Evidence for roles of radicals in protein oxidation in advanced human atherosclerotic plaque

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Oxidative damage might be important in atherogenesis. Oxidized lipids are present at significant concentrations in advanced human plaque, although tissue antioxidants are mostly present at normal concentrations. Indirect evidence of protein modification (notably derivatization of lysine) or oxidation has been obtained by immunochemical methods; the specificities of these antibodies are unclear. Here we present chemical determinations of six protein-bound oxidation products: dopa, *o*-tyrosine, *m*-tyrosine, dityrosine, hydroxyleucine and hydroxyvaline, some of which reflect particularly oxy-radical-mediated reaction pathways, which seem to involve mainly the participation of transition-metal ions. We compared the relative abundance of these oxidation products in normal intima, and in human carotid plaque samples with that observed after radiolytically generated hydroxyl radical attack on BSA *in vitro*. The close similarities in relative abundances in the latter two circumstances indicate that hydroxyl radical damage might occur in plaque. The relatively higher level of dityrosine in plaque than that observed after radiolysis suggests the additional involvement of HOCl-mediated reactions in advanced plaque.

INTRODUCTION

The theory that atherogenesis is initiated and/or propagated by oxidative events, notably lipoprotein oxidation, has gained some credence [1]. Thus the formation of lipid-laden macrophage-derived foam cells, an early hallmark of atherogenesis, does not occur when macrophages are exposed to low-density lipoprotein (LDL), apparently the main source of lipids in plaque, unless the LDL has been modified so that its cellular uptake is enhanced and unregulated [2]. This can occur after oxidation of LDL [3–5]. Furthermore, substantial quantities of oxidized lipids are present in plaque, although paradoxically these are accompanied by normal levels of certain antioxidants, in both the aqueous and lipid phases [6]. The underlying mechanism of enhanced lipid oxidation is not clear, although one possibility is that tocopherol-mediated peroxidation could be responsible [7] if co-antioxidation [8] were deficient.

The oxidative theory has gained strength from a range of observations that support plausible hypotheses for the evolution of oxidative damage into pathogenic processes [1]. For example, slightly oxidized LDL has a wide range of actions, including some on cell proliferation and cell triggering [9–11], which could have propagating roles in atherogenesis. Furthermore, some of the products of lipid oxidation, notably the oxysterol 7-ketocholesterol [12,13], can inhibit the pathways that normally cause lipid efflux from cells, and hence contribute to lipid homoeostasis in cells, a process that is clearly grossly aberrant in atherogenesis.

If oxidation is important in atherogenesis then one would expect that lipid oxidation would be accompanied by oxidation of other components, notably proteins. Until recently the evidence for this was largely indirect (reviewed in [14]) and often did not distinguish between two separate processes. On the one hand, derivatization of lysine residues on proteins, notably apoB of LDL, might occur as a consequence of oxidation of lipids, carbohydrates [15] or other molecules, giving rise to reactive aldehydes. This derivatization alters the surface charge, electrophoretic mobility and the surface binding and uptake of LDL by cells [16]; such derivatized LDLs (or other proteins) have been reported to be present in plaque (see, for example, [17]). On the other hand, direct oxidation of protein might occur, and should be distinguished from the processes just described.The nucleophilic non-radical oxidant, HOCl, is produced by triggered neutrophils and can also derivatize lysines; both immunological and chemical evidence has been obtained for the presence of HOCl-modified proteins in plaque [18,19]. Oxidation of proteins by HOCl is accompanied by a substantial formation of chlorotyrosine [19,20] and a lesser formation of dityrosine. Little direct evidence has so far been obtained for radical-oxidized proteins in plaque; Heinecke and co-workers [21] have, for example, reported that the levels of tyrosine derivatives resulting from oxy-radical attack on phenylalanine in LDL are not elevated in plaques.

In the present study we have investigated which oxidants might contribute products to advanced plaques, by using analytical methods for six protein-bound oxidation products. Hydroxyl radical attack (e.g. by metal-ion-catalysed Fenton chemistry) is known to generate hydroxylated derivatives of the aliphatic amino acids leucine [22] and valine [23], and the aromatic amino acids phenylalanine (*o*-tyrosine and *m*-tyrosine) and tyrosine (dopa) [24]. We demonstrate here that HOCl does not generate most of these species [14]. Assessment of such a wide spectrum of oxidation products is needed to determine the mechanism(s) of generation of such products; this has been discussed in detail in a recent monograph [25].

Dopa, and hydroxyvalines and hydroxyleucines represent two categories of long-lived reactive intermediates in protein oxidation [26,27]. Protein-bound dopa is capable of reducing both metal ions and metalloproteins; it might thereby propagate radical reactions by redox cycling of iron or copper ions, which could catalyse the Fenton reaction [24]. Protein hydroxides are

Abbreviations used: LDL, low-density lipoprotein; Leu.OH2 and Leu.OH3, the two stereoisomers of 5-hydroxyleucine; MPO, myeloperoxidase; OPA, *o*-phthaldialdehyde; Val.OH1, 3-hydroxyvaline.

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stable products arising from the reduction of protein hydroperoxides, which are formed on proteins during radical attack (e.g. by hydroxyl radicals) (reviewed in [28,29]). Protein hydroperoxides can give rise to further radicals that might react with other biological components [30], and hence propagate damage. The fact that these protein oxidation products are reactive does not preclude their accumulation, because this merely requires there to be a greater rate of generation than of removal [27]. We show here that these species, together with a spectrum of other products, accumulate in plaque. This is consistent with a major role for metal-ion-catalysed oxy-radical chemistry in atherogenesis.

MATERIALS AND METHODS

Materials

o-Phthaldialdehyde (OPA) crystals, OPA diluent [containing 3 % (w/v) KOH and 3 % (w/v) boric acid at pH 10.4] were from Pickering Laboratories. 2-Mercaptoethanol, BSA (fatty acid-free) was from Sigma. Sodium deoxycholate was from Difco Laboratories. All other chemicals were of AR grade. All organic solvents were of HPLC grade. Isotonic PBS, pH 7.4, was prepared in nanopure water and treated overnight with Chelex-100 resin (Bio-Rad) to remove contaminating metal ions [31].

Oxidation of BSA by radiolytically generated hydroxyl radicals

BSA (2 mg/ml in water) was exposed essentially as described previously [32] to ^{60}Co radiation (dose rate approx. 30 Gy/min), with top-gassing with oxygen. Doses of up to 1200 Gy were used; such a system generates known quantities of hydroxyl and superoxide radicals in a 1:1 ratio [33]. Superoxide radicals alone do not significantly oxidize BSA [14,32], so the oxidized amino acids generated in this system are largely attributable to initial hydroxyl radical attack and the species involved in the subsequent chain reactions of protein oxidation [30,34].

Preparation of intima and intimal homogenate

Normal iliac arteries were obtained from liver transplant donors and human plaques from patients undergoing carotid endarterectomy, as described previously [6]. Immediately after surgical removal, samples were placed in Chelex-treated and argon-flushed PBS containing butylated hydroxytoluene (100 μM) and EDTA (1 mM) and stored at −80 °C. The normal subjects were victims of either motor accidents or cerebral oedema and were aged from 22 to 50 years (Table 1); their samples showed no macroscopic evidence of atherosclerosis. The patients undergoing endarterectomy were 61–79 years of age; only small quantities of material could be obtained. All samples (also summarized in Table 1) represented advanced fibrofatty lesions; seven were calcified and two contained attached thrombus. As discussed previously [6], there is no available source of fresh vessel materials to provide the theoretically desirable controls for the endarterectomy samples, nor, conversely, diseased counterpart vessels for the iliacs. In this study we chose to avoid post-mortem diseased material because of the risk of artifact during the period before samples become available for analysis, and the difficulties of supply of such materials. All samples studied were obtained by qualified hospital staff, and all procedures were approved by the local human ethics committee.

After being thawed, intimas were dissected from adventitia and media under a dissecting microscope, rinsed, blotted with Whatman filter paper and weighed. PBS containing butylated hydroxytoluene and EDTA was then added to the tissue (0.05 g/ml) at 4 °C. The material was minced and homogenized at 4 °C in an Ultra-Turrax T8 homogenizer (IKA Labortechnik) at 3000–5000 rev./min for 1 min. The homogenate was subjected to delipidation and protein hydrolysis as described below.

Delipidation, amino acid removal and protein hydrolysis: controls for oxidation artifacts

A published method [22–24,28] was used for delipidation, removal of free amino acids and hydrolysis. In brief, the homogenate (700 μl) was transferred into a 1 ml brown glass autosampler vial (Alltech) and mixed with 0.3 % sodium deoxycholate (50 μl) and 50 % (w/v) trichloroacetic acid (100 μl). After centrifugation, the protein pellets were resuspended and washed twice with cold acetone and once with diethyl ether. All free amino acids were removed by this method, as confirmed by the recoveries of added modified amino acids (dopa, *o*-tyrosine and *m*-tyrosine).

The delipidated protein samples were subsequently freeze-dried and hydrolysed by using a standard gas-phase acid-catalysed method (HCl containing mercaptoacetic acid and phenol; anaerobic conditions) [22–24,28]. Hydrolysates were freeze-dried, redissolved in water (100 μl) and filtered through a 0.22 μm pore-size filter paper (Whatman) for HPLC analysis.

We determined whether protein-bound amino acid hydroperoxides were converted into hydroxides during tissue homogenization. Intima obtained from Plaque 4 was cut into two portions (approx. 200 mg of wet tissue in each). They were homogenized either with or without the prior addition of sodium borohydride. After delipidation the protein fraction was hydrolysed and analysed for the presence of hydroxyvaline and hydroxyleucine.

The extent of amino acid oxidation during acid hydrolysis was examined by determining the concentrations of dopa (from

Table 1 Characteristics of normal subjects and atherosclerotic patients

Control	Age (years)	Sex	Cause of death	Patient	Age (years)	Sex	Type of carotid plaque
Normal 1	50	M	Head injury	Plaque 1	79	M	Calcified
Normal 2	42	F	Cerebral oedema	Plaque 2	79	F	Calcified
Normal 3	26	M	Motor accident	Plaque 3	77	M	Calcified
Normal 4	22	M	Motor accident	Plaque 4	75	M	Slightly calcified with thrombus
Normal 5	22	F	Motor accident	Plaque 5	70	F	Slightly calcified
Normal 6	22	M	Motor accident	Plaque 6	68	F	Calcified
				Plaque 7	66	M	Fatty and calcified with thrombus
				Plaque 8	63	F	Calcified
				Plaque 9	61	F	Fatty

tyrosine), *o*-tyrosine and *m*-tyrosine (from phenylalanine) and hydroxyleucines and hydroxyvalines (from leucine and valine). Thus we subjected the free amino acids (tyrosine, phenylalanine, leucine and valine) both alone and in the presence of melittin (a protein that lacks tyrosine) to acid hydrolysis. These approaches confirmed the generation of low levels of tyrosine and phenylalanine oxidation products by the hydrolysis procedure (dopa, 130 μmol/mol of tyrosine; *m*-tyrosine, 10 μmol/mol of phenylalanine; *o*-tyrosine, 20 μmol/mol of phenylalanine). No generation of leucine or valine hydroxides was detected. Estimates of *o*-tyrosine and *m*-tyrosine in freshly isolated human plasma proteins (both approx. 1 per 10^4 phenylalanines) were respectively similar to or less than those reported by using enzymic hydrolysis and MS [35].

HPLC analysis

HPLC analysis was performed on an LC-10A HPLC system (Shimadzu), equipped with a column oven (30 °C; Waters). Peak integration and system operation was automated by Class LC-10 software (Shimadzu).

Two steps of HPLC were needed for the quantification of 3-hydroxyvaline (Val.OH1) [23] and the 5-hydroxyleucines (Leu.OH2, Leu.OH3) [22]. In the first step, the hydrolysate (25 μl) was fractionated on an LC-NH_2 column (25 cm × 4.6 mm, 5 μm particle size; Supelco) with a 2 cm Pelliguard column (Supelco) with 82 % (v/v) acetonitrile in 10 mM sodium phosphate buffer, pH 4.3, as eluent, at 1.5 ml/min and monitored at 210 nm. The eluate between 12 and 14 min corresponding to the elution of Val.OH1 (12.5 min), Leu.OH2 (12.8 min) and Leu.OH3 (13.5 min) was collected, freeze-dried and redissolved in water (50 μl). Aliquots (40 μl) were subjected to a second HPLC step, involving precolumn derivatization with freshly prepared OPA reagent. Typically, OPA crystals (50 mg) were dissolved in ethanol (5 ml) and added to deoxygenated OPA diluent (45 ml) [22] followed by the addition of 2-mercaptoethanol (200 μl). The sample fraction, after automated mixing with the OPA reagent (20 μl) by the autosampler, was chromatographed on a Zorbax ODS column (25 cm × 4.6 mm, 5 μm particle size; Rockland Technologies) as described previously [22,23,28]. Leu.OH3 could not be quantified in tissue samples owing to interference by a contaminant in the second HPLC step. The second HPLC step was also used to measure (unmodified) valine, leucine and phenylalanine in the hydrolysate (without any prior fractionation). Aliquots (40 μl) of a hydrolysate diluted 1:5000 usually provided an appropriate working concentration.

For detection of dopa, *m*-tyrosine, *o*-tyrosine and dityrosine, hydrolysate (10 μl) was chromatographed on a Zorbax ODS column with a Pelliguard guard column. The mobile phase was a gradient of solvent A [10 mM sodium phosphate buffer (pH 2.5)/100 mM sodium perchlorate] and solvent B [80 % (v/v) methanol] at a flow rate of 1 ml/min. The gradient change over a run was programmed as follows: isocratic elution with 100 % A for 12 min; then to 80 % A in 8 min; further elution at 80 % A for 3 min before changing to 50 % A in 3 min; isocratic elution at 50 % A for another 3 min; then re-equilibration with 100 % A for 10 min. The eluate was monitored in series by both a UV detector (Shimadzu) set at 280 nm and a fluorescence detector (Hitachi F-1080). After excitation at 280 nm, the fluorimetric emission of the eluate was monitored at 320 nm for the first 20 min for the detection of dopa (retention time 7.6 min), *m*-tyrosine (14.7 min), and *o*-tyrosine (19.0 min) and at 410 nm thereafter for the detection of dityrosine (20.7 min). Unmodified *p*-tyrosine (10.2 min) was quantified by UV measurement because of its off-scale response in the fluorescence channel. 3-Chlorotyrosine and 3-nitrotyrosine eluted at 21.6 min and 22.5 min respectively; UV absorption permitted their detection in quantities greater than 10 pmol. Elution positions were defined on the basis of standards; identities were confirmed by UV absorption and fluorescence spectra.

RESULTS

Homogenization does not induce hydroxyl radical-mediated damage

It has previously been documented that hydroxyl radicals can generate many oxidation products from amino acids, peptides and proteins [14,25,33]. In the present study we measured the level of 6 oxidized amino acid derivatives, namely dopa, dityrosine, *o*-tyrosine, *m*-tyrosine, Val.OH1 (reduction product of 3-hydroperoxyvaline) and Leu.OH2 (reduction product of 5-hydroperoxyleucine) in intimal proteins from normal human arteries and human atherosclerotic plaques, and compared these values with those for proteins from human plasma.

To quantify oxidized amino acid residues in intimal proteins of human plaques, homogenization of the samples is necessary. Experiments were undertaken to examine the formation of these oxidized amino acids as artifacts during homogenization. Intimas were obtained from both normal subjects and patients (Normal 6 and Plaque 4). Amino acids (Tyr, Phe, Val, Leu) at final concentrations of 2 mM were added to intimal samples in PBS containing butylated hydroxytoleuene and EDTA (0.1 g/ml wet tissue). Samples were then homogenized as described in the Materials and methods section. After homogenization, trichloroacetic acid and sodium deoxycholate were added and centrifuged to precipitate proteins. The supernatant was analysed for dopa, dityrosine, *o*-tyrosine, *m*-tyrosine, Val.OH1 and Leu.OH2 by HPLC methods. Oxidized amino acids were not observed and amino acid recoveries were 95–100 %.

Homogenization converts protein-bound hydroperoxides into hydroxides quantitatively

Protein hydroperoxides are reduced to the corresponding hydroxides in biochemical and cellular systems [36]. However, the fate of protein hydroperoxides during homogenization of biological samples has not been studied. Therefore hydroperoxides (50 μM from radiolysis of aqueous valine) were added to buffer (control) or intimal samples (Plaque 4). After homogenization and the precipitation of proteins, the supernatants were assayed for valine hydroperoxides by HPLC and post-column chemiluminescence detection [22,23,28,36]. The recovery of valine hydroperoxides was 100 % from controls and less than 5 % from the samples homogenized with plaque tissue. Separate HPLC analysis of the same samples after derivatization with OPA (see the Materials and methods section) revealed that the valine hydroperoxides destroyed during homogenization with plaque tissue were converted quantitatively into the corresponding hydroxyvalines (results not shown), suggesting that any hydroperoxides present in the starting plaque samples were reduced to the corresponding hydroxides.

An alternative approach was to measure the level of hydroxyvalines/leucines from plaque material (Plaque 4) treated with borohydride before homogenization and then compare the values with those obtained from the same sample but without treatment with borohydride. We have reported previously [36] that acid-catalysed protein hydrolysis destroys the majority of amino acid hydroperoxides, producing something other than the corresponding hydroxides. If homogenization of intimas did not

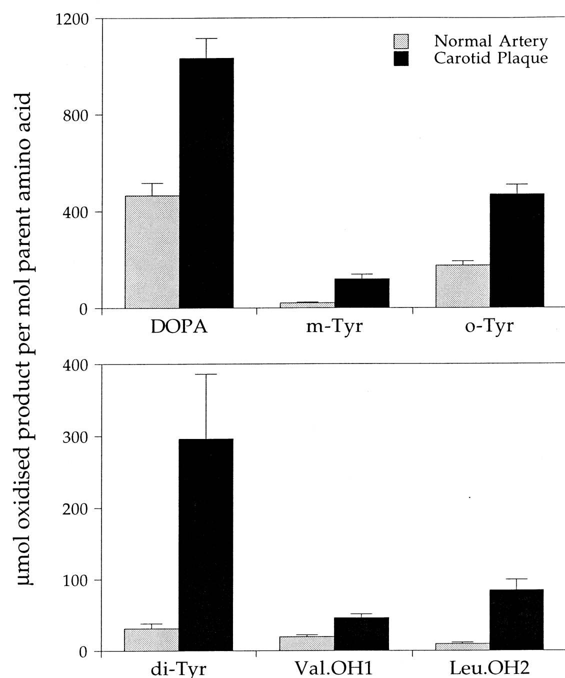

Figure 1 Levels of oxidized amino acids present in intimal proteins of normal human arteries and human atherosclerotic plaques

Results are expressed as μmol of oxidized product per mol of parent amino acid and are means ± S.E.M. ($n = 6$ normal subjects; $n = 9$ plaques). The absolute masses of each oxidized amino acid (pmol/mg wet weight of intimal tissue, means ± S.D.; values for normal samples, then for plaque samples) were as follows: dopa, 9.02 ± 1.98, 14.26 ± 3.80; dityrosine, 0.63 ± 0.41, 4.75 ± 5.17; *m*-tyrosine, 0.71 ± 0.17, 3.53 ± 1.88; *o*-tyrosine, 5.90 ± 1.77, 13.53 ± 4.19; Val.OH1, 1.07 ± 0.33, 2.01 ± 0.68; Leu.OH2, 0.53 ± 0.24, 3.76 ± 2.14.

convert protein hydroperoxides quantitatively into the corresponding hydroxides, as does sodium borohydride [36], then a difference in the level of hydroxyvalines/leucines should be expected between the two samples. Our experiment revealed no difference between samples: Val.OH1, 26.1 ± 4.0 and 21.6 ± 1.8 μmol/mol, and Leu.OH2, 50.6 ± 2.7 and 49.4 ± 2.6 μmol/mol for determinations with or without treatment with borohydride respectively (means ± range of duplicates). This result confirms that homogenization converts protein hydroperoxides into hydroxides, and that the latter survive the subsequent protein hydrolysis. Accordingly we cannot determine whether all hydroperoxides generated *in vivo* are converted into hydroxides in plaque or whether the hydroxides are generated by homogenization. In either case, homogenization does not affect the measurement of amino acid hydroxides (representing previously formed hydroperoxides and hydroxides) in oxidized proteins.

Dopa is recovered fully from homogenization

Protein-bound dopa is a useful marker for studying protein oxidation, and also a potential contributor to further oxidative reactions because of its reducing capacity. Dopa has been shown to undergo decomposition and further oxidation under neutral or basic conditions [37–39]; therefore its recovery after homogenization required clarification. Dopa (50 μM) or albumin-bound dopa (generated by radiolysis) was added to a plaque sample (Plaque 4) and subjected to the standard homogenization procedure. After protein precipitation, the supernatant was analysed for dopa. A recovery of 100 % was observed.

Occurrence and relative abundance of oxidized amino acids in intimal proteins from normal arteries and atherosclerotic plaques

Oxidized amino acids were measured in normal arteries and advanced atherosclerotic plaques. The intimal proteins were selectively concentrated from the homogenates by delipidation and protein precipitation. After protein hydrolysis, the hydrolysates were analysed by HPLC. The levels of oxidized amino acids (dopa, dityrosine, *m*-tyrosine, *o*-tyrosine, Val.OH1 and Leu.OH2) in the intimal proteins from each individual sample are detailed in Figure 1 (expressed as molar ratios of oxidized amino acid to parent amino acid, and absolute quantities). No correlation was observed by regression analysis between the level of oxidized amino acids and age in either group (normal arteries and plaques). Figure 1 shows clearly that the levels of all six oxidized amino acids measured in this study were significantly elevated in plaque samples compared with those in normal arteries. In terms of product per parent amino acid, dopa was present at the highest ratio, with Val.OH1 and Leu.OH2 giving the lowest values. This relative abundance is in accord with the known greater reactivity of aromatic amino acids than aliphatic amino acids with radicals such as hydroxyl radicals.

It was interesting to compare the levels in the diseased and normal arteries with those in normal plasma samples, because plasma contains proteins that are readily exchanged with most extracellular pools in the body and are efficiently turned over. Plasma would thus be expected to be relatively resistant to the accumulation of proteins containing oxidized amino acids. For $n = 4$ freshly processed normal human plasma samples we determined the following levels of protein-bound oxidized amino acids (μmol/mol of parent amino acid; mean ± S.D.): dopa, 195 ± 23; *m*-tyrosine, 87 ± 8; *o*-tyrosine, 81 ± 16; Val.OH1, 14 ± 4; Leu.OHs, 11 ± 3; dityrosine, 12 ± 5. These values are all significantly lower than the corresponding values in the plaque samples, confirming that there is an elevation of radical-damaged species there; for dopa and *o*-tyrosine, products from the amino acids most sensitive to oxidation among those studied, the levels in normal arteries were also elevated compared with the normal plasmas, suggesting an exaggerated radical damage, whereas for the other species the values were similar. Perhaps most striking is the fact that even in the physiological plasma samples the relative abundances of the different oxidized amino acids are in the same sequence as in the diseased samples, suggesting that a common oxidative mechanism such as hydroxyl radicals might be involved.

Relative abundance of oxidized amino acids in BSA after radiolytic hydroxyl radical attack or oxidation by HOCl *in vitro*

To obtain further indications of whether oxygen-centred radicals and/or HOCl might be responsible for the observed relative abundances of oxidized products, we undertook a series of model experiments. First the relative abundances of the oxidized amino acids in plaque samples were compared with those in BSA that had been oxidized by radicals generated from ^{60}Co radiolysis. The relative abundances of the individual oxidized amino acids in radiolysed BSA were found to be essentially independent of the extent of oxidation (Figure 2). The range of substitution levels achieved (product/parent amino acid) overlaps those observed in plaque, at the lowest end of the radiolysis dose, confirming that this independence is relevant to plaque. In both radiolysed BSA and plaque, dopa was the predominant oxidation product, followed by *m*-tyrosine and *o*-tyrosine, with hydroxy-

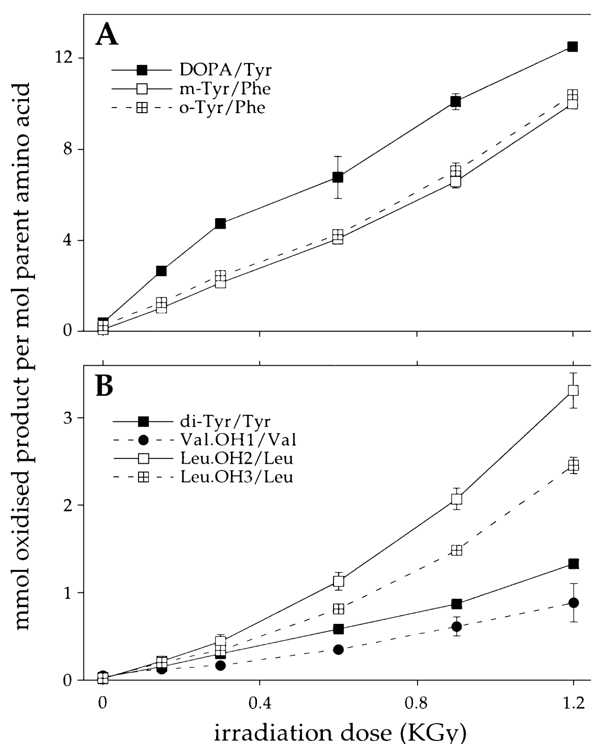

Figure 2 Levels of six oxidized amino acids detected on BSA after attack by radiolytically generated hydroxyl radicals *in vitro*

BSA (2 mg/ml in water) was exposed to a ^{60}Co source of γ-radiation with top-gassing with oxygen. The oxidized BSA solutions were treated with sodium borohydride (1 mg/ml) and freeze-dried. After hydrolysis of protein, the hydrolysates were analysed with HPLC as described in the Materials and methods section. Results are means $\pm$ S.D. for a single experiment ($n=3$) representative of several.

valine and hydroxyleucine giving the lowest values; this is consistent with the involvement of hydroxyl radicals in their generation in plaque. The levels of dityrosine relative to other oxidized amino acids observed in plaques are much higher than those observed in BSA after oxidation *in vitro*. In other work we have shown that during Fenton chemistry initiated by iron complexes, the relative abundance of oxidation products generated on proteins is similar to that which we have detailed here for radiolytic hydroxyl radical attack (S. Fu, R. T. Dean, M. Southan and R. Truscott, unpublished work).

In contrast, the relative levels of oxidation products generated by exposure of the protein to HOCl (Table 2) bore little relation to those observed in plaque. The most abundant product (in terms of mol/mol of parent amino acid) was chlorotyrosine, whose levels were almost 100-fold those of the next most abundant product, dopa, which was generated only to a very limited degree by this oxidant. In contrast, chlorotyrosine levels in the atherosclerotic samples were much lower than those of dopa or the other species under study. Although the levels of dityrosine were increased approx. 10-fold by the exposure of albumin to HOCl, the levels of substitution reached were still modest (approx. 0.1 mmol/mol Tyr). The generation of *o*-tyrosine by the reaction of HOCl was just detectable, whereas there was no detectable generation of the hydroxylated aliphatic amino acids.

DISCUSSION

Our observation that levels of hydroxyleucine and hydrovaline are increased in advanced plaque points directly to a role for oxy-radicals in their generation, and hence possibly for roles of metal-ion-catalysed Fenton chemistry, and/or other radicals involved in this such as peroxyl and alkoxyl radicals. At present there are no known alternative explanations for the generation of these species, and we show here that they are not products of oxidation by HOCl. If hydroxyl radicals are generated in the intima during atherogenesis, then the observed greater elevations of dopa, *o*-tyrosine and *m*-tyrosine can also be explained by the same pathways, as shown by the present studies of defined hydroxyl radical attack on BSA. It is also notable that the oxidized protein-bound amino acids measured here are detectable in fresh human plasma, at lower levels but with similar relative abundances. This might indicate ongoing basal oxy-radical flux in normal physiological conditions.

The levels of dityrosine observed in normal intima and plasma are very low in comparison with the other aromatic amino acid products, and are comparable with those of the less reactive aliphatic amino acids, as might be expected from low-flux oxy-radical damage. In contrast, dityrosine was relatively elevated in the plaque proteins, to levels comparable with those of *o*-tyrosine and *m*-tyrosine, but somewhat lower than those for dopa. This might be indicative of a contribution from at least one other oxidation pathway. Previous studies have implicated HOCl, which can be generated from the reaction of H_2O_2, chloride and myeloperoxidase (MPO), and/or other MPO-catalysed reactions that give rise to tyrosine phenoxyl radicals [40,41], in the formation of dityrosine. MPO is secreted by activated neutrophils and is present in active forms in atherosclerotic plaque [42]. 3-Chlorotyrosine is present in plaque at levels elevated in comparison with normal tissue, again consistent with a role for HOCl/MPO in atherogenesis [19]. In the present study we have also examined the presence of 3-chlorotyrosine and 3-nitro-

Table 2 Generation of oxidized amino acids on BSA after reaction with reagent HOCl

BSA (2 mg/ml in water) was reacted with HOCl (final concentration 750 μM) at room temperature for 20 min. BSA alone (without addition of HOCl) was also included in the experiment as a negative control. After the addition of $NaBH_4$ (1 mg/ml), the reaction solution was mixed with 5% (w/v) trichloroacetic acid and centrifuged at 4000 ***g*** for 2 min. The protein pellet thus obtained was dried and subjected to protein hydrolysis. The hydrolysates were analysed by HPLC as described in the Materials and methods section. Results are mmol of product/mol of parent amino acid, given as means with range from duplicate determinations.

Sample	Cl-Tyr	Dopa	Dityrosine	*o*-Tyrosine	Val.OH1	Leu.OH2
Control	0.69 ± 0.11	0.51 ± 0.03	0.02 ± 0.01	0.12 ± 0.01	0.026 ± 0.003	0.010 ± 0.001
+ HOCl	58.3 ± 2.3	0.72 ± 0.06	0.12 ± 0.01	0.15 ± 0.01	0.025 ± 0.002	0.011 ± 0.001

tyrosine by HPLC by detection with UV; the levels in all samples were below our detection limit (0.4 mmol/mol of parent). This limit slightly exceeds the elevated levels of chlorotyrosine described earlier [19], so our results and those of Heinecke and coworkers are mutually consistent, as are those for phenylalanine derivatives in the 'normal' samples; the literature is unresolved as to whether nitrotyrosine is elevated in plaque [43–45]. Thus besides oxy-radical/Fenton systems, HOCl/MPO are plausible additional influences in protein oxidation in plaque, although the degree of conversion of tyrosine into the putative products of HOCl oxidation is significantly lower than into dopa, or for phenylalanine into *o*-tyrosine and *m*-tyrosine. In particular, as shown by our determination of a wide range of oxidation products on protein molecules after exposure to HOCl, if it were a dominant influence in plaque, a much greater relative abundance of chlorotyrosine would be expected than is observed in our advanced plaque samples.

It is interesting to compare the results obtained here for plaque with those obtained for lens proteins from eyes at various defined stages of cataractogenesis ([14,22], and S. Fu, R. T. Dean, M. Southan and R. Truscott, unpublished work). During cataract formation, dopa, *o*-tyrosine and *m*-tyrosine, and hydroxyleucine and hydroxyvaline, increase progressively with the evolution of the disease; this increase is more pronounced than for atherosclerotic plaque. However, dityrosine levels remain comparable with those of the hydroxy aliphatic amino acids, as in normal plasma, normal intima and also in normal lenses. Thus in lens cataractogenesis there is little indication of a role for HOCl, and a metal ion/hydroxyl radical system could suffice to explain the observations because we have provided evidence arguing against oxidation being due to the modest irradiation with UV at more than 300 nm that the lens experiences.

Some information on the stage dependence of protein oxidation in atherogenesis has been presented by Heinecke and coworkers. By using post-mortem human materials they have distinguished vicinal sections of arteries as advanced, intermediate, and so on, according to conventional morphological criteria, and suggested that *o*-tyrosine and *m*-tyrosine are not elevated in total plaque proteins at least until the stage of advanced lesions [19,21], whereas dityrosine and other components might be. It is not known whether intermediate lesions in a person who also has advanced lesions are similar to intermediate lesions in one who lacks advanced, but the results are consistent with ours in indicating that the relative abundance of dityrosine (per tyrosine in protein) is lower than that of dopa, and only in the same range as those of *o*-tyrosine and *m*-tyrosine. The elevation of 3-chlorotyrosine observed by these authors might indicate a role for HOCl and/or MPO in early lesions, consistent with studies on HOCl-oxidized proteins [18]. It is not yet known whether the hydroxylated aliphatic amino acids, which seem to be the best fingerprints for oxy-radical/Fenton systems, are elevated in such lesions, and so it is not yet clear what role these oxidative systems might have.

It should be noted that dityrosine arises from the dimerization of tyrosine phenoxyl radicals, and therefore the levels of this material are critically dependent on the radical flux and will not increase in a linear manner (as observed with the other radical-oxidized products measured here) with increasing steady-state radical concentrations. Thus differing levels of dityrosine per parent tyrosine might give valuable information on the radical flux in various tissues. The formation of tyrosine phenoxyl radicals can occur both through the action of MPO, or through the formation of oxy-radicals (such as hydroxyl radicals), which might be generated by metal-ion-catalysed reactions. The available levels of free tyrosine will also influence these pathways, because exogenous (free amino acid) tyrosine phenoxyl radical can form dityrosines with tyrosine phenoxyl radicals on proteins [41]. The results available cannot distinguish between these possibilities, and it is not yet clear whether any of the dityrosines observed in plaque involve free tyrosine.

Thus it seems necessary to invoke oxy-radical fluxes as substantial contributors to protein oxidation in advanced plaques. It must, however, be borne in mind that all these determinations of tissue levels of oxidized protein components merely reflect levels at particular time points; these levels might be transient and do not necessarily indicate the relative input and output rates, which are influenced by factors such as protein turnover. Nevertheless it is likely that the elevations of protein oxidation products identified here are largely consequent on atherogenesis rather than simply on aging, for several reasons. The strongest is that even in long-lived proteins such as those of the lens, where the rate of oxidation is reflected most directly, with little input from proteolysis and excretion, it is quite clear that phenylalanine oxidation products do not show significant age-related accumulation [46]. In agreement with this, the analyses of age relationships within our limited range of materials are not indicative of any significant correlation with age; this has also been argued from other published data (see, for example, [14,19,21]). Although protein carbonyls have been claimed to accumulate with age in several circumstances, these are not usually measured as individual defined entities, and the assays can be confounded by other carbonyls such as those on sugars, DNA and lipids (reviewed in [14]). Evidence for the accumulation of specific protein oxidation products in aging is therefore limited at present.

The protein oxidation products detected here might contribute further reactions in plaque. The absence of protein-bound leucine aldehydes (which would have been detected after reduction with borohydride as an increment in hydroxyleucines [22]) might well be due to the formation of Schiff bases by these materials and/or to other reactions. This would parallel the propensity of these leucine aldehydes, when formed on free leucine, to cyclize [22]. This reaction is analogous to the intermolecular reactions of protein-dopa and protein-hydroperoxides that we have described [27,29,36,47]. Evidence *in vitro* of the capacity of protein-dopa to oxidize DNA, and to undergo redox cycling, has been obtained [48,49]; and similarly it is known that protein-hydroperoxides can give rise to further radicals and damage other target molecules [29,30,36].

We conclude that reactive products of protein oxidation have to be considered as possible contributors to atherogenesis, and that oxy-radical fluxes, possibly involving metal-ion-catalysed reactions, might be an important component of the mechanisms that lead to their deposition in advanced human atherosclerotic plaque.

This work was supported in part by the Australian Research Council, and the National Health and Medical Research Council, Australia.

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Received 26 February 1998/16 April 1998; accepted 23 April 1998