Glutathione-Related Antioxidant Defenses in Human Atherosclerotic Plaques

Domenico Lapenna, MD; Sergio de Gioia, MD; Giuliano Ciofani, Tch; Andrea Mezzetti, MD; Sante Ucchino, MD; Antonio M. Calafiore, MD; Antonio M. Napolitano, MD; Carmine Di Ilio, PhD; Franco Cuccurullo, MD

Background—Oxidative stress, resulting from an antioxidant/prooxidant imbalance, seems to be crucial in atherogenesis. Recent evidence has emerged, however, of a surprisingly high content of low-molecular-weight antioxidants in human atherosclerotic plaques, although other antioxidant systems have not been investigated in these lesions.

Methods and Results—We studied glutathione-related antioxidant defenses (which play a key role in tissue antioxidant protection) in carotid atherosclerotic plaques of 13 patients subjected to endarterectomy and in normal internal mammary arteries of 13 patients undergoing coronary artery bypass surgery. Selenium-dependent glutathione peroxidase activity was undetectable in the plaques of 7 patients; the other 6 patients with plaques showed a mean enzymatic activity \( \approx 3.5 \)-fold lower than that of mammary arteries. Glutathione reductase activity was also markedly lower in the plaques than in the arteries. Glutathione transferase instead had comparable activity in the two tissues. Remarkably, 5 of the 7 patients with an undetectable selenium-dependent glutathione peroxidase activity but none of the 6 with a detectable one were characterized by multivascular atherosclerotic involvement (3 patients) or stenosis of the contralateral carotid artery (2 patients).

Conclusions—A weak glutathione-related enzymatic antioxidant shield is present in human atherosclerotic lesions. Although the cause of this phenomenon remains to be determined, the present data suggest that a specific antioxidant/prooxidant imbalance operative in the vascular wall may be involved in atherogenic processes in humans. *(Circulation. 1998;97:1930-1934.)*

Key Words: atherosclerosis n antioxidants n enzymes

A large body of evidence has implicated free radicals and oxidative stress in atherogenic processes. Indeed, oxidant-mediated LDL oxidation and vascular injury are crucial in atherogenesis.\(^1\) The endogenous antioxidant capacity of arterial tissues seems relevant in atherosclerosis because, given the strong antioxidant properties of plasma, LDL oxidation may occur in sequestered domains of the arterial wall, where a low antioxidant potential and/or a high prooxidant activity could be operative.\(^2\) It has recently been shown, however, that human atherosclerotic plaques are endowed with a surprisingly high content of low-molecular-weight antioxidants, such as vitamin E, ascorbate, and urate, despite the occurrence of massive plaque lipid oxidation.\(^3\) Yet investigations dealing with the enzymatic antioxidant defenses of human atherosclerotic plaques are, to date, apparently lacking. This is an important issue, considering the pivotal role of peculiar enzymatic systems, such as the glutathione-related ones, in biomolecular antioxidant protection, especially in vascular parietal cells.\(^4\) In the present article, we have studied glutathione-related antioxidant defenses in human atherosclerotic plaques surgically removed from stenosed carotid arteries and in sub- tially normal human arteries, namely internal mammary arteries, obtained from other patients undergoing coronary artery bypass surgery. We provide here experimental evidence for a weak glutathione-related enzymatic antioxidant shield in human atherosclerotic plaques.

Methods

Patient Population

For the enzymatic study, 13 carotid atherosclerotic plaques were obtained from 13 patients (11 men and 2 women; age, 62.3±6.1 years) after elective endarterectomy surgery performed at Istituto di Patologia Chirurgica, Università “G. D’Annunzio” (Chieti, Italy). The extra length of internal mammary arteries not used for coronary grafting\(^5\) was also studied as a normal arterial tissue in another 13 patients (11 men and 2 women; age, 58.2±6.7 years) undergoing coronary artery bypass surgery at Cattedra di Cardiochirurgia, Università “G. D’Annunzio.” These artery specimens showed no macroscopic evidence of atherosclerosis. The two groups of patients were also matched for diabetes mellitus, arterial hypertension, dyslipidemia, smoking habit, and drug use, and they came from the same geographical area (Chieti, Abruzzo, Italy). No patient took...
antioxidants, included selenium, vitamin E, or thiols. Procedures were approved by the local human ethics committee.

Biological Analyses

Reagents were obtained from Sigma Aldrich srl. Immediately after tissue samples were surgically removed, they were placed in ice-cold, Chelex-100–treated and argon-flushed 50 mmol/L [Tris(hydroxy-methyl)aminomethane] HCl buffer, pH 7.4, plus 0.8 mmol/L EDTA and 1.0 mmol/L methionine (buffer A), and repeatedly washed with the same buffer. It is noteworthy that Tris per se is characterized by scavenging antioxidant properties against oxidizing species, included the chlorinated ones.12 Blotted samples were stored in liquid nitrogen until processed (usually within 2 weeks). In previous experiments, no loss of enzymatic activity was noted after storage for at least 2 months. Samples were homogenized in ice-cold buffer A by use of an Ultra-Turrax apparatus (Tecmar Co); differential centrifugation with ultracentrifugation at 80 000g was then performed, and the relative cytosolic supernatant used to measure the specific activity of glutathione-related enzymes. Glutathione peroxidase activity was determined according to the method of Paglia and Valentine,13 as previously described.11,14,15 To determine the fractions of GSH-Px and GST-Px, the enzymatic activities were recorded by use of both H$_2$O$_2$ (0.25 mmol/L) and cumene hydroperoxide (1.2 mmol/L), respectively, as substrates.14–16 Reaction mixtures contained 50 mmol/L potassium phosphate buffer, pH 7.0, 1.0 mmol/L EDTA, 1.5 mmol/L Na$_2$O$_2$, 1.0 mmol/L GSH, 0.16 mmol/L NADPH, 4 μg glutathione reductase, various cytosol amounts, and 0.25 mmol/L H$_2$O$_2$ or 1.2 mmol/L cumene hydroperoxide. Reaction rate was recorded following spectrophotometrically the NADPH-related decrease of absorbance values at 340 nm; specific activity was expressed as milliunits per mg protein, 1 mU representing 1 nmol GSH oxidized per minute. For the assay of GSSG-Red activity, appropriate cytosol aliquots were added to a reaction mixture containing 0.1 μmol/L potassium phosphate buffer, pH 7.4, 1.0 mmol/L EDTA, 0.16 mmol/L NADPH and 1.0 mmol/L GSSG.11,14,15 Blanks were without GSSG, and NADPH disappearance was followed at 340 nm.11,14,15 Results were calculated as milliunits (nmoles NADPH oxidized per minute) per mg protein.

GST activity was measured basically according to the method of Habig et al.,13 as previously reported.11,14,15 Assay mixtures contained 0.1 μmol/L potassium phosphate buffer, pH 6.5, 1.0 mmol/L EDTA, 2.0 μmol/L GSH, 1.0 mmol/L 1-chloro-2,4-dinitrobenzene as the substrate, and suitable cytosol amounts. Specific activity was expressed as milliunits per mg protein, each 1 mU representing 1 nmol substrate conjugated with GST per minute.11,14,15,17

Enzymatic activities were assessed in duplicate and at least at two different protein concentrations. The value for a blank reaction with the enzyme source replaced by buffer was subtracted for each enzymatic assay.

Recovery of enzymatic activities was performed in previous separate experiments with both plaques and mammary arteries by adding known amounts of native enzymes to sample-containing buffer A or homogenates before homogenization or ultracentrifugation, respectively. Recovery was also performed with some of the samples belonging to the effective series of cases reported in the Table (four plaques and three mammary artery specimens, characterized by a greater size and weight). In this latter case, samples were chopped into smaller pieces, which were weighed. About one half of the tissue weight was used for endogenous enzymatic activity measurement; the remaining half was used for recovery experiments.

### Table

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<th>Atherosclerotic Plaques</th>
<th>Mammary Arteries</th>
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<td>GSH-Px</td>
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Mean±SD of 13 endarterectomy-derived carotid plaques and 13 internal mammary artery specimens. 1 mU=1 nmol of GSH oxidized per minute for GSH-Px and GST-Px activities, 1 nmol of NADPH oxidized per minute for GSSG-Red activity, and 1 nmol of substrate (1-chloro-2,4-dinitrobenzene) conjugated per minute for GST activity. *Mann-Whitney U test.

GST-Px activity of 3 plaques with GSH-Px activity (specific activity undetectable in the other 7 plaques).

GST activity of 6 plaques (specific activity undetectable in the other 7 plaques).

(C owing to the limited amount of tissue available, in these experiments native enzymes were added to buffer A containing the samples only before the homogenization step.)

Cytosol protein concentrations were assayed by Bradford’s method.

Tissue DNA content was assessed through the recognized colorimetric reaction of DNA with cysteine (which is more specific than diphenylamine),19 after sample freezing-thawing to favor cell lysis and Ultra-Turrax homogenization in cold 5% TCA.20 DNA was extracted from the resulting protein-DNA pellet with 5% TCA for 30 minutes at 90°C,21,22 and the relative supernatant was subjected to cysteine reaction for DNA in the presence of 75% sulfuric acid. After 24 hours of incubation at room temperature, absorbance values at 490 nm were recorded spectrophotometrically against appropriate blanks. A standard curve of calf thymus DNA treated in a similar manner was also obtained to perform calculations.

### Statistics

Data were calculated as mean±SD and analyzed statistically by the Mann-Whitney U test and Fisher’s exact test when appropriate.23

### Results

Recovery of native enzymes was almost total, ruling out the possibility of some artifactual enzyme inactivation as a result of sample workup procedures.

As shown in the Table, the atherosclerotic plaques had a lower specific glutathione-related antioxidant capacity than the mammary arteries. In particular, the activity of GSH-Px was undetectable in the atherosclerotic tissue of seven patients; this means that even with quite high cytosolic amounts added to specific assay mixtures, the enzyme-related NADPH oxidation of these plaques was apparently equal to or lower than the spontaneous low level of NADPH autoxidation of blanks. The other six plaques showed a mean GSH-Px activity ≃3.5-fold lower than that of arteries (the Table). GSSG-Red activity also was lower in the plaques than in the arteries ($P<.0001$), whereas GST activity was similar in the two tissues (the Table). Protein content tended to be higher in the arterial than in the atherosclerotic tissue (67±24.7 versus 58.7±21.5 mg/g tissue, $P=NS$), and the activities of both GSH-Px (when detectable) and GSSG-Red relative to tissue weight were even lower in the plaques than in the arteries (209.5±124 versus 798±254 mU/g tissue and 63±23.7
versus 215±80.5 mU/g tissue, respectively; \( P<.0001 \); GST activity, however, continued to be not significantly different between the plaques and arteries (1235±312.7 versus 1505±402 mU/g tissue, \( P=NS \)). Interestingly, in three of the six plaques with GSH-Px activity, we could detect an activity of GST-Px (which belongs to GST isoenzymes); GST-Px activity was instead undetectable in the mammary arteries (the Table). This phenomenon indicates the appearance in some atherosclerotic lesions of a new enzymatic activity, apparently unexpressed in a normal vascular tissue, conceivably to withstand tissue oxidant load.

We sought to normalize enzymatic activities on the basis of, besides cytosol protein levels, tissue DNA content. However, we have observed that samples, especially plaques, have to be homogenized directly in TCA for proper extraction and recovery of DNA. A similar approach has been used for DNA assay in mammalian arterial tissue.29 Yet sample homogenization in TCA results in protein precipitation and does not allow enzymatic activity measurement. Division of samples into different parts, with assessment of enzymatic activities and DNA content separately in such parts, has not been possible, especially with mammary artery specimens, because of the limited amount of tissue usually available for specific analytical purposes. Thus, we could not measure simultaneously and adequately the enzymatic activities and DNA content in plaques and mammary artery specimens. It is conceivable that at least under an analytical profile, the most satisfactory way to express specific enzymatic activities in these tissues is in terms of cytosol protein content, because both enzymatic activities and proteins can be properly measured in the cytosol fraction of the same vascular sample. However, in separate experiments based on direct TCA tissue homogenization, DNA content was assayed specifically in other seven endarterectomy-derived carotid plaques and seven mammary artery specimens, which were macroscopically similar to those used for the measurement of glutathione-related enzymatic activities (these samples were obtained from patients substantially comparable with those considered for the enzymatic study). DNA content of plaques was not significantly different from that of mammary arteries (1932 ± 1197 versus 328.5±393 mU/g tissue, respectively; \( P=NS \)). Interestingly, in three of the six plaques with GSH-Px activity, we could detect an activity of GST-Px (which belongs to GST isoenzymes); GST-Px activity was instead undetectable in the mammary arteries (the Table). This phenomenon indicates the appearance in some atherosclerotic lesions of a new enzymatic activity, apparently unexpressed in a normal vascular tissue, conceivably to withstand tissue oxidant load.

Remarkably, although the patients with and without plaque GSH-Px activity were comparable for the presence of major atherosclerosis risk factors, three of the latter but none of the former had evidence of atherosclerotic involvement of two or more vascular districts (ie, peripheral vascular disease and/or ischemic heart disease). Moreover, two patients with undetectable GSH-Px activity had atherosclerotic stenosis of the contralateral carotid artery (one of these patients agreed to be subjected to further endarterectomy). Thus, under an anatomicoincidental profile, five of the seven patients with an undetectable plaque GSH-Px activity showed evidence of more severe atherosclerosis, whereas such evidence was apparently lacking in the patients with plaque GSH-Px activity. When analyzed by Fisher’s exact test, with the aforementioned five patients considered as a whole group, the anatomicoincidental differences between the patients with and without plaque GSH-Px activity were statistically significant (\( P<.025 \)).

Discussion

The “control” vascular tissue of the atherosclerotic plaques deserves specific comments. It should be noted that as suggested by Smith and coworkers,4 in absolute terms such a “control” tissue does not exist, which may render accurate biochemical comparisons between atherosclerotic plaques and normal vessels difficult, especially in quantitative terms. In this context, it is noteworthy that in some investigations dealing with the problem of oxidative stress in atherosclerotic lesions, a control tissue has not been used at all.4,24 In a comparative study of the low-molecular-weight antioxidant content of human carotid and femoral atherosclerotic plaques, Suarna and associates6 have recently used as control an intimal preparation of normal iliac arteries of young liver transplant donors who were accident victims. Even though such a preparation could appear rational because the atherogenetic processes begin in the intima, its use may have some limitations. Indeed, the very thin intima of normal human arteries is virtually free of smooth muscle cells, containing essentially endothelium, basement membrane, and few collagen and/or elastic fibers, whereas the major cell component of the atherosclerotic plaques is right smooth muscle cells.25 Moreover, when an human intimal preparation is used, it appears virtually impossible to have a sufficient tissue quantity, especially of arteries obtained in vivo, to assay specific enzymatic activities and to match adequately atherosclerotic patients and control subjects; for example, as reported in the study by Stocker et al,8 the mean age of patients is more advanced than that of control subjects (71 versus 23 years), and age is a relevant factor influencing the endogenous antioxidant/prooxidant balance. For comparative purposes, in our enzymatic investigation, we had to study normal arteries obtainable in vivo, so that postmortem proteolytic processes affecting specific enzymatic activities could be avoided. Because healthy arterial tissue is not removed during endarterectomy, comparisons between diseased and normal tissue of the same vascular bed were not possible. In light of the aforementioned considerations, we decided to study normal internal mammary arteries, which are characterized by the intrinsic presence of smooth muscle cells and can be obtained in the cardiac surgery setting.11 It is indeed worth noting that in the present investigation, both atherosclerotic plaques and mammary arteries were obtained in vivo in matched patients.

A striking finding of our study is that GSH-Px activity is often absent in atherosclerotic tissue. The absence of an enzymatic activity such as that of GSH-Px, which is the main antioxidant enzyme especially in vascular parietal cells, is not only a “quantitative” but also a “qualitative” phenomenon that is not expected in normal tissues. In this regard, we have never observed the absence of GSH-Px activity in normal human vessels obtainable in vivo, such as, besides internal mammary arteries, gastroepiploic arteries and patches of ascending aorta, as well as saphenous veins.11 It should be noted that our data do not permit us to determine whether an
inactivation of the enzyme or, rather, a true lack of the GSH-Px protein is operative in the plaques; further specific studies could be designed to address this issue. However, it is known that GSH-Px, as well as GSSG-Red, can be inactivated by oxidant species. For instance, GSH-Px is particularly susceptible to inactivation by myeloperoxidase-derived hypochlorous acid. Remarkably, the presence of myeloperoxidase as an oxidative catalyst and of hypochlorous acid-modified proteins has been proven in human atherosclerotic lesions. 

Moreover, 4-hydroxynonenal, an aldehydic by-product of lipid peroxidation generated during LDL oxidation conceivably in the vascular wall, has been reported to inactivate cell GSH-Px, whereas GST is resistant to inactivation. Interestingly, GST activity is similar in the plaques and in the arteries, despite the absence or the low level of GSH-Px activity in the plaques themselves. It appears feasible, therefore, that specific antioxidant enzyme inactivation occurs in atherosclerotic lesions, which may be regarded as prooxidant environments with lipoperoxide burden. However, the cause of plaque enzyme activity depression remains to be determined. On the other hand, atherosclerotic plaques and mammary arteries do not have a different cytosol protein content, indicating that inadequate specific protein concentrations are not responsible for plaque enzymatic activity depression. Furthermore, the presence of a similar activity of GST in the plaques and arteries, as well as the appearance of GST-Px activity in some atherosclerotic lesions, argues against a nonspecific depression of plaque GSH-Px (and GSSG-Red) activity owing to a peculiar protein array with enzyme deficiency or to a paucity of the endogenous cell glutathione peroxidase. 

In the arteries, despite the absence or the low level of GSH-Px activity, the cause of plaque enzyme activity depression remains to be determined. On the other hand, atherosclerotic plaques and mammary arteries do not have a different cytosol protein content, indicating that inadequate specific protein concentrations are not responsible for plaque enzymatic activity depression. Furthermore, the presence of a similar activity of GST in the plaques and arteries, as well as the appearance of GST-Px activity in some atherosclerotic lesions, argues against a nonspecific depression of plaque GSH-Px (and GSSG-Red) activity owing to a peculiar protein array with enzyme deficiency or to a paucity of the endogenous cell components. In this regard, it is noteworthy that DNA content is not significantly lower (by %35) in carotid atherosclerotic tissue than in mammary artery specimens, whereas the activities of GSH-Px (when present) and that of GSSG-Red are significantly lower (by %230 and %200, respectively) in the plaques than in the arteries (by %280 and %240 when expressed as DNA content per tissue weight). In any event, GSH-Px dysfunction should be critical in conditioning oxidative stress in atherosclerotic lesions. Indeed, GSH-Px, which is essential for the removing of inorganic and organic peroxides, represents the key antioxidant enzyme of the mammalian cell. Peroxides are cytotoxic for vascular cells, especially in the presence of reduct-oxidative transitions metals, which are available in a catalytically active form in human atherosclerotic plaques. The pivotal role of GSH-Px in vascular antioxidant protection is further pointed out by the findings that catalase activity is lacking in human vascular cells and that superoxide dismutase is poorly effective against human cell oxidant damage. Thus, the deficient GSH-Px and glutathione redox cycle status of the atherosclerotic tissue may significantly weaken its antioxidant potential favoring oxidative stress and atherogenic processes, even in the presence of an apparently adequate property of low-molecular-weight scavenging antioxidants. Consistently, our data indicate that the lack of GSH-Px activity in atherosclerotic lesions may be associated with a more severe expression of atherosclerosis in humans.

In conclusion, a weak glutathione-related enzymatic antioxidant shield is present in human atherosclerotic plaques, suggesting that a specific antioxidant/prooxidant imbalance operative in the vascular wall may be involved in atherogenic processes in humans.

References


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