The relation among glutathione-related enzyme activities, thiobarbituric acid–reactive substances of the human aorta and internal mammary artery, and serum lipids was studied in 40 male patients undergoing coronary revascularization. Glutathione peroxidase and glutathione reductase activities were significantly higher in the internal mammary artery, whereas glutathione transferase activity was elevated in the aortic wall. Moreover, non–selenium-dependent glutathione peroxidase activity was detectable only in the aorta. The levels of thiobarbituric acid–reactive substances were significantly higher in the aorta. A positive correlation was found among the activity of glutathione peroxidase, glutathione reductase, and thiobarbituric acid–reactive substances in the internal mammary artery and total cholesterol, low density lipoprotein cholesterol, and triglycerides. In the aortic wall, a positive correlation among the activity of glutathione peroxidase, glutathione transferase, thiobarbituric acid–reactive substances, and the previously mentioned serum lipids was evident. In contrast, high density lipoprotein cholesterol was inversely related to enzymatic activities and thiobarbituric acid–reactive substances in both the internal mammary artery and aorta. In conclusion, significant differences in the levels of glutathione-related enzyme activities and thiobarbituric acid–reactive substances in the internal mammary artery and aorta were found, suggesting a different ability of the two tissues to counteract oxidative stress: the glutathione-related antioxidant properties and the level of lipid peroxidation in the arterial tissue seem to be specifically influenced by serum lipids. (Arteriosclerosis and Thrombosis 1992;12:92–98)

Free radical–induced lipid peroxidation has been proposed as an etiologic factor in atherosclerosis. Increased levels of lipid peroxides have been found in the blood and arterial wall of patients with hyperlipidemia and/or atherosclerotic disease. Moreover, a high-fat, cholesterol-rich diet has recently been shown to induce significant changes in the antioxidant enzymatic defenses and lipid peroxidation in the rabbit aorta. In particular, an increase in selenium-dependent glutathione peroxidase (GSH-px) activity has been observed, apparently paralleling plasma cholesterol levels.

Glutathione (GSH)-related enzymes are generally considered to play an important role in the elimination of toxic products of lipid peroxidation. Variations in the regional distribution of GSH-related antioxidant defenses and thiobarbituric acid–reactive substances (TBARS) have been shown in the normal rabbit aorta, thus suggesting the possibility of different degrees of oxidative stress. In this context, different antioxidant properties have also been demonstrated in the human internal mammary artery (IMA) and saphenous vein. Even though a positive correlation between plasma and tissue lipid peroxides and plasma cholesterol and triglycerides levels has been reported, little is known concerning possible relations among blood lipids and enzymatic antioxidant defenses and lipid peroxidation in the human arterial tissue.

To address this issue, the specific activities of GSH-related enzymes as well as the levels of TBARS in the IMA and ascending aorta, which show different susceptibility to atherosclerosis, were investi-
gated. The relation among tissue activities of GSH-related enzymes, TBARS, and the levels of serum lipids was also evaluated.

Methods

Patient Population and Serum and Tissue Samples

The study was performed with 40 male patients who were undergoing aortocoronary bypass surgery at the Department of Cardiovascular Surgery of the University of Chieti. The mean (±SD) age of the patients was 54±8.7 years (range, 39–68 years). Informed consent to the study was obtained from each patient. Only patients with mild to moderate essential hypertension were included in the study. No patient had been taking lipid-lowering drugs or substances with a known antioxidant effect for at least 1 month. In all patients, blood samples for serum lipid determination were drawn 48 hours before bypass surgery.

The extra length of IMA not used for coronary grafting and the biopsies routinely taken while attaching the grafts to the ascending aorta were used in this study. To exclude possible atherosclerotic involvement of the arterial tissues, histological screening was performed, and samples from plaque areas or complicated lesions were excluded from the study.

Lipoprotein and Lipid Determinations

The blood was obtained by venous puncture into Vacutainer tubes (Becton Dickinson, Mountain View, Calif.) after an overnight fast (12 hours). Serum levels of total cholesterol (TC) and triglycerides (TGs) were measured by the use of Monotest (CHOD-PAP) and Peridichrom (GPO-PAP) kits from Boehringer Mannheim, Mannheim, FRG.15 High density lipoprotein cholesterol (HDL-C) was measured in serum after precipitation of apolipoprotein (apo) B-containing lipoproteins with polyethylene glycol-6000.16 The low density lipoprotein cholesterol (LDL-C) content was calculated by the Friedewald formula17: TC-HDL-C-(TGs/5).

Tissue Processing

The freshly taken samples were immediately rinsed and placed in ice-cold 0.1 M KH₂PO₄ buffer with 1 mM EDTA (pH 7.4). Then the perivascular tissue was carefully excised, and the adventitia was dissected and discarded. The samples were blotted dry and stored in liquid nitrogen until processed. No loss of enzymatic activity was noted after storage for at least 2 months. Each sample was weighed, minced, and homogenized with two 20-second bursts of an Ultraturrax homogenizer, while allowing 30-second rests between bursts in 10 mM KH₂PO₄ buffer (1:12, w/vol), pH 7, containing 1 mM EDTA. The homogenate was first centrifuged at 800g to remove cellular debris. The resultant supernatant was used to assess tissue content of TBARS. Another aliquot was further centrifuged at 50,000g for 60 minutes to obtain the cytosolic fraction, which was used for determination of enzymatic activities.

Glutathione-Related Enzyme Activity Determination

GSH-px activity was measured essentially according to the method of Paglia and Valentine.18 To determine the fractions of the selenium-dependent and selenium-independent GSH-px, the enzymatic activities were recorded by using both hydrogen peroxide and cumene hydroperoxide, respectively, as substrates.19 The final concentration of peroxides was 0.25 mM for H₂O₂ and 1.2 mM for cumene hydroperoxide. The assay solution contained 50 mM KH₂PO₄ buffer (pH 7.0), 1 mM EDTA, 1.5 mM NaN₃, 1 mM GSH, 0.16 mM NADPH, 4 μg glutathione reductase (GSSG-rx), and a suitable sample (0.04–0.08 mg) of the enzyme solution. After 5 minutes of preincubation, the reaction was started with the addition of either peroxide. The value for a blank reaction (with the enzyme source replaced by water) was subtracted for each assay. The rate of reaction was recorded at 25°C by following the decrease in absorbance at 340 nm. Specific activity was expressed as milliunits per milligram protein; 1 milliunit represents 1 nanomole GSH oxidized per minute per milligram protein.

Glutathione transferase (GST) activity was measured by the method of Habig et al.20 The assay mixture contained 0.1 M KH₂PO₄ buffer (pH 6.5), 1 mM EDTA, 2 mM GSH, 1 mM 1-chloro-2,4-dinitrobenzene, and a suitable amount of enzyme source. The reference cuvette contained the complete assay mixture, with the enzyme having been replaced by water. Enzymatic assay was carried out at 25°C. Specific activity was expressed as milliunits per milligram protein, each milliunit representing 1 nanomole GSH conjugated per minute per milligram protein. GSSG-rx activity was determined as previously described.11,12 The assay mixture contained 0.1 M KH₂PO₄ buffer (pH 7.4), 1 mM EDTA, 1 mM GSSG, 0.16 mM NADPH, and an appropriate amount of enzyme source. The blank did not contain GSSG. Enzyme activity was determined at 25°C by measuring the disappearance of NADPH at 340 nm and was expressed in milliunits per milligram protein. One milliunit is 1 nanomole NADPH oxidized per minute per milligram protein. All enzymatic activities, in a final volume of 1 ml, were performed in duplicate and at two or more different protein concentrations. The method of Bradford22 was employed for the determination of protein concentration of the cytosolic fraction, with gamma globulin as the protein standard.

Thiobarbituric Acid–Reactive Substances

TBARS were measured by the modified method of Uchiyama and Mihiara.23 An aliquot volume of the vessel homogenate, prepared as previously described, was added to 3.0 ml 1% H₃PO₄, 1 ml 0.6% thiobarbituric acid in aqueous solution, and 0.2 ml 8.1% sodium dodecyl sulfate. The mixture was boiled at 95°C for 45 minutes. After cooling, the resultant chromogen was extracted with 4.0 ml n-butanol, and
a brief centrifugation was performed to separate the phases. The absorbance of the organic layer was measured at 532 and 520 nm with a double-beam Varian DMS 200 spectrophotometer (Varian, Sunnyvale, Calif.) against an appropriate blank. The calculations were based on the difference in absorbances at 532 and 520 nm to avoid background problems. The values were calculated as nanomoles TBARS per gram wet tissue, with $1.56 \times 10^{-3} M^{-1} \times cm^{-1}$ as the molar extinction coefficient.

Statistics

The results are expressed as mean±SEM. Differences between the two arterial tissues were compared by paired t test, with $p<0.05$ considered significant. The relations between tissue GSH-related enzyme activities and TBARS with serum levels of HDL-C, TC, LDL-C, and TGs were estimated and tested for statistical significance by the regression calculation technique.

Results

The serum levels of HDL-C, TC, LDL-C, and TG are reported in Table 1. The IMA levels of GSH-px activity (Table 2) were significantly higher than those in the aortic samples ($12.1 \pm 0.6$ versus $4.6 \pm 0.35$ milliunits/mg, respectively, $p<0.0001$); the aortic GSH-px activity assessed with cumene hydroperoxide was found to be significantly higher than that with hydrogen peroxide (Table 2), suggesting the existence of a non-selenium-dependent GSH-px activity ($4.6 \pm 0.35$ versus $2.71 \pm 0.20$ milliunits/mg, $p<0.0001$). GSSG-rx activities were also significantly lower in the ascending aorta than in the IMA ($4.6 \pm 0.35$ versus $2.7 \pm 0.20$ milliunits/mg, $p<0.0025$).

There was a strong inverse correlation between serum HDL-C (Table 3) and the IMA’s levels of total GSH-px ($r=0.67$, $p<0.0001$) and GSSG-rx ($r=0.41$, $p<0.0008$). On the contrary, a significant direct correlation between TC, LDL-C, and TG, and GSH-px ($r=0.41$, $p<0.0009$; $r=0.38$, $p<0.0002$; and $r=0.38$, $p<0.013$, respectively) or GSSG-rx ($r=0.47$, $p<0.0023$; $r=0.43$, $p<0.0005$; and $r=0.44$, $p<0.0005$, respectively) was found (Table 3). When we analyzed the relations between HDL-C, TC, LDL-C, and TG and the enzyme activities of the aortic samples, the following correlations were found (Table 3). HDL-C was inversely related to GSH-px ($r=0.58$, $p<0.0001$), GSSG-rx ($r=0.58$, $p<0.0001$), and GST ($r=0.43$, $p<0.0053$); in contrast, TC, LDL-C, and TG were directly correlated to GSH-px ($r=0.33$, $p<0.036$; $r=0.41$, $p<0.0077$); and $r=0.33$, $p<0.04$, respectively.

Table 2. Glutathione-Related Enzyme Activities and Thiobarbituric Acid-Reactive Substances in the Ascending Aorta and Internal Mammary Artery in 40 Male Patients Undergoing Coronary Artery Revascularization

<table>
<thead>
<tr>
<th>Enzymes</th>
<th>Ascending aorta</th>
<th>Internal mammary artery</th>
</tr>
</thead>
<tbody>
<tr>
<td>GSH-px</td>
<td>H$_2$O$_2$ 2.7±0.20</td>
<td>12.4±0.60*</td>
</tr>
<tr>
<td></td>
<td>CHP 4.6±0.35§</td>
<td>12.1±0.58†</td>
</tr>
<tr>
<td>GSSG-rx</td>
<td>2.6±0.20</td>
<td>3.3±0.16*</td>
</tr>
<tr>
<td>GST</td>
<td>47±2.53</td>
<td>24.6±1.53$</td>
</tr>
<tr>
<td>TBARS</td>
<td>3.09±0.14</td>
<td>2.62±0.11†</td>
</tr>
</tbody>
</table>

Values are mean±SEM.

GSSG-rx was positively and significantly correlated only to LDL-C ($r=0.32$, $p<0.04$). GST activity was significantly higher in aortic samples than in IMAs ($47±2.53$ versus $24.6±1.53$ milliunits/mg, respectively, $p<0.0001$). A significant correlation between IMA GST and serum HDL-C, TC, LDL-C, or TG was not present. In contrast with the IMA, a significant inverse relation between GST and HDL-C was present ($r=0.43$, $p<0.0051$) for aortic samples (Table 3). Furthermore, aortic GST activities were directly and significantly correlated with serum TC, LDL-C, and TG ($r=0.50$, $p<0.001$; $r=0.58$, $p<0.0001$; and $r=0.31$, $p<0.05$, respectively). TBARS levels (Table 2) were significantly higher in the ascending aorta than in the IMA ($3.09±0.14$ versus $2.62±0.11$ nmol/g, respectively, $p<0.0005$). TBARS, both in the IMA and ascending aorta, were positively correlated to serum TC, LDL-C, and TG (IMA: $r=0.55$, $p<0.0003$; $r=0.54$, $p<0.0003$; and $r=0.36$, $p<0.024$, respectively; in the ascending aorta, $r=0.58$, $p<0.0001$; $r=0.49$, $p<0.0011$; and $r=0.45$, $p<0.0035$, respectively), whereas they were inversely correlated to HDL-C (IMA: $r=0.48$, $p<0.0015$; ascending aorta: $r=0.39$, $p<0.012$) (Figures 1 and 2). Because the antioxidant enzymatic system may be influenced by aging, possible correlations with age were evaluated, but no relation was found.

Discussion

The present study shows significant quantitative and qualitative differences in the levels of GSH-related enzyme activities between the IMA and the ascending aorta suggesting, a different ability of the two tissues to counteract oxidative stress. Aortic total GSH-px activity was found to be significantly lower compared with that of the IMA, but this lower total GSH-px value was associated with a higher value of GST and the appearance of a non–selenium-depen-
TABLE 3. Correlations Between Reduced Glutathione-Related Enzyme Activities and Thiobarbituric Acid- Reactive Substances Levels in Arterial Walls and Serum Lipid Values in 40 Male Patients Undergoing Coronary Revascularization

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Serum</th>
<th>HDL-C</th>
<th>TC</th>
<th>LDL-C</th>
<th>TG</th>
</tr>
</thead>
<tbody>
<tr>
<td>Internal mammary artery</td>
<td>GSH-px</td>
<td>$r = -0.67$</td>
<td>$p &lt; 0.0001$</td>
<td>$r = 0.55$</td>
<td>$p &lt; 0.014$</td>
</tr>
<tr>
<td></td>
<td>GSSG-rx</td>
<td>$r = -0.41$</td>
<td>$p &lt; 0.009$</td>
<td>$r = 0.43$</td>
<td>$p = 0.44$</td>
</tr>
<tr>
<td></td>
<td>GST</td>
<td>$r = -0.11$</td>
<td>$p &lt; 0.0023$</td>
<td>$r = 0.056$</td>
<td>$r = 0.13$</td>
</tr>
<tr>
<td></td>
<td>TBARS</td>
<td>$r = -0.48$</td>
<td>$p &lt; 0.0015$</td>
<td>$r = 0.54$</td>
<td>$p &lt; 0.024$</td>
</tr>
<tr>
<td>Ascending aorta</td>
<td>GSH-px</td>
<td>$r = 0.58$</td>
<td>$p &lt; 0.0001$</td>
<td>$r = 0.34$</td>
<td>$p &lt; 0.41$</td>
</tr>
<tr>
<td></td>
<td>GSSG-rx</td>
<td>$r = 0.58$</td>
<td>$p &lt; 0.036$</td>
<td>$r = 0.27$</td>
<td>$p = 0.32$</td>
</tr>
<tr>
<td></td>
<td>GST</td>
<td>$r = 0.43$</td>
<td>$p &lt; 0.0001$</td>
<td>$r = NS$</td>
<td>$p &lt; 0.04$</td>
</tr>
<tr>
<td></td>
<td>TBARS</td>
<td>$r = 0.39$</td>
<td>$p &lt; 0.0051$</td>
<td>$r = 0.49$</td>
<td>$p &lt; 0.45$</td>
</tr>
</tbody>
</table>

HDL-C, high density lipoprotein cholesterol; TC, total cholesterol; LDL-C, low density lipoprotein cholesterol; TG, triglycerides; GSH-px, glutathione peroxidase; GSSG-rx, glutathione reductase; GST, glutathione transferase; TBARS, thiobarbituric acid-reactive substances.

dent GSH-px activity. These data are intriguing and difficult to explain because the IMA and the ascending aorta are exposed to the same oxygen blood content and pressure. The presence in the aortic tissue of non–selenium-dependent GSH-px activity, which was not detectable in IMAs but which was associated with significantly higher values of GST activity, may represent a compensatory phenomenon.

FIGURE 1. Scatterplots showing relations between thiobarbituric acid–reactive substances (TBARS) levels (nmol/mg wet tissue) in the ascending aorta and serum values of high density lipoprotein (HDL) cholesterol, total cholesterol, low density lipoprotein (LDL) cholesterol, and triglycerides (all in mg/dl) in 40 male patients undergoing coronary revascularization.
to the lower selenium-dependent GSH-px activity. In fact, it has been reported that when selenium-dependent GSH-px activity is depressed, activation of non-selenium-dependent GSH-px (which belongs to specific GST isoenzymes) occurs. However, this phenomenon may not be merely compensatory but may also represent exposure of the tissues to different levels of oxidative stress and lipoperoxidation. In this context, it should be noted that the aortic specimens come from a segment characterized by higher mechanical stress and tissue metabolic level. Because free-radical generation seems to be influenced by mechanical processes and tissue metabolic activity, greater radical generation could occur in the upper aorta, potentially activating GST and non-selenium-dependent GSH-px. In this regard, it is remarkable that higher GST activity and TBARS content have recently been found in the aortic arch of normal rabbits compared with other segments.

It is worth noting that non-selenium-dependent GSH-px and GST are particularly active against by-products of lipid peroxidation such as organic hydroperoxides and hydroxalkenals. These diffusible compounds originate from the peroxidative breakdown of polyunsaturated fatty acids, such as linoleic and arachidonic acids. There is experimental evidence that 4-hydroxynonenal appears during LDL oxidation and may be partly responsible for the cytotoxicity of oxidized LDL. Such oxidative events seem not to occur in the circulation because plasma inhibits oxidation, but it could occur in the arterial wall. Furthermore, it has been shown that intracellular levels of GSH play a crucial role in protecting endothelial cells against the toxic effect of oxidized LDL. In this context, GSH-related antioxidant defenses of the arterial wall could limit lipid peroxide generation and LDL oxidation. A similar defensive role could also be exerted against very low density lipoprotein, which, like LDL, may undergo oxidative modification and exert cytotoxic effects. Thus, the presence of non-selenium-dependent GSH-px activity in the ascending aorta, together with higher values of GST, could indicate that this tissue is subjected to a higher oxidative stress with an increased organic peroxide production. Accordingly, significantly higher TBARS levels have been found in the ascending aorta, even though the TBARS assay should be cautiously considered as an index of lipoperoxidation in complex biologic samples. It is remarkable that the ascending aorta is admittedly more prone to atherosclerosis, whereas the IMA is considered protected.

Furthermore, our study shows the existence of significant correlations among tissue activity of GSH-related enzymes, TBARS levels, and serum lipids. Accordingly, recent studies have shown a positive correlation between plasma and arterial lipid peroxide concentration and TC or TG levels. These results seem to confirm the pro-oxidant role of lipids, particularly cholesterol, in vivo. Moreover, it has recently been suggested that lipid peroxides may interfere with metabolic pathways. In particular, Wada et al demonstrated that lipid peroxides inhibit lipoprotein lipase activity and thereby, plasma triglyceride hydrolysis.
An inverse correlation among arterial levels of GSH-related enzyme activities, TBARS, and HDL-C was detected (Table 3). Recent studies, apparently in contrast with our data, have shown a positive correlation between serum levels of selenium and selenium-dependent GSH-px and serum HDL-C and LDL-C.42,43 However, these studies were carried out in young normolipidemic subjects, and the GSH-px activity was determined in serum and not in arterial tissue, which is the target of atherosclerosis. Furthermore, the serum GSH-px is immunologically distinct from the tissue form, and its antioxidant role has yet to be determined.44,45 HDLs have recently been reported to inhibit or prevent LDL-induced cytotoxicity in cultures of vascular cells.37 Moreover, it has been suggested that HDL exerts antioxidant properties and may protect LDLs from peroxidation.4,46

It is well known that HDLs have a protective role against atherosclerosis, probably mediated by reverse cholesterol transport.47 It could be hypothesized that HDL, by transporting cholesterol away from the arterial wall, significantly reduces the opportunities for tissue lipoperoxidation and LDL oxidative changes. Therefore, in patients affected by coronary atherosclerotic disease, higher arterial levels of GSH-related enzyme activities could represent a compensatory phenomenon in the presence of lower serum HDL levels.

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