomethane, and converted to o-trimethylsilyl ether derivatives, and 1-μl aliquots were analyzed by gas chromatography equipped with a DB-1 capillary column and a flame ionization detector. Chromatographic analyses were performed with Axxi-chrom 747 analytical chromatography software (Axxiom Chromatography, Inc., Moorpark, Calif.). Oxysterol identity was confirmed by gas chromatography and mass spectrometry.

Plasma. Briefly, six volumes of chloroform/methanol (2:1, vol/vol) containing 0.01% butylated hydroxytoluene were added to 1 ml plasma, mixed vigorously for 5 minutes, and centrifuged to separate the organic phase, which was collected and saved. The aqueous phase was reextracted as above with three volumes of solvent, and the organic phase was pooled. The lipid extracts were evaporated to dryness under argon and then redissolved in 1 ml argon-saturated toluene/ethyl acetate (1:1, vol/vol). As an internal standard, 100 μg 5α-cholestanol (5 mg/ml in toluene) was added to each sample. The samples were applied to diol extraction columns as described previously.

The amount of each oxidation product was calculated as either micrograms per aorta or micrograms per deciliter plasma. Alternately, the levels of each cholesterol oxide were calculated as a percentage of total cholesterol in the sample.

Statistical Analysis

In all tests of significance the null hypothesis was rejected when its probability was 5% or less. Because of the nonnormality of the data, nonparametric procedures were applied. For more general comparison of cholesterol and blood pressure determinations among NT/NL, NT/HL, NL/HT, and HT/HL groups, an analysis of variance of the Kruskal-Wallis test was used, coupled with application of the Bonferroni adjustment when appropriate. Within a group changes from baseline were tested using the Wilcoxon signed-rank test for repeated measures. The relation of blood pressure change and total cholesterol change to the depth of immunohistochemical localization of AEs was analyzed
Results

The animals remained in good health and consumed their respective diets well throughout the experimental period except for one rabbit of the HT/HL group that died of congestive heart failure due to excessive coarctation. The amount of food consumed and the weight gain were not significantly different among the experimental groups, nor was there any characteristic difference in the growth pattern. At the termination of the experiment, the animals had gained between 568 and 683 g.

Blood Pressure and Plasma Cholesterol

Table 1 lists the recordings of mean systolic blood pressures and mean total plasma cholesterol levels in the four experimental groups of rabbits during a period of 6 weeks. The hypertension was mild but statistically significant ($p<0.01$) in the coarcted rabbits at the time the animals were killed compared with preoperative baseline (as well as with NT animals; see below) with the following mean values±SEM: 83±11 and 721±120 in the NT/NL and 83±11 and 804±100 in the HT/HL group. A relatively moderate increase in the total cholesterol levels, despite feeding the animals a 2% cholesterol and 10% peanut oil–rich diet for 6 weeks, could partially be due to the irregular eating pattern of the rabbits during the postoperative convalescence period.

Cholesterol Oxide

Table 2 shows the amounts of cholesterol and cholesterol oxides in the plasma and aortic arch of New Zealand White rabbits representing the four experimental groups at the time of autopsy. The levels of cholesterol oxides, particularly cholesterol epoxides and cholestane triols, were significantly elevated both in the plasma and aortic arch of HT and atherosclerotic animals. The increase in the level of plasma cholesterol oxides was more than twofold in NT/HL, threefold in HT/NL, and fourfold in HT/HL compared with that of the control NT/NL rabbits. Of particular interest is the finding of cholesterol-5β,6β-epoxides as the major cholesterol oxide in nearly all samples, suggesting that a major portion of the cholesterol oxides were generated through free-radical chain-propagation reactions. Although the level of circulating cholesterol oxides in HT/NL and HT/HL groups ($5.4±0.1\%$ versus $6.1±0.1\%$, respectively) appears to be similar as shown in Table 2, this observation can easily be explained on the basis that cholesterol oxides were computed as a percentage of total cholesterol, which is much higher in HT/HL.

by calculating simple correlation coefficients followed by applying multiple linear-regression analysis to test the interactions between the independent variables and to find a model that best fit the data. All analyses were done with the aid of the Statistical Analysis System (SAS). Values are mean±SEM.

Blood pressures were determined weekly for all animals, and a mean of at least five concordant measurements was recorded. Plasma cholesterol levels were obtained biweekly. Group comparison of systolic blood pressure data at 6 weeks revealed statistically significant difference ($p<0.01$).

<table>
<thead>
<tr>
<th>Parameter</th>
<th>$n$</th>
<th>Baseline</th>
<th>6-Week</th>
<th>Change</th>
</tr>
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<tr>
<td>Blood pressure (mm Hg)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NT/NL*</td>
<td>6</td>
<td>63±3</td>
<td>64±2</td>
<td>1±1</td>
</tr>
<tr>
<td>HT/NL</td>
<td>7</td>
<td>67±3</td>
<td>83±3*</td>
<td>16±3*</td>
</tr>
<tr>
<td>NT/HL</td>
<td>8</td>
<td>63±2</td>
<td>63±2</td>
<td>-2±2</td>
</tr>
<tr>
<td>HT/NL</td>
<td>6</td>
<td>65±2</td>
<td>76±3*</td>
<td>11±7*</td>
</tr>
<tr>
<td>Total cholesterol (mg/dl)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NT/NL*</td>
<td>6</td>
<td>39±5</td>
<td>43±5</td>
<td>4±1</td>
</tr>
<tr>
<td>HT/NL</td>
<td>7</td>
<td>48±4</td>
<td>33±3</td>
<td>-15±3</td>
</tr>
<tr>
<td>NT/HL</td>
<td>8</td>
<td>49±7</td>
<td>593±66§</td>
<td>544±98§</td>
</tr>
<tr>
<td>HT/HL</td>
<td>6</td>
<td>83±11</td>
<td>804±100§</td>
<td>721±120§</td>
</tr>
</tbody>
</table>

Values are mean±SEM.

Blood pressures were determined weekly for all animals, and a mean of at least five concordant measurements was recorded. Plasma cholesterol levels were obtained biweekly. Group comparison of systolic blood pressure data at 6 weeks revealed statistically significant difference ($p<0.01$).

NT, normotensive; NL, normolipidemic; HT, hypertensive; HL, hyperlipidemic.

*Sham operated.

$t p<0.05$, Wilcoxon rank-sum test, compared with NT animals in NL and HL groups.

$tp<0.05$, Wilcoxon signed-rank test for repeated measures; one-sided.

$\$p<0.001$, Wilcoxon rank-sum test, compared with NL animals in NT and HT groups.
Morphological and Immunohistochemical Results

Gross examination of the aortic arch from all the animals did not reveal any visible plaques. Immediately frozen sections were examined in this study because paraffin-embedded sections revealed a suboptimal immunohistochemical staining, possibly due to poor antigen preservation. Multiple serial cryosections of aortic arch (three sections per slide) of each animal in different groups were evaluated under optimally defined conditions for the immunohistochemical demonstration of primary AEs. Sections incubated with preimmune sera, secondary antibodies, or sera adsorbed with Cu-Zn SOD, GSH-Px, or CAT revealed no immunostaining. Sections from liver showed intense positive immunoreactivity of all three AEs.

**Normotensive/normolipidemic group.** Figure 1A exhibits the architecture of a normal arterial wall. Immunoreactive native Cu-Zn SOD was predominantly localized in the aortic endothelium of NT/NL rabbits (Figure 1B). At higher magnification, granular aggregates of the immunopositive Cu-Zn SOD conjugates were seen in the cytoplasm of the endothelial cells and in the superficial SMC layer of the inner media. However, the rest of the tunica media appeared devoid of the immunostain. An identical distribution pattern of enzyme reactivity was observed for GSH-Px and CAT (Figures 1C and 1D).

**Hypertensive/normal lipoprotein group.** The arterial wall appeared to be considerably thickened in the HT/NL rabbits compared with that of the NT/NL group. Focal intimal thickenings were noted at several areas around the intimal circumference (Figure 2A). The early HT lesions displayed scattered collagen bundles and some intimal neolastica formation as revealed by Verhoeff’s-Sirius red staining. The internal elastic lamina was fragmented and deranged. Increased cellularity was seen in the HT lesions. The arterial media also showed apparently thicker collagen bundles. These intimal and medial changes appeared to contribute to the substantially thicker appearance of aortic wall in HT animals.

By comparison, the antioxidant immunohistochemical staining of the adjacent representative cross sections of HT/NL rabbit aortic arch revealed a greater proportion of arterial wall with intense Cu-Zn SOD, GSH-Px, and CAT immunoreactivity (Figures 2B–2D). Diffuse dense intracellular as well as extracellular granular deposits of the specific immunostains were evident in the endothelial cells and in SMCs deep into the arterial media, especially at HT intimal thickenings. Some staining around the internal elastic lamina was also noted. The shoulders of the lesion revealed comparatively decreasing cellular and extracellular staining of the AEs. The nonlesion-adjacent areas of the HT/NL arterial wall also exhibited a relatively greater distribution of AEs compared with NT/NL rabbit aorta. The staining pattern of CAT, although enhanced when compared with that of NT/NL animals, was relatively weaker (Figure 2D).

**Normotensive/ hyperlipidemic group.** The morphological structure of the aortic arch in NT/HL animals exhibited a typical foam cell lesion formation (Figure 3A). The intima was markedly raised because of accumulation of a large number of foam cells and some minor deposits of collagen and elastin as revealed by the Verhoeff’s–Sirius red stain.

The subsequent consecutive section showed intense immunoreactivity of Cu-Zn SOD and GSH-Px (Figures 3B and 3C). The positive immunostain deposits were associated with cells as well as with the extracellular matrix of the lesion. However, in contrast to HT/NL animals, the arterial media under the lesion also showed scattered AE activity. The nonlesion areas revealed a general increase in immunostaining compared with those of NT/NL rabbit aortas. The CAT immunostaining again was relatively less intense (Figure 3D).

**Hypertensive/ hyperlipidemic group.** Figure 4A exhibits a characteristic fibrous-fatty lesion as seen in HT/HL rabbit aortas. Increased collagen bundles were evident in the region as demonstrated by Sirius red stain, while foam cells were strikingly few compared with the fatty streak lesions seen in NT/HL animals. The internal elastic lamina was totally fragmented and deranged, and the formation of neolastica was evident in the lesion intima.

Immunohistochemical localization of the AEs in the adjacent serial cryostat sections revealed extensive en-

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**Table 2. Concentrations of Cholesterol and Cholesterol Oxide Levels in Serum and Aortic Arch of Rabbits in Different Experimental Groups at Sacrifice**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>NT/NL* (n=6)</th>
<th>HT/NL (n=7)</th>
<th>NT/HL (n=8)</th>
<th>HT/HL (n=6)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cholesterol (mg/dl)</td>
<td>91±5</td>
<td>96±8</td>
<td>667±33‡</td>
<td>918±55‡</td>
</tr>
<tr>
<td>Aorta (μg/aorta)</td>
<td>151±4</td>
<td>181±15</td>
<td>231±11‡</td>
<td>413±8‡</td>
</tr>
<tr>
<td>Cholesterol oxides</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Serum (% cholesterol)</td>
<td>1.5±0.4</td>
<td>5.4±0.1‡</td>
<td>3.2±0.2‡</td>
<td>6.1±0.1‡</td>
</tr>
<tr>
<td>Aorta (% cholesterol)</td>
<td>1.9±0.8</td>
<td>5.2±0.7‡</td>
<td>7.1±0.5‡</td>
<td>3.6±0.3‡</td>
</tr>
</tbody>
</table>

Values are mean±SEM.

Severity of disease in hypertensive as well as in hyperlipidemic rabbits correlated with sharply greater amounts of cholesterol epoxides (expressed as a percentage of cholesterol) compared with NT/NL control animals. It is of interest that cholesterol oxides are substantially elevated in HT/NL rabbits despite the fact that cholesterol levels are within the normal range. Levels of cholesterol and cholesterol oxides in the aortic arch essentially reflect those measured in serum.

NT, normotensive; NL, normolipidemic; HT, hypertensive; HL, hyperlipidemic.

Sham operated.

†Cholesterol oxides consisted of cholesterol-5β,6β-epoxide, cholesterol-5α,6α-epoxides, cholesterol triol, 7-ketocholesterol, and 7-hydroxycholesterol.

‡p<0.001 vs. NT/NL animals, tested by Wilcoxon rank-sum test. α=0.02, Bonferroni adjustment.
zyme reactivity of Cu-Zn SOD and GSH-Px in the fibrocellular plaque (Figures 4B and 4C). In the lesion, dense granular cytoplasmic enzyme deposits were seen in the cells of the lesion intima, including endothelial cells and medial SMCs. Some positive immunostain products also were seen associated with the extracellular lesion matrix. In some lesion areas the localization of Cu-Zn SOD and GSH-Px enzymes was sharply demarcated, whereas in other areas massive accumulations were diffuse and confluent. In the nonlesion areas there was an overall increase of the AE distribution throughout the tunica media and adventitia. The staining pattern of CAT immunoreactivity once again seemed milder in intensity compared with Cu-Zn SOD and GSH-Px, but CAT was much stronger than that seen in the normal arteries of NT/NL rabbits (Figure 4D).

Morphometric Image Analysis
The results of morphometric image analysis for the depth of immunolocalization of AEs across the arterial wall (Figure 5) in the four experimental groups are
FIGURE 2. Comparative staining patterns of consecutive cryostat sections of mid-aortic arch of a representative hypertensive, normolipidemic rabbit. Panel A: Section exhibits a typical hypertensive lesion, showing thickened intima and tunica media. Note the deranged internal elastic lamina (IEL) and neoelastica formation in the thickened intima as well as wider elastic interlamellar spaces in the media due to increased collagen formation (Verhoeff’s–Sirius red stain). Panel B: Superoxide dismutase; panel C: Glutathione peroxidase; and panel D: Catalase, avidin–biotin–immunoperoxidase staining. These sections reveal the distribution patterns of the three antioxidant enzymes. Note the markedly increased depth of immunostaining of the three antioxidant enzymes in the hypertensive lesion. Arrow shows extracellular pools of immunostaining. The intensity but not the depth of catalase immunostaining was relatively milder than that of the other antioxidant enzymes. Bar=100 μm.

summarized as a bar graph in Figure 6. In each animal group the mean value of the depth of immunohistochemical staining as well as total intimal–medial thickness of the arterial wall was determined. Compared with normal controls (NT/NL), the relative proportion of the depth of intimal–medial immunohistochemical localization of the three AEs was much greater in all HT and HL groups with the following order of depth of involvement in the arterial wall: HT/HL>NT/HL>HT/NL>NT/NL.

Statistical Analysis
Correlations between differences in absolute depth of intimal–medial AE immunostaining and blood pressure
FIGURE 3. Serial cryostat sections through midaortic arch of a normotensive, hyperlipidemic rabbit. Panel A: Section shows a typical large foam cell/fatty streak lesion, displaying marked thickening of intima mainly due to increased cellularity of fat-laden cells and very few collagen strands. Tunica media revealed normal collagen deposits, which were relatively less marked compared with those from the hypertensive aortic arch (Verhoeff's–Sirius red stain). Panel B: Superoxide dismutase; panel C: Glutathione peroxidase; and panel D: Catalase, avidin–biotin–immunoperoxidase staining. These serial cross sections reveal the extensive increase in the immunohistochemical expression of the three antioxidant enzymes across the arterial wall. Note the increase in immunostaining throughout the media as compared with the hypertensive, normolipidemic rabbit aortic arch. Catalase immunostaining is relatively less intense. Bar=100 μm. IEL, internal elastic lamina.

change in treatment subgroups at 6 weeks are summarized in Table 3. A statistically significant positive correlation was observed between blood pressure change and the depth of immunolocalization of AEs at 6 weeks of induction of hypertension ($p<0.05$). Similarly, a much stronger positive correlation ($p<0.0001$) was observed between the change of total cholesterol and the depth of immunolocalization of the three AEs across the arterial wall. Obviously, the greater depth of AE immunostaining in the aortic arch of HL animals was due to the relatively larger foam cell/fatty streak lesions compared with smaller HT lesions.

Multiple linear-regression analysis was done to evaluate the relation between change of blood pressure and change of cholesterol with the depth of intimal–medial immunolocalization of three AEs, and the results are summarized in Table 4. We found that more than 70% of the variability in the depth of immunohistochemical localization of three AEs could be explained by the increase in blood pressure and elevation in total plasma
Figure 4. Sequential cryostat sections through the midaortic arch of a representative hypertensive, hyperlipidemic rabbit. Panel A: Section reveals a typical fibrous-fatty lesion showing marked cellularity due to foam cell accumulation and abundant deposits of collagen. The fragmented and deranged internal elastic lamina and substantially thickened tunica media are evident (Verhoeff’s-Sirius red stain). Panel B: Superoxide dismutase; panel C: Glutathione peroxidase; and panel D: Catalase, avidin-biotin-immunoperoxidase staining. Sections exhibit an extensive immunoexpression of the three antioxidant enzymes in early stages of combined hypertension and hyperlipidemia. Simultaneous diffuse extracellular (open arrows) and cell-associated staining (solid arrow) in the lesion is noted. Bar=100 μm.

Discussion

The vascular endothelium clearly faces an oxidative challenge of relative luminal hyperoxia and abluminal hypoxia. In addition to this generally hostile oxidative environment of arteries, reactive oxygen intermediates such as $\cdot O_2$, $H_2O_2$, $OH$, $RO$, and singlet molecular oxygen ($O_2$) are generated, as in any biologic system, even under normal physiological conditions via several pathways, both enzymatic and nonenzymatic. Endogenous sources of free radicals include those produced during oxidative phosphorylation in the mitochondria, from the activity of cyclooxygenase, lipoxygenase peroxidases and dehydrogenase, auto-oxidation, flavins,
To adapt to this oxidative stress, not only endothelial cells but also possibly all cells have evolved a complex AE defense system. The in vivo immunohistochemical demonstration of Cu-Zn SOD, GSH-Px, and CAT in the endothelial cells of the NT/NL control rabbit aortic arch suggests that the vascular endothelium is well equipped in its armamentarium to defend against the highly toxic oxygen free radicals. The importance of the glutathione redox cycle as a potent antioxidant defense system has previously been demonstrated by in vitro studies in human endothelial cells.

Our finding of an identical immunohistochemical expression of the three AEs reflects a harmonious interrelation among Cu-Zn SOD, GSH-Px, and CAT for the detoxification of oxygen metabolites under normal physiological conditions. We postulate that the delicate balance between pro-oxidants and antioxidants could be easily altered during the early stages of hypertension and/or hypercholesterolemia.

In the HT/NL rabbits a greater proportion of the arterial wall displayed Cu-Zn SOD and GSH-Px immunostaining in the early HT lesions, around the lesion shoulders as well as in the nonlesion areas. The CAT reactivity, although increased compared with that of NT/NL, was relatively less intense. We found intracellular as well as extracellular immunohistochemical distribution of the AEs: over the cells of the lesion intima as well as over the extracellular matrix of the lesion, including along the deranged internal elastic lamina. These results may be interpreted to suggest that extensive oxygen free-radical formation occurs during the early stages of hypertension following membrane lipid peroxidation, leading to the release of lysosomal and hydrolytic enzymes. These hydrolytic enzymes not only bring about cell necrosis but also induce drastic modifications in the chemistry of the extracellular matrix.

Membrane lipid peroxidation has been extensively reviewed. The unsaturated fatty acids and phospholipids present in the membrane and the oxidizable amino acids present in transmembrane proteins are the potential targets of oxyradical damage. Significant changes in membrane chemistry impair the functional characteristics, including membrane permeability, fluidity, the activity of membrane-associated enzymes, and finally, cell death. Moreover, it must be emphasized that cell membrane lipids are not the only components at risk from injury by the endogenously generated free radicals. In addition, the extracellular matrix such as glycosaminoglycan and especially collagen also is sensitive to free-radical damage.

There is ample evidence that HT stimuli in experimental animals lead to early arterial wall hypermetabolism and augmented oxygen consumption. Crawford and Kramsch have demonstrated relative subintimal mural hypoxia in the HT rabbit aorta. Similarly, Seidel and Strong observed a clear increase in oxygen consumption in aortic rings from rats with spontaneous, renal, and deoxycorticosterone salt hypertension. These changes in oxygen distribution in the HT arterial wall, with areas of relative hypoxia adjacent to areas of normal oxygen tension, could favor the formation of oxygen free radicals in a fashion analogous to reperfusion after myocardial infarction.

Another condition in which AEs were found distributed in the intima, subintima, and media was in athero-
sclerotic aortas of NT animals fed an atherogenic diet for 6 weeks. We observed an even greater depth of the immunohistochemical distribution of three AEs in the atherosclerotic arterial wall of NT/HL rabbits compared with HT/NT animals. One explanation of this finding could be that the intimal lesions in purely HL rabbits are large fatty streaks composed of many layers of lipid-laden foam cells, while the purely HT lesions are relatively thin fibromuscular thickenings of the intima. As recently demonstrated in our laboratory, Boccio et al,20 who have recently reported an increase in macrophages in the pathogenesis of atherosclerosis. Additionally, Hen-riksen et al2 demonstrated an increase in the activity of Cu-Zn SOD in whole aortas of cholesterol-fed rabbits. Bjornheden and Bondjers12 concluded that, in early rabbit atherosclerosis, aortic mural oxygen consumption is increased because of foam cell activity, thereby causing abluminal hypoxia. Heughan et al49 reported that oxygen tension in the arterial wall of HL rabbits was 10 mm Hg compared with 30–40 mm Hg in normal controls.

The statistical analysis of the data using simple correlation coefficients revealed that the change in total cholesterol was relatively more significant (p < 0.0001) than the change in blood pressure (p < 0.05) in relation to the depth of immunohistochemical localization of three AEs across the arterial wall at the 6-week time point of the experiment. The results of multiple linear-regression analysis revealed that more than 70% of the variability in the depth of the intimal–medial distribution of the three AEs in the arterial wall could be explained by the elevation in blood pressure and the rise of total cholesterol. In addition, significant interaction between the change in blood pressure and the change in cholesterol in relation to the depth of AE distribution suggests that these two variables have a strong predictive capacity during the early stages of HT and atherosclerotic lesion development.

There is now considerable experimental evidence3,5,49,50 indicating the importance of the roles of oxidized LDL and recruitment of monocytes/macrophages in the pathogenesis of atherosclerosis. Additional evidence that oxidized LDL could be generated in vivo comes from studies of the immunohistochemical colocalization of malondialdehyde-conjugated LDL3 or 4-hydroxy-2-nonenal LDL with unmodified LDL. In light of the available data, we can interpret our results of increased AE activity as evidence of increased free-radical formation in the altered sequestered microenvironment of the arterial intima and subintima in HT and also hypercholesterolemic rabbits, with a compounding

### Table 3. Relation Between Differences in Absolute Depth of Intimal–Medial Antioxidant Enzyme Immunostaining and Blood Pressure Change in Treatment Subgroups

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Normolipidemic</th>
<th>Hyperlipidemic</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cu-Zn SOD</td>
<td>NT/NT: 6.8±0.7, n=6&lt;br&gt;HT/NT: 66.4±8.9, n=7&lt;br&gt;p=0.003, r=0.67</td>
<td>NT/HL: 134.8±6.3, n=8&lt;br&gt;HT/HL: 321.0±26.3, n=6&lt;br&gt;p=0.002, r=0.63</td>
</tr>
<tr>
<td>GSH-Px</td>
<td>NT/NT: 8.2±1.3, n=6&lt;br&gt;HT/NT: 67.6±8.6, n=7&lt;br&gt;p=0.003, r=0.67</td>
<td>NT/HL: 137.6±7.0, n=8&lt;br&gt;HT/HL: 320.8±24.8, n=6&lt;br&gt;p=0.002, r=0.66</td>
</tr>
<tr>
<td>CAT</td>
<td>NT/NT: 8.5±1.0, n=6&lt;br&gt;HT/NT: 69.4±9.0, n=7&lt;br&gt;p=0.003, r=0.67</td>
<td>NT/HL: 136.8±5.7, n=8&lt;br&gt;HT/HL: 321.8±25.6, n=6&lt;br&gt;p=0.002, r=0.67</td>
</tr>
</tbody>
</table>

Depth values are mean±SEM of antioxidant enzyme immunostaining.

NT, normotensive; NL, normolipidemic; HT, hypertensive; HL, hyperlipidemic; BP, blood pressure; Cu-Zn SOD, superoxide dismutase; GSH-Px, glutathione peroxidase; CAT, catalase.

1 Wilcoxon rank-sum test, comparing the depth of arterial wall antioxidant enzyme immunostaining between NT and HT animals in NL and HL groups, respectively.

2 Correlation coefficient (Corr coeff) between BP change and depth of arterial wall antioxidant enzyme immunostaining in normolipidemic and hyperlipidemic groups. All are statistically significant (p < 0.05).

### Table 4. Multiple Regression Models Linking Blood Pressure Increase and Total Cholesterol Elevation to Extent of Immunohistochemical Localization of Antioxidant Enzymes Across the Arterial Wall at 6 Weeks

<table>
<thead>
<tr>
<th>Dependent variables</th>
<th>Independent variables</th>
<th>Parameter estimate SEM p Total R²</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cu-Zn SOD</td>
<td>TC (mg/dl)</td>
<td>0.115 0.046 0.02</td>
</tr>
<tr>
<td></td>
<td>BP (mm Hg)</td>
<td>1.113 1.749 0.53</td>
</tr>
<tr>
<td></td>
<td>TC×BP</td>
<td>0.010 0.004 0.03 0.71</td>
</tr>
<tr>
<td></td>
<td>Intercept</td>
<td>44.08</td>
</tr>
<tr>
<td>GSH-Px</td>
<td>TC (mg/dl)</td>
<td>0.117 0.043 0.01</td>
</tr>
<tr>
<td></td>
<td>BP (mm Hg)</td>
<td>1.200 1.667 0.48</td>
</tr>
<tr>
<td></td>
<td>TC×BP</td>
<td>0.010 0.004 0.02 0.73</td>
</tr>
<tr>
<td></td>
<td>Intercept</td>
<td>44.01</td>
</tr>
<tr>
<td>CAT</td>
<td>TC (mg/dl)</td>
<td>0.118 0.045 0.01</td>
</tr>
<tr>
<td></td>
<td>BP (mm Hg)</td>
<td>1.428 1.707 0.41</td>
</tr>
<tr>
<td></td>
<td>TC×BP</td>
<td>0.009 0.004 0.03 0.72</td>
</tr>
<tr>
<td></td>
<td>Intercept</td>
<td>43.25</td>
</tr>
</tbody>
</table>

Cu-Zn SOD, superoxide dismutase; GSH-Px, glutathione peroxidase; CAT, catalase; TC, total cholesterol; BP, blood pressure.

*All the independent variables are changes from baseline to 6 weeks.

Interaction term between blood pressure increase and total cholesterol elevation in relation to intimal–medial depth of antioxidant enzyme immunostaining.
of this effect when both conditions are present simultaneously. Indirect in vitro evidence suggests that oxidation of LDL may be mediated by endothelial cells, SMCs, and macrophages. Native LDL appears to be non-toxic to arterial cells. However, one of the newer hypotheses is that LDL is trapped in the arterial wall by forming complexes with several matrix proteins, and then it could become peroxidated during a prolonged residence time in the vessel. Oxidative modification of LDL has been considered a key step in the generation of macrophage-derived foam cells and in the initiation of the atherosclerotic process; however, the precise mechanisms and the exact sequence of events remain speculative. Steinbrecher et al reported a clear connection between lipid peroxidation and oxidative modification of LDL. We postulate that increased oxygen free-radical formation occurs in the subintima and inner media of HT arteries as well as in arteries exposed to hyperlipidemia, which could be responsible for the oxidative modification of LDL and the subsequent generation of oxidation-specific lipid protein adducts derived from LDL in the atherosclerotic lesions.

Therefore, it becomes clear that the surrounding endothelial cells and SMCs must acquire abundant AEs to shield against the cytotoxic effects of oxyradicals, lipid hydroperoxides, and oxidized LDL. The invasion of macrophages to scavenge oxidized LDL through their scavenger receptors appears to be a reasonably benign function in the early stages of lesion formation. However, later on when the macrophages are overloaded with oxidized LDL due to continuous hyperlipidemia and/or hypertension, they become potentially pathogenic, producing a vicious cycle of respiratory burst, oxyradicals, lipid peroxidation, cytotoxicity, necrosis, and chemotaxis. Steenberg et al contended that oxidative modification is not likely to occur in the circulation and seems to be a local event in the microenvironment of the vascular wall. We found, biochemically, a threefold increase in the levels of cholesterol oxides in the arteries and sera of hypertensive rabbits as well as in rabbits with hypercholesterolemia. This provides additional supportive evidence that the arterial wall is a site of formation of lipid peroxidation products, some of which may have escaped into the bloodstream to account for their presence in the sera.

We conclude that intense oxygen free-radical generation is one possible process in the arterial wall of HT and atherosclerotic animals and that this occurs even in the presence of considerable amounts of antioxidant mechanisms.

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References
Antioxidant Enzymes in Hypertension/Atherosclerosis


Immunolocalization of native antioxidant scavenger enzymes in early hypertensive and atherosclerotic arteries. Role of oxygen free radicals.
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