Antioxidant Enzyme Activity in Human Abdominal Aortic Aneurysmal and Occlusive Disease (44342)

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Abstract. **The present study further investigates evidence for lipid peroxidation in atherosclerotic aortic tissue by determining the activity of antioxidant enzymes and concentrations of lipid peroxide fluorochromes in abdominal aortas from 15 patients with abdominal aortic aneurysms (AAA), an additional 7 patients with ruptured abdominal aneurysms, and 12 patients with atherosclerotic occlusive disease (AOD). Aortas from nonatherosclerotic organ donors served as nondiseased controls. Cu,Zn-superoxide dismutase (Cu,Zn-SOD) activities in AAA and AOD tissues were 16% and 25% of control activity, respectively. Mn-SOD activity in diseased aortae were about 65% of controls. CuZn-SOD protein in AAA and AOD was 56% and 100% of controls, respectively, resulting in significantly lower CuZn-SOD specific activity in these tissues. Ruptured AAA tissue also had low SOD activity and protein. Glutathione peroxidase (GPx) activity in AAA and AOD aortas was 70% and 65% of controls, respectively, and glutathione reductase (GR) activity in AAA and AOD aortas was 80% and 65% of control activities, respectively. These results were associated with significantly higher lipid peroxide fluorochromes, expressed as U/g aorta, in both groups of atherosclerotic aortas than in controls. AOD aortas had 33% higher fluorescence than AAA aortas, but the highest levels were seen in ruptured AAA. These data further support the involvement of free radicals and lipid peroxidation in atherosclerotic aortic disease, but do not indicate that these mechanisms are specifically involved in aneurysm formation versus development of occlusive disease.** [P.S.E.B.M. 1999, Vol 220]

Atherosclerotic disease of the infrarenal aorta is most commonly associated with either progressive stenosis or dilation and aneurysm formation. Abdominal aortic aneurysms (AAA) and atherosclerotic occlusive disease (AOD) have been characterized by distinct differences in their morphology, particularly in their connective tissue metabolism (1). For example, in AAA the aortic wall is attenuated due to a reduction in the smooth muscle and elastin content, whereas AOD is characterized by increased thickness of the aortic wall. Despite these differences, AAA and AOD share many of the manifestations of atherosclerosis, including risk factors such as cigarette smoking, hypertension, and dietary and genetic factors (2, 3). However, these risk factors can account for only 50%–60% of the incidence of atherosclerosis (4). Therefore, other as yet unidentified risk factors must contribute to the pathogenesis of the disease.

Recent evidence suggests that free radical mechanisms are involved in the etiology of atherosclerosis (4–7). It has been shown that the oxidative modification of low-density lipoprotein (LDL) can be induced by free radicals and can

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lead to the pathophysiological events associated with plaque formation in the arterial wall (8, 9). In addition, epidemiological and animal experimental studies suggest that antioxidant status may mediate the development of ischemic heart disease and atherosclerosis (10, 11). It would appear that free radical mechanisms may also be involved in the development and progression of AAA and AOD. Studies in our laboratory observed abnormal copper, zinc, and iron metabolism, low ascorbic acid levels, and markedly lower superoxide dismutase activity in AAA and AOD tissue when compared with controls (12–14). Most recently we have observed that human atherosclerotic aortas contained higher vitamin E concentrations and higher levels of conjugated dienes than aortas from organ donors (15). The present study further tests our hypothesis that free radicals play a role in the progression of AAA and AOD and that indices of oxidative stress should be highest in the ruptured AAA group. In addition, this study reports, for the first time, glutathione peroxidase (GPx) and glutathione reductase (GR) activities and Cu,Zn-superoxide dismutase (Cu,Zn-SOD) protein levels in aneurysmal and occlusive disease of the infrarenal aorta, including ruptured aneurysms.

Materials and Methods

Human Aortic Tissue. Aortic tissue from the infrarenal aorta was obtained at surgery from patients with AAA or AOD. Aortic tissue from the patients with ruptured abdominal aneurysms was obtained at the time of emergency surgery. This protocol was approved by the Human Subjects Review Committee of the University of Arizona. All specimens were taken from the abdominal aorta at least 2 cm below the origin of the renal arteries and included the entire thickness of the aortic wall. Specimens were then carefully stripped of surrounding periadventitial fat and surface thrombus, snap frozen, weighed, and stored at −70°C until analyzed. As in previous studies, AOD tissue served as the atherosclerotic control to AAA (12, 14). Tissue removed from the same region of the aorta from organ donors served as nondiseased controls.

Potential risk factors for atherosclerosis were recorded. These included smoking history, age, and the presence of hypertension. Hypertension was defined as a blood pressure exceeding 140/90 mm Hg with or without antihypertensive therapy (14). In addition, a piece of each aortic sample was fixed in phosphate-buffered formalin and processed for light microscopy according to standard technique. Sections were stained with hematoxylin and eosin.

Biochemical Studies. Aortae were homogenized (9:1, v/w) in 0.25 *M* sucrose/10 m*M* Tris-HCl (pH 7.0), sonicated, and centrifuged at 4°C at 10,000 rpm for 30 min as described previously (12). Enzyme assays were conducted on the supernatant. Se-dependent glutathione peroxidase (GPx) activity in aorta was determined indirectly by measuring the rate of oxidation of NADPH in the presence of H_2O_2, GSH, and GSH-reductase as described by Lawrence and Burk (16). Glutathione reductase (GR) activity was measured spectrophotometrically by the method described by Rogers and Augusteyn (17). Data were expressed as nmol NADPH oxidized/min/mg protein (mUnits/mg protein). Superoxide dismutase (SOD) activity was determined by the method of Marklund and Marklund (18). Mn-SOD activity was distinguished from total SOD activity by the addition of 1 m*M* KCN in the assay buffer. Cu,Zn-SOD activity was then calculated by subtracting the Mn-SOD activity from total activity, as previously described (12). CuZn-SOD protein was determined by ELISA following the general approach used by DiSilvestro and David (19) for human ceruloplasmin. The interassay Coefficient of Variation for the SOD ELISA assay was 2.4%. The coating antibody was sheep anti-human Cu,Zn-SOD (Binding Site, San Diego, CA), and the sandwich antibody was rabbit anti-human Cu,Zn-SOD developed in Dr. DiSilvestro's laboratory. The human Cu,Zn-SOD protein used to prepare this antibody gave a single band in SDS polyacrylamide gel electrophoresis upon analysis of 150 μg of protein. The antibody gave a single precipitin band in Oucterlony interaction with Cu,Zn-SOD protein or human erythrocyte extract. Specific activity of Cu,Zn-SOD was expressed as units/mg enzyme protein. Protein concentrations were determined using a commercially available dye-binding kit (BioRad Laboratories, Richmond, CA).

Estimates of lipid peroxidation in aortic tissue were determined fluorometrically as described by Tappel (20) with slight modification (13). Aortic tissue underwent a 2:1, v/v chloroform:methanol extraction of the lipid fraction. After 20 hr, the extracts were centrifuged for 15 min at 900*g*. The lipid fraction was isolated and lipid peroxidation estimated fluorometrically (excitation 355 nm, emission 435 nm) as described (13, 15).

Statistics. Data are expressed as mean ± SD and were analyzed by analysis of variance. If a significant F-statistic was obtained, a Newman-Keuls test was performed to determine statistical significance from the controls. A $P < 0.05$ was considered significant.

Results

Patients with AAA, ruptured AAA, or AOD were of similar age and had similar smoking histories (Table I). As expected, the ages of the organ donors were younger in order to obtain normal aorta essentially free of grossly visible atherosclerotic disease. Hypertension was found in about 30% of the AOD and over 50% of the AAA patients. A majority of patients in the AOD and AAA groups also smoked. Histologic sections of the aortae from AOD and AAA tissue contrast these two forms of atherosclerotic disease (Fig. 1). Whereas the AOD aortic specimen showed increased thickness of the aortic wall with cholesterol deposits, the abdominal aortic aneurysmal specimen showed marked attenuation of the intimal and medial layers of the aortic wall. In comparison, histologic sections of abdominal aorta from an organ donor showed only mild intimal thickening with intact media (Fig. 1).

Table I. Demographic Data from Organ Donors and Patients with Aortic Disease

Tissue	Organ donors	Occlusive disease	Aneurysms	Ruptured aneurysms
n	15	12	15	7
Age[a] (years)	38.6 ± 14.7	65.3 ± 7.6	70.2 ± 5.8	70.3 ± 8.5
Age Range	15–65	51–76	56–79	56–80
Male	12	10	14	5
Female	3	2	1	2
Hypertension	—	4	8	2
Smokers	6	6	10	2

[a] Data are expressed as mean ± SD.

To determine whether the data obtained from the organ donors simply reflected their younger age when compared with the AOD and AAA patients, organ donors were divided into those under 40 years old (n = 8) and those over 40 years old (n = 7). No significant differences in Se-dependent glutathione peroxidase (GPx) nor glutathione reductase (GR) activities, expressed as mU/mg protein, were observed between the two age groups (GPx mean ± SD: 11.63 ± 2.05 vs 11.95 ± 2.44 and GR: 2.10 ± 0.81 vs 1.89 ± 0.47, respectively. Mn- and Cu,Zn-SOD activities tended to be sightly higher in the younger than older controls, but the differences were not statistically significant (Mn-SOD: 2.74 ± 1.49 vs 1.88 ± 0.91 and Cu,Zn-SOD: 3.48 ± 1.85 vs 2.99 ± 2.62, respectively). Lipid peroxide fluorochrome concentrations were also not significantly different between younger and older controls (1.89 ± 0.38 vs 2.02 ± 0.49 × 10^4 FU/g). As a consequence, control data were pooled into a single group to compare with the diseased aortas.

In general there were no significant differences in antioxidant enzyme activities between AAA and AOD tissues. Activities of antioxidant enzymes in diseased aorta, however, were significantly lower than in nondiseased control aorta. Mn-SOD activities in AOD and AAA tissue were both about 65% of control aorta, whereas Mn-SOD activity in ruptured AAA was only 32% of controls (Fig. 2). Cu,Zn-SOD activities in AOD and AAA tissue were 25% and 16% of controls, respectively, and Cu,Zn-SOD activity in ruptured AAA tissue was 35% of control activity (Fig. 2).

Cu,Zn-SOD enzyme protein concentrations in AOD tissue were similar to controls. Enzyme protein concentrations in AAA and ruptured AAA were about 57% of control levels. (Fig. 3, top). Taken together, these data resulted in significantly lower ratios of Cu,Zn-SOD activity to SOD protein (specific activity) in both AOD and AAA tissues compared with controls (Fig. 3, bottom). Interestingly, Cu,Zn-SOD specific activity in ruptured AAA was similar to controls (Fig. 3, bottom).

GPx and GR activities are presented in Figure 4. GPx activity was 65% and 70% of control activity in AOD and AAA tissue, respectively. GPx activity in ruptured AAA was 42% of controls. GR activity in AOD and AAA tissue was 43% and 33% lower than controls, respectively, but in ruptured aneurysms, GR activity was similar or slightly higher than in controls (Fig. 4).

Concentrations of lipid peroxide fluorochromes showed a tendency to be higher than controls in all diseased aortas assayed. Fluorochrome levels were 66%, 90%, and 141% higher than controls in AOD, AAA, and ruptured AAA tissues, respectively (Fig. 5). However, due to variation in the diseased tissue, only AAA tissue achieved statistical significance when compared with controls.

Discussion

A review of the current literature supports the complexity of the etiology of abdominal aortic aneurysms. In addition to genetic factors (21, 22) and the involvement of inflammatory mediators (23, 24), the presence of atherosclerosis (25, 26) appears to be a consistent finding. AAA ranks as the 13th leading cause of death in the United States (27, 28), and AAA's age- and sex-adjusted incidence has tripled over the past 30 years (29) despite decreases in mortality from coronary artery disease and cerebrovascular insults (27). Therefore, it is important to understand as many aspects of the disease pathophysiology as possible. It should be noted that the histologic assessment of the abdominal aorta from patients in the current study is consistent with the well-defined morphology associated with aneurysmal and occlusive disease of the abdominal aorta (30).

Although recent studies have linked free radicals to atherosclerotic disease, the majority of studies supporting this hypothesis have focused on the role of oxidized LDL in the disease process (5–9). However, few studies have examined whether free radical processes also may occur in other aspects of atherosclerotic disease. The present study furthered our investigations into the role of free radical mechanisms in the expression of aortic occlusive and abdominal aneurysmal disease.

In the present study, we observed markedly lower Cu,Zn-SOD activity in both AAA and AOD tissue compared with controls. These data are in agreement with our previous preliminary observations in disease aorta (12, 14). In addition, we observed that although Cu,Zn-SOD protein concentrations were significantly lower in AAA tissue than controls, Cu,Zn-SOD concentration in AOD tissue was similar to controls. These data indicate a low Cu,Zn-SOD specific activity (Units/mg SOD protein) in both aneurysmal and occlusive disease. In AAA tissue, the present results suggest that the low enzyme activity is due, at least in part, to the low concentration of apoenzyme. In contrast, in AOD tissue, the concentration of apoenzyme is similar to

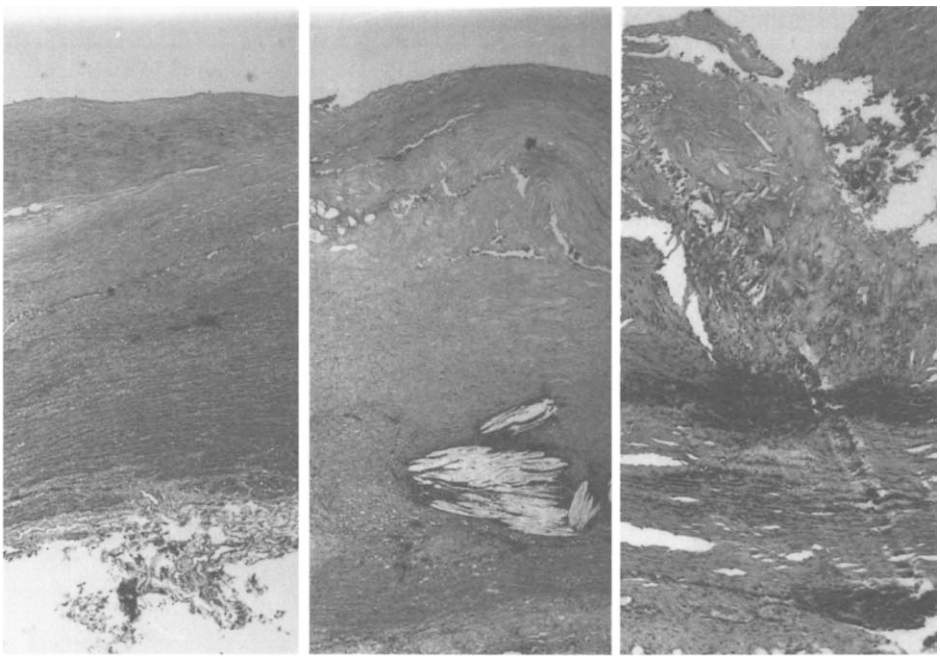

Figure 1. Histologic sections of the aortic wall from a (left panel) representative organ donor and patients with (center panel) AOD and (right panel) AAA. (Left panel) There is mild intimal thickening with an intact media. (Center panel) Increased thickening of the aortic wall with typical features of an occlusive plaque is present. Plaque region shows cholesterol clefts in a lipid core. (Right panel) There is marked attenuation of the intimal and medial layers of the aortic wall, with an inflammatory cell infiltrate in the media and adventitia. Hematoxylin and eosin. 80×.

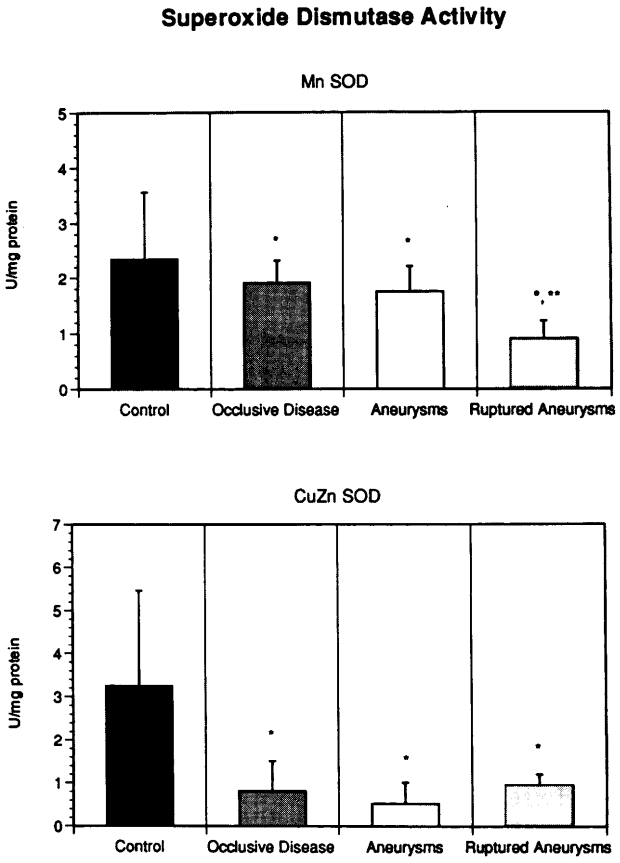

Figure 2. Mn- and Cu,Zn-superoxide dismutase activity in control and diseased abdominal aortae. Data, as Units/mg aortic protein, represent the mean ± SD. For nondiseased controls and AAA groups, $n = 15$, for AOD group $n = 12$, and for ruptured AAA group, $n = 7$. $^{*}P < 0.05$ from control. $^{**}P < 0.05$ from occlusive disease and aneurysms groups.

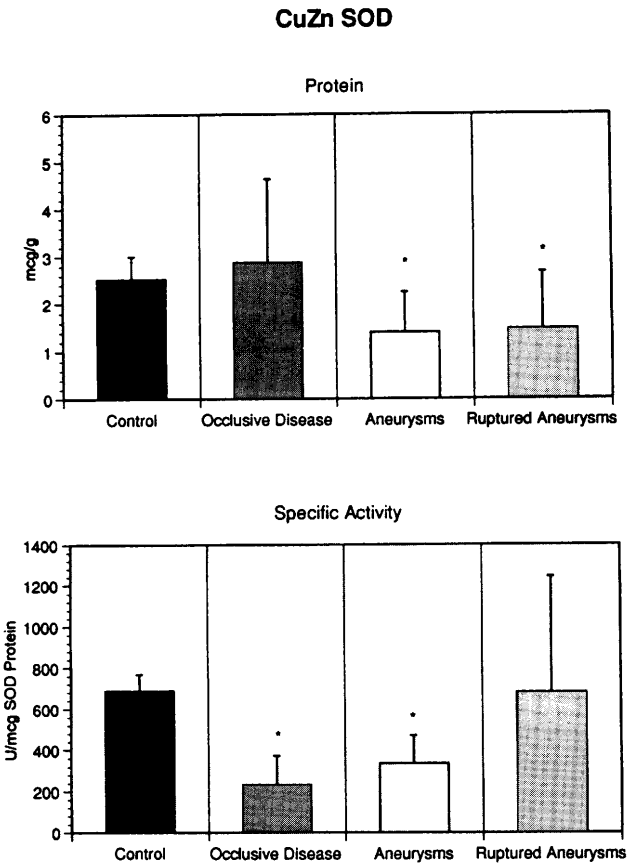

Figure 3. Cu,Zn-superoxide dismutase protein concentration and specific activity in control and diseased abdominal aorta. Data represent the mean ± SD. Number of samples per group are as stated in the legend to Figure 2. $^{*}P < 0.05$ from control.

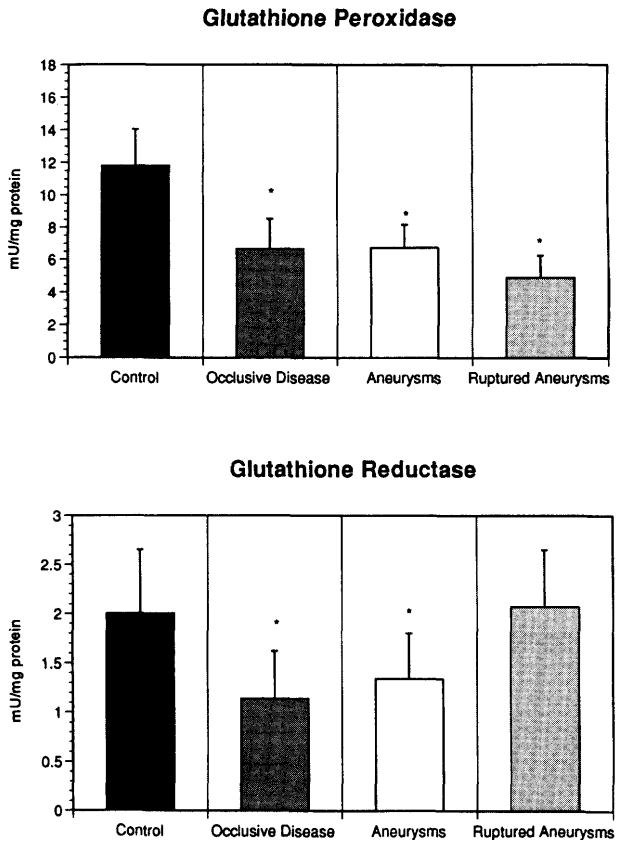

Figure 4. Glutathione peroxidase and glutathione reductase activity in control and diseased abdominal aorta. Data are expressed as the mean ± SD. Number of samples per group are as stated in the legend to Figure 2. $^*P < 0.05$ from control and ruptured aneurysms.

controls, but enzyme activity is low. With respect to Cu,Zn-SOD, a low enzyme activity to protein ratio suggests impaired copper status in these tissues. In rats deficient or marginal in copper status, DiSilvestro and Marten (31) reported low SOD activity to protein ratios. However, our previous studies found that AAA and AOD tissue actually had higher copper concentrations than did control aortas (12). In addition work by us (32) and others (33) indicated that most of the aortic Cu in diseased tissue was in the plaque rather than the surrounding tissue. It should also be mentioned that Tilson (34) observed markedly lower hepatic Cu levels in AAA patients when compared with control subjects. Taken together, these data suggest more of a functional or compartmental copper deficiency in diseased aorta than a deficiency based solely on low tissue levels. For example, in AOD tissue, there could be a defect in Cu transport into the SOD apoprotein. Nevertheless, the present data indicate that aneurysmal tissue expresses both low Cu,Zn-SOD activity and protein concentrations, whereas occlusive diseased aortas expressed low enzyme activity but normal enzyme protein levels. Considering previous studies on the potential role of adverse Cu metabolism in cardiovascular disease (35), further studies into Cu utilization seem warranted to elucidate the apparently different mechanisms responsible for the overall low SOD activity in AOD versus AAA tissue.

In addition, results from the present study showed that Mn-SOD activity was significantly lower in both AAA and AOD specimens compared to controls when activity was expressed per milligram protein. This observation differed from our previous findings that suggested that Mn-SOD activity, when expressed per gram wet weight, was similar or even slightly higher in diseased aortas than in controls (12, 14). The present results reflected the observation that diseased aortas have 70%–75% higher protein concentrations than control aortas. This could account, at least in part, for the differences observed among the different studies.

The present study also observed significantly lower GPx and GR activity in diseased aortas compared with controls. To our knowledge this is the first report on activity of these enzymes in AAA and AOD tissue. Interestingly, GR activity in ruptured aneurysms was similar to controls and significantly higher than in nonruptured AAA tissue. These data indicate that although ruptured AAA also generally

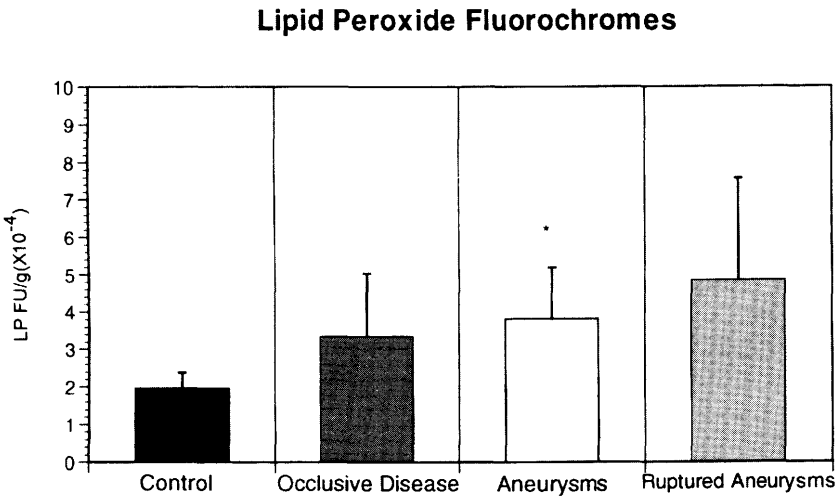

Figure 5. Lipid peroxide fluorochrome levels in control and diseased abdominal aortae. Data are expressed as the mean ± SD. Number of samples per group are as stated in the legend to Figure 2. $^*P < 0.05$ from control.

have low antioxidant enzyme activity, they do not have the lowest as we hypothesized. Since rupture remains the most serious outcome of aneurysms, future studies are needed to determine how the present findings interplay with the various risk factors associated with ruptured aneurysms (36) and with previous studies suggesting that ruptured aneurysms express higher collagenase activity than nonruptured AAA (1, 36, 37).

Finally, the present study showed that lipid peroxide fluorochrome concentrations in AAA and AOD tissue were significantly elevated over control levels, with the highest concentrations observed in ruptured aneurysms. Although this method as an index of lipid peroxidation is less frequently used than the thiobarbituric acid (TBA) assay, previous investigations suggest that this method detects more products of lipid peroxidation associated with atherosclerosis than the TBA assay (13). This method has been employed successfully to detect lipid soluble products of oxidative damage in studies of aging, ozone exposure, and dietary antioxidant manipulations (38). Employing this method in previous studies, we observed high lipid peroxide fluorochrome concentrations in atherosclerotic vessels (13, 15), in agreement with others who observed increased lipid peroxides in plasma from patients with atherosclerosis (7). It is possible that the higher lipid peroxidation observed in diseased aortas reflects the higher iron concentrations observed in these tissues compared with nondiseased controls (12, 14). Iron is a well-known catalyst in the formation of hydroxyl radicals *via* the Haber-Weiss or Fenton reactions. However, although total iron is higher in diseased aortas than in controls, it is unknown whether any of the iron exists in a free state or labile pool that could contribute to lipid peroxidation. In addition, since iron concentrations were higher in AAA than in AOD tissue (12, 14, 32), it would seem that other mechanisms are also involved in the generation of lipid peroxidation in these tissues.

In addition, key questions remain as to the source of the free radicals and their mechanistic role in these disease processes. We have discussed previously the significance of hypertension as a major risk factor in atherosclerosis and AAA development, and that free radical mechanisms are associated with hypertension (14, 15). Smoking is also a well-known major risk factor associated with atherosclerosis and AAA (2, 39, 40), and studies have shown that AAA development is related to the number of cigarettes smoked and the depth of inhalation (41). Cigarette smoking has long been associated with the generation of oxidants and free radicals (42), and a number of studies have shown that cigarette smokers have lower serum antioxidant vitamin concentrations than nonsmokers (43, 44). Nevertheless the results of the current and previous studies (15) indicate that free radical mechanisms independent of hypertension and cigarette smoking are also associated with atherosclerotic disease of the infrarenal aorta, but these mechanisms do not distinguish between aneurysm formation or development of occlusive disease. At present it remains unknown whether free radicals are involved in the initiation or progression of the disease, or are simply a passive marker of damaged tissue. Whatever the initiating factor of atherosclerotic disease, experimental evidence suggests that disease progression may be slowed by antioxidants (6, 45). This would suggest that free radicals are important for the progression of atherosclerotic disease, possibly interfering with repair of the vascular tissue as the disease progresses. The present observations of similar levels of Cu,Zn-SOD protein in AOD and control aortas and higher protein concentrations in diseased aortas, suggest that the antioxidant status of AAA and AOD reflect the disease process and are not just a consequence of the disease.

In summary, results of the present study indicate that AAA and AOD tissue are associated with lower antioxidant enzyme activity and increased lipid peroxidation when compared to nondiseased control aortic tissue. Although the controls were younger than the diseased group, the present data from the organ donor group divided into those older than 40 versus those younger than 40, failed to detect any significant differences in the variables determined. Thus, these data agree with our previous studies that no age-related correlation in antioxidant enzyme activity or protein was detected (12–15). Therefore, the data in the present study do not simply represent age-related differences. Further, we suggest that both forms of atherosclerotic aortic disease express a similar, but not identical, "oxidative injury" profile. Interestingly, this profile varies somewhat in ruptured aneurysms. In addition, a tendency toward lower SOD protein concentrations in AAA than in AOD tissue may indicate mechanistic differences that result in a similar expression of lower SOD activity in these tissues. Further studies are needed to investigate whether apoprotein levels of the other antioxidant enzymes measured are also differentially affected among the various forms of atherosclerotic aortic disease.

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