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 \( \equiv 3.5 \)-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 • antioxidants • 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. 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. 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. 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.

In the present article, we have studied glutathione-related antioxidant defenses in human atherosclerotic plaques surgically removed from stenosed carotid arteries and in substantially 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\pm6.1 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 was also studied as a normal arterial tissue in another 13 patients (11 men and 2 women; age, 58.2\pm6.7 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 thiol. Procedures were approved by the local human ethics committee.

Biochemical Analyses
Reagents were 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)ammonomethane] 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 at 80 000 g was then performed, and the relative cytosolic supernatant used to measure the specific activity of glutathione-related enzymes. Glutathione peroxidase activity was measured according to the method of Paglia and Valentine,13 as previously described.14–16 Reaction mixtures contained 0.1 mol/L potassium phosphate buffer, pH 7.4, 1.0 mmol/L NADPH, 0.16 mmol/L NADPH, 4 μg glutathione reductase, various cytosol amounts, and 0.25 mmol/L H2O2 (0.25 mmol/L) and cumene hydroperoxide (1.2 mmol/L), and GST-Px, the enzymatic activities were recorded by use of both H2O2 and 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 mol/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.

GSH-Px activity was measured basically according to the method of Habig et al.,11 as previously reported.11,14,15 Assay mixtures contained 0.1 mol/L potassium phosphate buffer, pH 6.5, 1.0 mmol/L EDTA, 2.0 mmol/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 mmol substrate conjugated with GSH 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 (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.

\[
\begin{array}{|c|c|c|c|}
\hline
\text{GSH-Px} & \text{GSSG-Red} & \text{GST} & \text{GST-Px} \\
\text{mU/mg protein} & \text{mU/mg protein} & \text{mU/mg protein} & \text{mU/mg protein} \\
3.55 & 1.07 & 20.9 & 6.4 \\
\hline
\end{array}
\]
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, \( P = \text{NS} \)). 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 mammmary 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.20 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 mammmary 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 (890.6±393 versus 1197±328.5 \( \mu \)g DNA/g tissue, respectively; \( P = \text{NS} \)), suggesting that the depressed GSH-Px and glutathione redox cycle status of the atherosclerotic tissue may not be due to a quantitative deficiency of the endogenous cell components.

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 anatomoclinical 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 anatomoclinical 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 atherogenic 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.26–29 For instance, GSH-Px is particularly susceptible to inactivation by myeloperoxidase-derived hypochlorous acid.27 Remarkably, the presence of myeloperoxidase as an oxidative catalyst and of hypochlorous acid-modified proteins has been proved in human atherosclerotic lesions.30,31 Moreover, 4-hydroxynonenal, an aldehydic by-product of lipid peroxidation generated during LDL oxidation conceivably in the vascular wall,32 has been reported to inactivate cell GSH-Px, whereas GST is resistant to inactivation.29 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.4–6,24,30,31 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 GSH-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, indicating that inadequate specific protein concentrations are not responsible for plaque enzymatic activity depression. In conclusion, a weak glutathione-related enzymatic antioxidant defense against lipid hydroperoxide- and iron-mediated reactions. J Lipid Res 1993;34:479–490.


References


Glutathione-Related Antioxidant Defenses in Human Atherosclerotic Plaques
Domenico Lapenna, Sergio de Gioia, Giuliano Ciofani, Andrea Mezzetti, Sante Ucchino, Antonio M. Calafiore, Antonio M. Napolitano, Carmine Di Ilio and Franco Cuccurullo

Circulation. 1998;97:1930-1934
doi: 10.1161/01.CIR.97.19.1930
Circulation is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 1998 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7322. Online ISSN: 1524-4539

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circ.ahajournals.org/content/97/19/1930

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Circulation can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Circulation is online at:
http://circ.ahajournals.org/subscriptions/