Detection of HOCl-mediated protein oxidation products in the extracellular matrix of human atherosclerotic plaques

Alan A. WOODS, Stuart M. LINTON and Michael J. DAVIES[1]

The Heart Research Institute, 145 Missenden Road, Camperdown, Sydney, New South Wales 2050, Australia

Oxidation is believed to play a role in atherosclerosis. Oxidized lipids, sterols and proteins have been detected in early, intermediate and advanced human lesions at elevated levels. The spectrum of oxidized side-chain products detected on proteins from homogenates of advanced human lesions has been interpreted in terms of the occurrence of two oxidative mechanisms, one involving oxygen-derived radicals catalysed by trace transition metal ions, and a second involving chlorinating species (HOCl or Cl_2), generated by the haem enzyme myeloperoxidase (MPO). As MPO is released extracellularly by activated monocytes (and possibly macrophages) and is a highly basic protein, it would be expected to associate with polyanions such as the glycosaminoglycans of the extracellular matrix, and might result in damage being localized at such sites. In this study proteins extracted from extracellular matrix material obtained from advanced human atherosclerotic lesions are shown to contain elevated levels of oxidized amino acids [3,4-dihydroxyphenylalanine (DOPA), di-tyrosine, 2-hydroxyphenylalanine (*o*-Tyr)] when compared with healthy (human and pig) arterial tissue. These matrix-derived materials account for 83–96 % of the total oxidized protein side-chain products detected in these plaques. Oxidation of matrix components extracted from healthy artery tissue, and model proteins, with reagent HOCl is shown to give rise to a similar pattern of products to those detected in advanced human lesions. The detection of elevated levels of DOPA and *o*-Tyr, which have been previously attributed to the occurrence of oxygen-radical-mediated reactions, by HOCl treatment, suggests an alternative route to the formation of these materials in plaques. This is believed to involve the formation and subsequent decomposition of protein chloramines.

Key words: atherosclerosis, chloramine, extracellular matrix, hypochlorite, myeloperoxidase, protein oxidation.

INTRODUCTION

The onset and progression of atherosclerosis is a complex multifactorial phenomenon in which oxidation is believed to play a role [1]. Thus oxidized lipids, sterols and proteins have been detected in early, intermediate and advanced human atherosclerotic plaques at statistically elevated levels over control tissue (reviewed in [2,3]), together with decreased levels of antioxidants, although the latter are not completely depleted [4]. Both covalent modification and oxidation of low-density lipoproteins (LDLs), a major source of the lipids present in plaque, are known to trigger degeneration in cellular control over LDL uptake, leading to lipid accumulation and the formation of macrophage-derived foam cells [5–8]. It is also well established that oxidatively modified LDLs can have other effects, including effects on endothelial cell viability, cytokine expression and smooth-muscle cell proliferation ([9–11], reviewed in [3]).

In addition to modification of LDLs and cellular materials in atherosclerotic lesions, evidence has also been presented for alterations in the materials that comprise the extracellular matrix (ECM) of the vascular wall. It is well established that the internal elastic lamina of the artery wall is altered in human atherosclerotic plaques, with both structural modification of the lamina and the accumulation of lipids at such sites having been reported [12–14]. Other ECM components, including elastin and proteoglycans, are also believed to undergo fragmentation and/or physicochemical alteration during atherogenesis [12,15,16]. Elastin isolated from plaques has been reported to contain fluorophores that have been postulated to be derived from oxidized amino acids [17]. This fluorescent material has been ascribed to phenolic cross-links, such as 3,3′-di-tyrosine (di-Tyr), the C-3–C-3′ cross-linked species formed on dimerization of two Tyr phenoxyl radicals [18]. Physicochemical changes to ECM materials are known to affect endothelial cell adhesion [19,20] and smooth muscle cell migration and proliferation [21], processes that are implicated in atherogenesis.

A wide spectrum of oxidized amino acid side chains have been detected in proteins extracted from human atherosclerotic plaques; these materials are present at elevated levels when compared with control tissue and, at least in some cases, increase in concentration during lesion development [22–24]. The formation of these well-characterized products [3,4-dihydroxyphenylalanine (DOPA), di-Tyr, 2-hydroxyphenylalanine (*o*-Tyr), 3-hydroxyphenylalanine (*m*-Tyr), 3-chlorotyrosine (3-chloroTyr), valine and leucine alcohols] has been interpreted in terms of the occurrence of two oxidative mechanisms. The first of these involves oxygen-radical formation catalysed by trace transition-metal ions; the second involves chlorinating and oxidizing species, such as HOCl [the physiological mixture of hypochlorous acid and its anion (^{-}OCl) present at pH 7.4] or Cl_2, generated by the haem enzyme myeloperoxidase (MPO) [22,23,25]. DOPA, di-Tyr, *o*- and *m*-Tyr, valine and leucine alcohols are known products of hydroxyl-radical-mediated oxidation of Tyr, Phe, Val and Leu side chains, whereas di-Tyr and 3-chloroTyr are well-defined products of Tyr oxidation induced by MPO-derived species ([22,26,27], reviewed in [28]). MPO, released from activated leucocytes, catalyses the formation of HOCl from physiological concentrations of Cl^- ions and H_2O_2 (generated via the oxidative burst of leucocytes as well as other cells) [29]. It has been shown that both active MPO, and the products of its action,

Abbreviations used: 3-chloroTyr, 3-chlorotyrosine; di-Tyr, 3,3′-di-tyrosine; DOPA, 3,4-dihydroxyphenylalanine; ECM, extracellular matrix; LDL, low-density lipoprotein; MPO, myeloperoxidase; *m*-Tyr, 3-hydroxyphenylalanine; OPA, *o*-phthalaldehyde; *o*-Tyr, 2-hydroxyphenylalanine.

[1] To whom correspondence should be addressed (e-mail m.davies@hri.org.au).

© 2003 Biochemical Society

are present in various grades of human atherosclerotic lesion [22,24,30], and that the levels of this enzyme are strongly associated with coronary artery disease [31–33].

MPO is a strongly basic (cationic) protein at physiological pH [29] and binds, via electrostatic interactions, to negatively charged materials such as the polyanionic glycosaminoglycans of the ECM [34], and LDLs [35]. Positively charged transition-metal ion complexes would likewise be expected to associate with matrix materials. Such binding might be expected to make the material to which the oxidizing system is bound a major site of damage, and this has been confirmed in some cases [34,36]. Thus both MPO and some metal ion complexes can oxidize materials to which they are bound in a site-specific manner [37,38]. Evidence has been obtained for the modification of ECM components by MPO-derived oxidants in human atherosclerotic lesions by use of a monoclonal antibody (HOP-1) raised against HOCl-damaged LDLs. This antibody does not recognize proteins oxidized by a wide variety of other oxidants *in vitro* [39]. Application of this antibody resulted in significant staining of both intra- and extra-cellular locations, particularly in the intimal area and at cholesterol clefts, with little staining detected in normal human artery samples [39]. Recent studies have demonstrated that the intensity of staining induced by this antibody correlates with intimal thickening in lesions of differing severity, suggesting that the MPO-induced damage plays a role in lesion development [40].

In the light of the above data, which are consistent with MPO-mediated oxidation of matrix materials, and previous studies that have shown that modified and oxidized amino acid side chains accumulate primarily on long-lived (primarily extracellular) proteins which undergo slow turnover (reviewed in [41]), we hypothesized that the elevated levels of side-chain oxidation products detected in human atherosclerotic lesion material might be present primarily on long-lived ECM materials, rather than on cellular proteins. Furthermore, we have examined whether the elevated levels of some protein oxidation products (e.g. DOPA, *o*- and *m*-Tyr) detected in plaques, and previously ascribed to radical-mediated oxidation catalysed by transition-metal ions, might arise via further reactions of materials such as chloramines/chloramides [RNHCl and R-NCl-C(O)R′ species respectively] generated by MPO-catalysed reactions, as these intermediates have been shown previously to give rise to radicals in transition-metal-ion-catalysed reactions [42–44].

EXPERIMENTAL

Materials

o-Phthalaldehyde (OPA) crystals and OPA diluent were obtained from Pickering Laboratories (Mountain View, CA, U.S.A.). 2-Mercaptoethanol and mercaptoacetic acid were from Merck (Kilsyth, Vic, Australia). Sodium deoxycholate, BSA (minimum 98 %), Dulbecco's PBS, EDTA, methionine, sodium borohydride and trichloroacetic acid were from Sigma (St. Louis, MO, U.S.A.). Chelex 100 resin was from Bio-Rad (Hercules, CA, U.S.A.).

Isolation of ECM

Human atherosclerotic plaques were obtained from patients undergoing carotid endarterectemy as described previously [4]. Macroscopically normal human artery tissue was obtained as tissue 'buttons' removed from unaffected artery regions to allow attachment of new sections during heart-bypass surgery. All samples were collected after informed patient consent, and approval by the local human ethics committee. Surgical samples were immediately placed in Chelex-treated, argon-flushed, PBS containing butylated hydroxytoluene (100 μM) and 1 mM EDTA (buffer A) and stored at -80 °C. Healthy pig aortae were obtained from 16-week-old male Large White × Landrace pigs fed on a standard commercial diet. Immediately following death the thoracic aorta was removed, placed in ice-cold buffer A, washed with this buffer and subsequently stored at -80 °C.

ECM was isolated by the extraction of soluble macromolecules using 1 M NaCl as described previously [14]. Intimal tissue was dissected from the adventitia and the media of the aorta, frozen in liquid N_2 and subsequently pulverized under an atmosphere of N_2 to a powder using a pestle and mortar. Care was taken to ensure that the sample remained frozen during the process by regular addition of liquid N_2. The resulting powder was subsequently mixed with buffer B (Chelex-treated and nitrogen-flushed PBS containing 1 M NaCl, 1 mM EDTA, 0.1 mM butylated hydroxytoluene, 0.2 M methionine and 0.1 mM azide). The last two reagents were included to scavenge any pre-existing HOCl or chloramines in the sample, and to inhibit any active MPO, respectively [39], and thereby prevent any artifactual oxidation during sample processing. The samples were incubated for 2 h at 4 °C with constant agitation in a closed sample tube that was top-gassed with N_2. The samples were then centrifuged at 2000 ***g*** for 5 min and the supernatants removed. The process was repeated twice more; in excess of 90 % of soluble protein was found to be removed in the first incubation. The remaining material, which is designated as ECM from here on, was washed with buffer A (see above), then stored under N_2 at -80 °C until used.

The protein and polysaccharide contents of ECM samples were determined after complete digestion of the insoluble material with 1 M NaOH at 37 °C overnight. The protein concentration was then determined using the bicinchoninic acid (BCA) assay (Pierce, Rockford, IL, U.S.A.), using BSA as a standard. The polysaccharide content of this material was estimated by measuring the uronic acid content with the carbazole assay [45]. These analyses gave a yield of 0.4 ± 0.01 g of protein and 5.31 ± 0.13 mg of uronic acid/g dry weight of ECM.

Extraction and measurement of soluble protein

Soluble protein extracted from five endarterectomy samples, using the method described above, was concentrated using the following procedure. Supernatant from the first incubation of processed plaque in buffer B was concentrated above a 3000 Da molecular-mass-cut-off filter (YM3; Millipore Corporation, Bedford, MA, U.S.A.) under N_2 gas pressure, in a stirred ultrafiltration cell (model 8050; Amicon, Beverly, MA, U.S.A.) at 4 °C. The concentrated supernatant was then exchanged into buffer A using a PD-10 desalting column (Amersham Biosciences, Uppsala, Sweden). The protein concentration was then determined using the BCA assay, using BSA as the reference standard. Proteins were concentrated by precipitation with trichloroacetic acid (5 % final concentration, w/v), and centrifugation at 10 000 ***g*** before hydrolysis.

Hydrolysis of ECM and soluble protein for HPLC analysis

A previously described method [23,28] was used for delipidation and hydrolysis with slight modification. To a 1 ml brown glass autosampler vial (Alltech Associates, Baulkham Hills, NSW, Australia) was added either soluble proteins extracted from processed plaque samples or BSA in 650 μl of Chelex-treated PBS or, intimal ECM powder (about 2 mg) to which was added the same buffer. To this solution was added 10 μl of sodium borohydride in nanopure water (10 mg · ml^{-1}). Each sample was then mixed with 0.3 % sodium deoxycholate (50 μl) and 50 %

© 2003 Biochemical Society

trichloroacetic acid (100 μl), centrifuged and washed twice with cold acetone and once with diethyl ether. The delipidated samples were then freeze-dried and hydrolysed using a standard gas-phase acid-catalysed method for 16 h at 110 °C under vacuum [23,28]. Hydrolysates were subsequently freeze-dried, re-dissolved in 200 μl of nanopure water and filtered through a 0.45 μm-pore-size filter contained in a centrifugal device (Nanosep; Pall Life Sciences, Ann Arbor, MI, U.S.A.) before HPLC analysis. Previous studies have confirmed that no detectable artifactual oxidation occurs during these processes [23,28], and this is confirmed by the low levels of oxidation products detected from the healthy pig aortae samples (see below).

HPLC analysis of protein side chains

Samples were chromatographed on an LC-10A HPLC system (Shimadzu, South Rydalmere, NSW, Australia), which was equipped with a column oven set at 30 °C (Waters Corporation, Milford, MA, U.S.A.). Peak areas were determined using Class LC-10 software (Shimadzu).

The phenylalanine concentration in ECM hydrolysates was determined by pre-column derivatization with OPA reagent and separation on a Zorbax ODS column (4.6 mm × 25 cm, 5 μm particle size; Rockland Technologies, Newport, DE, U.S.A.) using a previously described method [23,46–48]. DOPA, Tyr, *m*-Tyr, *o*-Tyr, di-Tyr and 3-chloroTyr were assayed in ECM and soluble-protein hydrolysates by separation on a Zorbax ODS column preceded by a Pelliguard guard column. The mobile phase ($1\ ml \cdot min^{-1}$) consisted of a two-solvent system; solvent A being 10 mM phosphoric acid with 100 mM sodium perchlorate (pH 2.0) and solvent B being 80 % (v/v) methanol in nanopure water. The gradient involved isocratic elution, with 0 % solvent B for the first 35 min, then an increase to 6 % solvent B in 10 min, with further elution at 6 % solvent B before changing to 50 % solvent B in 2 min and washing with 50 % solvent B for 10 min and re-equilibration at 0 % solvent B for 10 min. The elution profile was monitored in series with a UV detector (λ 280 nm), a fluorescence detector and a dual-channel electrochemical detector (ESA Coulochem II; ESA, Chelmsford, MA, U.S.A.). Parent Tyr (retention time, 23.1 min) was quantified by UV absorbance. Fluorescence detection was carried out using a $\lambda_{excitation}$ of 280 nm and a $\lambda_{emission}$ of 320 nm (for $t = 0$–45 min) for the detection of DOPA (12.9 min), *m*-Tyr (17.2 min) and *o*-Tyr (38.2 min), and subsequently a $\lambda_{emission}$ of 410 nm for the detection of di-Tyr (59 min). 3-ChloroTyr (53.1 min) was separated and detected, in most samples, by the oxidation of interfering co-eluting peaks at +500 mV in the first electrode in the electrochemical detector, and measurement of the required species at +600 mV in the second electrode. In experiments involving BSA, 3-chloroTyr was also measured by its absorbance at 280 nm. Elution positions were determined with standards, the identities of compounds being confirmed previously by UV absorption and fluorescence spectra [23]. The level of each oxidized amino acid is expressed relative to the parent species to compensate for any loss during sample processing and analysis [28].

Statistical analyses

All statistical analyses were performed using GraphPad Prism version 3.0a for the Macintosh (GraphPad Software, San Diego, CA, U.S.A.). One-way ANOVA was conducted on the levels of oxidized amino acids in different arterial intimal samples (i.e. pig versus normal human, pig versus plaque and normal human versus plaque) and on the effect of HOCl on oxidized amino acid levels in ECM and BSA. *Post-hoc* statistical analysis of these samples were performed with the Tukey–Kramer multiple-comparison test, except HOCl-treated BSA samples, which were analysed with Dunnet's multiple-comparison test. Following subtraction of control values obtained in the absence of HOCl, the HOCl-treated BSA samples were also analysed with two-way ANOVA for interaction between HOCl concentration and incubation time. For all statistical analysis, significance was assumed at probability (P) levels below 0.05.

RESULTS

Levels of oxidized amino acids in ECM isolated from advanced atherosclerotic plaques, normal human aortae and normal pig aortae

Oxidized amino acids were quantified in the hydrolysates of intimal ECM proteins obtained from diseased and non-diseased arterial tissue, i.e. human atherosclerotic plaques, normal human aortic tissue and healthy pig aortae. HPLC analysis revealed significant levels of DOPA, *o*-Tyr and di-Tyr. Accurate quantification of the levels of *m*-Tyr could not be achieved, due to overlap with the parent Tyr peak in the fluorescence chromatogram. Levels of 3-chloroTyr could not be accurately determined in human plaque ECM, due to the presence of interfering, co-eluting, peaks of unknown origin. The levels of each measured oxidized product (DOPA, *o*-Tyr and di-Tyr) in each tissue preparation are reported in Figure 1.

The ratios of oxidized species relative to parent amino acid present in the soluble proteins of atherosclerotic lesions were also quantified so that the levels of these materials could be compared with those determined for the ECM (Table 1). Soluble proteins were extracted with 1 M NaCl from five human plaques (see the Experimental section) and the levels of the oxidized amino acids present assayed as described above. In these samples it was possible to quantify *m*-Tyr and 3-chloroTyr, in most cases, in addition to DOPA, *o*-Tyr and di-Tyr, due to the reduced complexity of the HPLC chromatograms obtained. When the ratio of *m*-Tyr/Tyr was too small to allow measurement of the oxidized amino acid (less than ≈ 700 μmol/mol of Phe) the level of this product was taken as zero. The levels of DOPA, *o*-Tyr and di-Tyr were not significantly different from the levels detected for the ECM when expressed per mol of parent amino

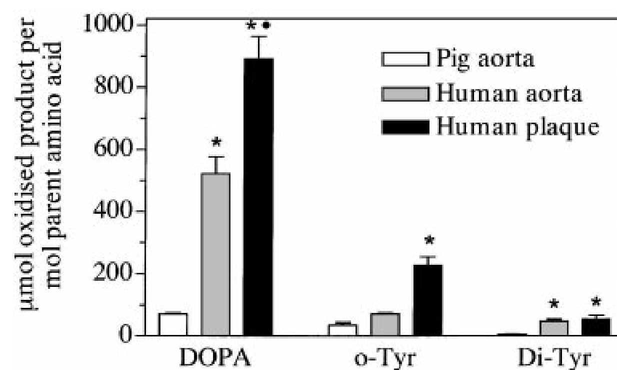

Figure 1 Levels of oxidized amino acids measured in proteins of the intimal ECM isolated from healthy pig aortae, human aortae and human atherosclerotic plaques

Results are expressed as μmol of oxidized amino acid/mol of parent amino acid and are means ± S.E.M. ($n = 6$ for pig aortae, $n = 8$ for human aortae, $n = 7$ for plaques). Statistical analysis using one-way ANOVA as described in the Experimental section revealed that the level of each oxidized product was significantly different between the different tissue types. Within each product group, the presence of a symbol (★, ●) indicates a significant difference (as judged by *post-hoc* analysis) from values in different tissue types without that symbol.

© 2003 Biochemical Society

Table 1 Levels of oxidized amino acids in the soluble and insoluble (ECM) proteins of human atherosclerotic plaques

Proteins were isolated and analysed by HPLC as described in the Experimental section. The level of each oxidized product is given as μmol/mol of parent amino acid ($\pm$S.E.M.; $n = 5$). Total levels of oxidized products were determined by calculating the relative distribution of the oxidized species in each protein fraction. This was achieved by comparing the amount of soluble protein extracted from the plaques in this study, 1.70 mg/g of atherosclerotic plaque intima, with the previously determined total value of 55.1 mg of protein/g of atherosclerotic plaque intima [4]. A dash indicates that the value could not be determined accurately, due to the presence of co-eluting materials in the HPLC chromatograms of the hydrolysates of the ECM proteins.

	Level of oxidized amino acids (μmol of oxidized amino acid/mol of parent amino acid)			Total oxidized product
Oxidized product	Soluble proteins	ECM proteins	Total proteins	in ECM (%)
DOPA	6290 $\pm$ 2872	987 $\pm$ 61.4	1151 $\pm$ 149	83 $\pm$ 5
m-Tyr	542 $\pm$ 337	–	–	–
o-Tyr	1060 $\pm$ 492	282 $\pm$ 45.8	306 $\pm$ 59.7	89 $\pm$ 15
3-ChloroTyr	3320 $\pm$ 568	–	–	–
Di-Tyr	52.1 $\pm$ 16.6	53.4 $\pm$ 16.6	53.6 $\pm$ 16.6	96 $\pm$ 30

acid. As the levels of *m*-Tyr measured in the soluble proteins are likely to be underestimates, as a result of the assumption of zero levels in some samples, it would appear that the levels of *m*-Tyr in this protein fraction are higher than the levels present in homogenates of whole plaques [23], consistent with the low level of this oxidized material in the ECM. The levels of 3-chloroTyr detected are markedly elevated above those reported for whole-plaque homogenates and LDLs isolated from whole plaque [22]. The variation in the levels of DOPA, *m*-Tyr and *o*-Tyr was in all cases much greater than that detected with 3-chloroTyr and di-Tyr; potential reasons for this variability are discussed in detail below.

Comparison of the level of oxidized species in the ECM and total protein fractions (Table 1) demonstrates that the vast majority of DOPA, *o*-Tyr and di-Tyr measured was present in the insoluble ECM fraction of the plaques assayed. Although it is possible that the soluble protein pool has been underestimated, the calculated overall levels of the oxidized products DOPA and *o*-Tyr are remarkably similar to the levels described in whole-plaque homogenates [23]. Di-Tyr levels were lower than reported in this previous study, although it should be noted that considerable variation was seen between samples in both studies.

Effect of HOCl on levels of oxidized amino acids in intimal ECM and BSA

In an attempt to gain further insight into the mechanism(s) of oxidative damage which give rise to the observed products, ECM extracted from the intima of healthy pig aortae was incubated with HOCl (0.6 μmol/mg of ECM). The ECM was then hydrolysed and analysed by HPLC for the levels of DOPA, *o*-Tyr, di-Tyr and 3-chloroTyr (Figure 2). In the treated samples 3-chloroTyr was the most abundant product, with this material present at up to 10-fold higher levels than DOPA, the next most abundant product.

As a previous investigation found little formation of DOPA on BSA treated with HOCl for 20 min [23], further experiments were carried out with BSA incubated with 0-, 10-, 20- or 40-fold molar excesses of HOCl for 1, 2 or 4 h at 37 °C. The treated BSA

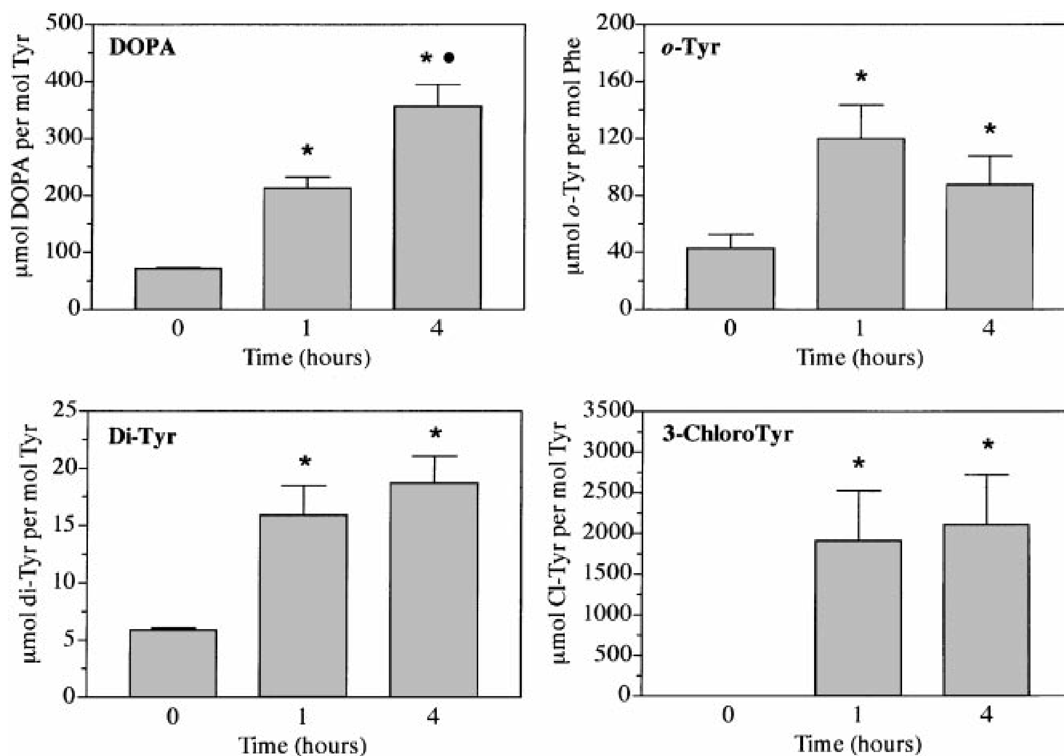

Figure 2 Levels of oxidized amino acids in the ECM of healthy pig aortae following incubation with HOCl for 1 or 4 h

ECM isolated from the intima of healthy pig aortae was incubated with HOCl (0.6 μmol/mg of ECM) and analysed by HPLC as described in the Experimental section. Results are expressed as μmol of oxidized amino acid/mol of parent amino acid and are means $\pm$ S.E.M. ($n = 6$). Statistical analysis of the data using one-way ANOVA as described in the Experimental section revealed that the level of each oxidized product was significantly different in each sample. Within each panel, the presence of a symbol denotes a significant difference from bars without that symbol (as judged by *post-hoc* analysis).

© 2003 Biochemical Society

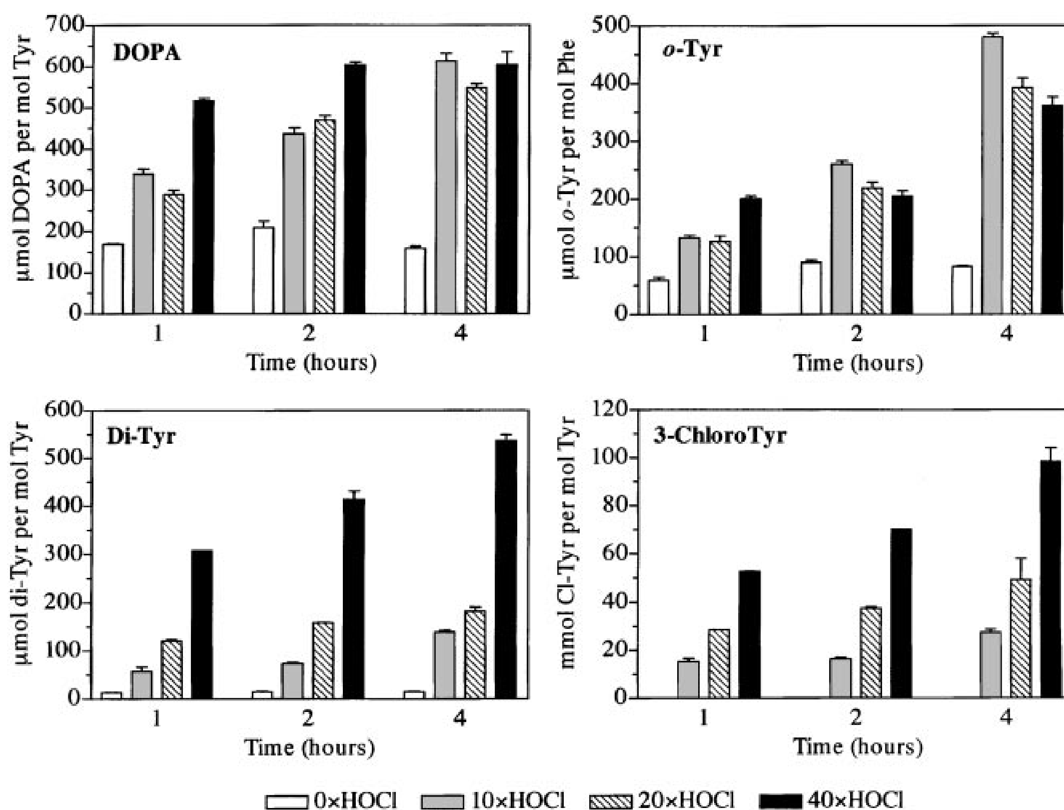

Figure 3 Levels of oxidized amino acids measured in BSA following incubation with HOCl

BSA (0.05 mM) was incubated with 0-, 10-, 20- or 40-fold molar excess of HOCl at 37 °C for 1, 2 or 4 h. DOPA, *o*-Tyr and di-Tyr are expressed as μmol of product formed/mol of parent amino acid and 3-chloroTyr is expressed as mmol/mol of parent (no 3-chloro-Tyr was detected in the control experiments). Results are expressed as means $\pm$ S.E.M. ($n = 3$). Statistical analysis of the data using one-way ANOVA as described in the Experimental section revealed that the means of each oxidized product were significantly different in each sample. *Post-hoc* analysis revealed that at all time-points the level of each oxidized amino acid was significantly higher in HOCl-treated samples than in BSA controls.

was then hydrolysed and analysed for DOPA, *o*-Tyr, di-Tyr and 3-chloroTyr as above. The most abundant product detected was 3-chloroTyr, which after 4 h at the highest HOCl concentration reached levels of one 3-chloroTyr molecule/10 Tyr molecules (Figure 3). As with 3-chloroTyr, di-Tyr was generated in a time- and concentration-dependent manner, though the absolute levels of this product were much lower ($\approx$ 200-fold less). Significant time-dependent generation of DOPA and *o*-Tyr was also observed, with the highest levels of these materials equivalent to those detected for di-Tyr. The yield of these two products did not increase in a linear manner with increasing HOCl concentration, in contrast with the behaviour of 3-chloroTyr and di-Tyr. Statistical analysis using two-way ANOVA revealed that, for each oxidized product, significant interaction between increasing concentrations of HOCl and incubation time occurred to produce more oxidized product than for each factor alone.

DISCUSSION

Oxidative damage to the ECM of the artery wall has been suggested to play a significant role in the development and progression of atherogenesis, although quantitative evidence for such oxidation is scarce, and the exact nature of the oxidized materials formed is poorly defined [12–18]. Furthermore, the slow rate of turnover of proteins present in the ECM, and the absence of extracellular repair or catabolic enzymes, is likely to result in the accumulation of such damage (reviewed in [41,49,50]). Two of the major mechanisms which have been suggested to play a role in the oxidation of materials present in artery wall, metal-ion catalysed processes and reactions induced by MPO [22,23,25,51], are also likely to occur extracellularly as a result of the avid binding of both metal ion complexes and MPO to extracellular components [34,36]. In both cases such binding would be expected to result in the preferential oxidation of the materials to which these catalysts are bound, due to the high reactivity, and hence small diffusion radius, of the oxidants produced (radicals, HOCl, Cl_2).

We have shown that specific markers of oxidative damage to protein side chains are elevated in ECM obtained from advanced human atherosclerotic plaques when compared with intimal ECM from healthy pig and normal human aortae (Figure 1). The levels of these oxidized products in plaque ECM are similar to those observed previously for whole-plaque homogenates [23], consistent with the observations made above that these oxidized protein-derived materials are present primarily on ECM proteins rather than intracellular proteins. The levels of these materials are also higher than those detected on lipoproteins (or the lipoprotein-containing fraction) extracted from plaques of varying degrees of development [24], again consistent with the accumulation of oxidative modifications primarily on (long half-life) matrix materials. The levels of these oxidized ECM-derived materials are also elevated when compared with total intimal proteins present in homogenates of normal human arteries [23] and the levels of these materials present in human plasma proteins [23].

© 2003 Biochemical Society

The levels of the individual protein side-chain oxidation products, DOPA, *o*-Tyr and di-Tyr, observed in plaque ECM and normal human aortae ECM are commensurate with the levels observed in proteins from homogenates of whole plaque and normal human arteries respectively [23]. In contrast, the levels of DOPA, *o*-Tyr and di-Tyr measured in proteins of healthy pig aortae are significantly lower than those observed in normal human aortae, and are close to the levels detected in fresh human plasma [23]. As the levels of oxidative products detected in ECM from human plaques are similar to the previous results for whole plaque [23], these data suggest that there is an increased accumulation of oxidized material in normal human aortae and iliac arteries when compared with healthy pig aortae, which might arise from either an increased rate of formation of oxidized materials or a decreased rate of removal; the current data do not allow these two possibilities to be differentiated. Such an accumulation is not surprising, however, given the relative ages of the groups (22–50 years for the human iliac arteries, 50–76 years for human aortae and 16 weeks for pigs).

The relative levels of the measured oxidized products DOPA, *o*-Tyr and di-Tyr in ECM isolated from human plaques are, like those previously detected in whole-plaque homogenates [23], inconsistent with the sole occurrence of transition-metal-ion-mediated, oxygen-radical-induced, damage. Thus oxidation of BSA by hydroxyl radicals has been shown to result in the formation of DOPA, *o*-Tyr and di-Tyr in proportions of $\approx 1.2:1:0.3$, which is markedly different from the proportions observed in the current studies. Furthermore, the detection of 3-chloroTyr on proteins derived from human plaques in both the current and previous studies [22] is consistent with the occurrence of oxidation mediated by HOCl (or other chlorinating species). Experiments involving the incubation of HOCl with BSA have demonstrated that this oxidant generates not only 3-chloroTyr, but also other oxidized products including di-Tyr and DOPA [23]. Due to the presence of co-eluting peaks it was not possible to quantify the levels of 3-chloroTyr in ECM extracted from plaques, despite varying a number of chromatographic parameters. This product could, however, be readily measured in the soluble protein fraction extracted from such plaques, even though this fraction contained only a small proportion ($\approx 20\,\%$) of the total oxidized amino acids detected. The level of 3-chloroTyr detected in soluble proteins isolated from plaque (3320 ± 568 μmol/mol of Tyr) is significantly higher than observed by Hazen and Heinecke in whole plaque (424 ± 26 μmol/mol of Tyr) [22]. However, this result is consistent with the elevated levels of other oxidized products (DOPA, *m*-Tyr, *o*-Tyr) that we observed when comparing soluble protein with whole plaque.

The extent of conversion of Tyr residues into 3-chloroTyr observed on reaction of BSA with HOCl provides an interesting contrast with the relative reactivity of Tyr predicted by kinetic modelling of the reaction of HOCl with human serum albumin [52]. This model, which takes into account the reactivity of the different amino acid side-chain groups and peptide bonds with HOCl and their relative abundance, predicts that there would be only minimal direct oxidation of Tyr residues by HOCl at the concentrations employed in the current study. Whereas the kinetic model is limited by an inability to take into consideration factors such as accessibility and electronic (charge) effects on the reaction rates, the high levels of Tyr chlorination observed on BSA in the current study suggests that there may be other routes to 3-chloroTyr generation than via direct reaction with HOCl. Whether such chlorination is direct or indirect, the level of modification detected in this study suggests that the HOCl flux required to generate the quantities of 3-chloroTyr detected in atherosclerotic plaques may be lower than that previously theorized [52], although it is clear that high levels of overall HOCl exposure must be occurring.

Previous studies have implicated the generation of Tyr phenoxyl radicals generated by MPO in the formation of di-Tyr [26,53]. The elevated levels of di-Tyr observed in plaque ECM are therefore consistent with the close juxtaposition of this enzyme with the ECM of arterial lesions. We did not observe significant additional formation of di-Tyr in ECM extracted from normal tissue incubated with HOCl. This may be due to either the absence of free Tyr that might cross-link to protein-bound Tyr residues, or the steric constraints that may be imposed on the dimerization of Tyr-derived phenoxyl radicals formed on matrix proteins by the three-dimensional structure of the matrix. A previous study from this laboratory has revealed that di-Tyr can be generated on treatment of the globular protein BSA with HOCl [54], suggesting that that steric factors play an important role in determining the relative yield of di-Tyr in different situations.

It is well established that reaction of HOCl with free Lys, and Lys residues on proteins, generates short-lived reactive chloramine (RNHCl) species [55]. These intermediates have been shown to decay to give rise to both carbonyl groups and radical species, with the latter having been postulated to play a role in the induction of protein fragmentation [43]. As these chloramine-derived radicals might potentially play a role in the observed formation of DOPA on ECM in the presence of HOCl, the potential formation of this oxidized product was monitored on samples of ECM that were exposed to HOCl. Under such conditions chloramines are known to be formed on the matrix materials ([19], and A. A. Woods and M. J. Davies, unpublished work). The increase in concentration of DOPA over time, which correlates with the time period over which chloramine decay occurs (A. A. Woods and M. J. Davies, unpublished work), supports the hypothesis that some of the DOPA detected in biological samples that have been exposed to HOCl as a result of chronic inflammation may arise via this pathway rather than via metal-ion-catalysed oxygen-radical attack [56,57]. The formation of DOPA on proteins is of potential importance, as this material can contribute to further oxidative reactions via its ability to redox cycle (e.g. [58,59]). The formation of both extracellular matrix-bound chloramines and DOPA within atherosclerotic plaques may contribute to further reactions and play a significant role in the development of atherosclerosis and plaque instability.

We thank Professor James May and colleagues for the collection of atherosclerotic plaque samples, Dr Paul Bannon and colleagues for the provision of normal arterial samples, and Professor Roger Dean for stimulating discussions. We are grateful to the National Health and Medical Research Council, the Australian Research Council and the Wellcome Trust for financial support.

REFERENCES

1 Steinberg, D., Parthasarathy, S., Carew, T. E., Khoo, J. C. and Witztum, J. L. (1989) Beyond cholesterol: modifications of low-density lipoprotein that increase its atherogenicity. N. Engl. J. Med. **320**, 915–924
2 Heinecke, J. W. (1998) Oxidants and antioxidants in the pathogenesis of atherosclerosis: implications for the oxidized low density lipoprotein hypothesis. Atherosclerosis **141**, 1–15
3 Dean, R. T. and Kelly, D. T. (eds) (2000) Atherosclerosis: Gene Expression, Cell Interactions and Oxidation, pp. 1–450, Oxford University Press, Oxford
4 Suarna, C., Dean, R. T., May, J. and Stocker, R. (1995) Human atherosclerotic plaque contains both oxidized lipids and relatively large amounts of α-tocopherol and ascorbate. Arterioscler. Thromb. Vasc. Biol. **15**, 1616–1624
5 Goldstein, J. L., Basu, S. K. and Brown, M. S. (1983) Receptor mediated endocytosis of low density lipoprotein in cultured cells. Methods Enzymol. **98**, 241–260
6 Steinbrecher, U. P., Parthasarathy, S., Leake, D. S., Witztum, J. L. and Steinberg, D. (1984) Modification of low density lipoprotein by endothelial cells involves lipid peroxidation and degradation of low density lipoprotein phospholipids. Proc. Natl. Acad. Sci. U.S.A. **81**, 3883–3887

© 2003 Biochemical Society

7 Steinbrecher, U. P., Zhang, H. and Lougheed, M. (1990) Role of oxidatively modified LDL in atherosclerosis. Free Radic. Biol. Med. **9**, 155–168
8 Jessup, W., Dean, R. T., de Whalley, V. C., Rankin, S. M. and Leake, D. S. (1990) The role of oxidative modification and antioxidants in LDL metabolism and atherosclerosis. Adv. Exp. Med. Biol. **264**, 139–142
9 Parhami, F., Fang, Z. T., Fogelman, A. M., Andalibi, A., Territo, M. C. and Berliner, J. A. (1993) Minimally modified low density lipoprotein-induced inflammatory responses in endothelial cells are mediated by cyclic adenosine monophosphate. J. Clin. Invest. **92**, 471–478
10 Rajavashisth, T. B., Yamada, H. and Mishra, N. K. (1995) Transcriptional activation of the macrophage-colony stimulating factor gene by minimally modified LDL. Involvement of nuclear factor-κB. Arterioscl. Thromb. Vasc. Biol. **15**, 1591–1598
11 Bolton, E. J., Jessup, W., Stanley, K. K. and Dean, R. T. (1994) Enhanced LDL oxidation by murine macrophage foam cells and their failure to secrete nitric oxide. Atherosclerosis **106**, 213–223
12 Kramsch, D. M., Franzblau, C. and Hollander, W. (1974) Components of the protein-lipid complex of arterial elastin: their role in the retention of lipid in atherosclerotic lesions. Adv. Exp. Med. Biol. **43**, 193–210
13 Robert, L., Jacob, M. P., Frances, C., Godeau, G. and Hornebeck, W. (1984) Interaction between elastin and elastases and its role in the aging of the arterial wall, skin and other connective tissues. A review. Mech. Ageing Dev. **28**, 155–166
14 Robert, L., Robert, A. M. and Jacotot, B. (1998) Elastin-elastase-atherosclerosis revisited. Atherosclerosis **140**, 281–295
15 Kramsch, D. M. and Hollander, W. (1973) The interaction of serum and arterial lipoproteins with elastin of the arterial intima and its role in the lipid accumulation in atherosclerotic plaques. J. Clin. Invest. **52**, 236–247
16 Wagner, W. D., Salisbury, B. G. J. and Rowe, H. A. (1987) A proposed structure of chondroitin 6-sulfate proteoglycan of human normal and adjacent atherosclerotic plaque. Arteriosclerosis **6**, 407–417
17 Rucker, R. B. and Tinker, D. (1977) Structure and metabolism of arterial elastin. Int. Rev. Exp. Pathol. **17**, 1–47
18 LaBella, F. S. (1974) Arterial mesenchyme and arteriosclerosis. Enzymic vs non-enzymic factors in the deterioration of connective tissue. Adv. Exp. Med. Biol. **43**, 377–402
19 Vissers, M. C. M. and Winterbourn, C. C. (1991) Oxidative damage to fibronectin. 1. The effects of the neutrophil myeloperoxidase system and HOCl. Arch. Biochem. Biophys. **285**, 53–59
20 Underwood, P. A., Bean, P. A. and Whitelock, J. M. (1998) Inhibition of endothelial cell adhesion and proliferation by extracellular matrix from vascular smooth muscle cells: role of type V collagen. Atherosclerosis **141**, 141–152
21 Underwood, P. A., Mitchell, S. M. and Whitelock, J. M. (1998) Heparin fails to inhibit the proliferation of human vascular smooth muscle cells in the presence of human serum. J. Vasc. Res. **35**, 449–460
22 Hazen, S. L. and Heinecke, J. W. (1997) 3-Chlorotyrosine, a specific marker of myeloperoxidase-catalysed oxidation, is markedly elevated in low density lipoprotein isolated from human atherosclerotic intima. J. Clin. Invest. **99**, 2075–2081
23 Fu, S., Davies, M. J., Stocker, R. and Dean, R. T. (1998) Evidence for roles of radicals in protein oxidation in advanced human atherosclerotic plaque. Biochem. J. **333**, 519–525
24 Upston, J. M., Niu, X., Brown, A. J., Mashima, R., Wang, H., Senthilmohan, R., Kettle, A. J., Dean, R. T. and Stocker, R. (2002) Disease stage-dependent accumulation of lipid and protein oxidation products in human atherosclerosis. Am. J. Pathol. **160**, 701–710
25 Fu, S., Dean, R. T., Davies, M. J. and Heinecke, J. W. (2000) in Atherosclerosis: Gene Expression, Cell Interactions and Oxidation (Dean, R. T. and Kelly, D., eds.), pp. 301–325, Oxford University Press, Oxford
26 Heinecke, J. W., Li, W., Daehnke, H. L. and Goldstein, J. A. (1993) Dityrosine, a specific marker of oxidation, is synthesized by the myeloperoxidase-hydrogen peroxide system of human neutrophils and macrophages. J. Biol. Chem. **268**, 4069–4077
27 Kettle, A. J. (1996) Neutrophils convert tyrosyl residues in albumin to chlorotyrosine. FEBS Lett. **379**, 103–106
28 Davies, M. J., Fu, S., Wang, H. and Dean, R. T. (1999) Stable markers of oxidant damage to proteins and their application in the study of human disease. Free Radic. Biol. Med. **27**, 1151–1163
29 Kettle, A. J. and Winterbourn, C. C. (1997) Myeloperoxidase: a key regulator of neutrophil oxidant production. Redox Rep. **3**, 3–15
30 Daugherty, A., Dunn, J. L., Rateri, D. L. and Heinecke, J. W. (1994) Myeloperoxidase, a catalyst for lipoprotein oxidation, is expressed in human atherosclerotic lesions. J. Clin. Invest. **94**, 437–444
31 Kutter, D., Devaquet, P., Vanderstocken, G., Paulus, J. M., Marchal, V. and Gothot, A. (2000) Consequences of total and subtotal myeloperoxidase deficiency: risk or benefit? Acta Haematol. **104**, 10–15
32 Nikpoor, B., Turecki, G., Fournier, C., Theroux, P. and Rouleau, G. A. (2001) A functional myeloperoxidase polymorphic variant is associated with coronary artery disease in French-Canadians. Am. Heart. J. **142**, 336–369
33 Zhang, R., Brennan, M. L., Fu, X., Aviles, R. J., Pearce, G. L., Penn, M. S., Topol, E. J., Sprecher, D. L. and Hazen, S. L. (2001) Association between myeloperoxidase levels and risk of coronary artery disease. J. Am. Med. Assoc. **286**, 2136–2142
34 McGowan, S. E. (1990) Mechanisms of extracellular matrix proteoglycan degradation by human neutrophils. Am. J. Respir. Cell. Mol. Biol. **2**, 271–279
35 Carr, A. C., Myzak, M. C., Stocker, R., McCall, M. R. and Frei, B. (2000) Myeloperoxidase binds to low-density lipoprotein: potential implications for atherosclerosis. FEBS Lett. **487**, 176–180
36 Upritchard, J. E. and Sutherland, W. H. (1999) Oxidation of heparin-treated low density lipoprotein by peroxidases. Atherosclerosis **146**, 211–219
37 Stadtman, E. R. (1993) Oxidation of free amino acids and amino acid residues in proteins by radiolysis and by metal-catalyzed reactions. Annu. Rev. Biochem. **62**, 797–821
38 Daphna, E. M., Michaela, S., Eynat, P., Irit, A. and Rimon, S. (1998) Association of myeloperoxidase with heparin: oxidative inactivation of proteins on the surface of endothelial cells by the bound enzyme. Mol. Cell. Biochem. **183**, 55–61
39 Hazell, L. J., Arnold, L., Flowers, D., Waeg, G., Malle, E. and Stocker, R. (1996) Presence of hypochlorite-modified proteins in human atherosclerotic lesions. J. Clin. Invest. **97**, 1535–1544
40 Hazell, L. J., Baernthaler, G. and Stocker, R. (2001) Correlation between intima-to-media ratio, apolipoprotein B-100, myeloperoxidase, and hypochlorite-oxidized proteins in human atherosclerosis. Free Radic. Biol. Med. **31**, 1254–1262
41 Linton, S., Davies, M. J. and Dean, R. T. (2001) Protein oxidation in ageing. Exp. Gerentol. **36**, 1503–1518
42 Hawkins, C. L. and Davies, M. J. (1999) Hypochlorite-induced oxidation of proteins in plasma: formation of chloramines and nitrogen-centred radicals and their role in protein fragmentation. Biochem. J. **340**, 539–548
43 Hawkins, C. L. and Davies, M. J. (1998) Hypochlorite-induced damage to proteins: formation of nitrogen-centred radicals from lysine residues and their role in protein fragmentation. Biochem. J. **332**, 617–625
44 Hawkins, C. L., Rees, M. D. and Davies, M. J. (2002) Superoxide radicals can act synergistically with hypochlorite to induce damage to proteins. FEBS Lett. **510**, 41–44
45 Blumencrantz, N. and Asboe-Hansen, G. (1973) New method for quantitative determination of uronic acids. Anal. Biochem. **54**, 484–489
46 Fu, S. L. and Dean, R. T. (1997) Structural characterization of the products of hydroxyl-radical damage to leucine and their detection on proteins. Biochem. J. **324**, 41–48
47 Fu, S., Hick, L. A., Sheil, M. M. and Dean, R. T. (1995) Structural identification of valine hydroperoxides and hydroxides on radical-damaged amino acid, peptide, and protein molecules. Free Radic. Biol. Med. **19**, 281–292
48 Dean, R. T., Fu, S., Gieseg, S. and Armstrong, S. G. (1996) in Free Radicals: a Practical Approach (Punchard, N. and Kelly, F. J., eds.), pp. 171–183, Oxford University Press, Oxford
49 Dean, R. T., Fu, S., Stocker, R. and Davies, M. J. (1997) Biochemistry and pathology of radical-mediated protein oxidation. Biochem. J. **324**, 1–18
50 Dunlop, R. A., Rodgers, K. J. and Dean, R. T. (2002) Recent developments in the intracellular degradation of oxidized proteins. Free Radic. Biol. Med. **33**, 894–906
51 Heinecke, J. W. (1999) Mechanisms of oxidative damage by myeloperoxidase in atherosclerosis and other inflammatory disorders. J. Lab. Clin. Med. **133**, 321–325
52 Pattison, D. I. and Davies, M. J. (2001) Absolute rate constants for the reaction of hypochlorous acid with protein side chains and peptide bonds. Chem. Res. Toxicol. **14**, 1453–1464
53 Heinecke, J. W., Li, W., Francis, G. A. and Goldstein, J. A. (1993) Tyrosyl radical generated by myeloperoxidase catalyzes the oxidative cross-linking of proteins. J. Clin. Invest. **91**, 2866–2872
54 Fu, S., Wang, H., Davies, M. J. and Dean, R. T. (2000) Reaction of hypochlorous acid with tyrosine and peptidyl-tyrosyl residues gives dichlorinated and aldehydic products in addition to 3-chlorotyrosine. J. Biol. Chem. **275**, 10851–10858
55 Weiss, S. J., Lampert, M. B. and Test, S. T. (1983) Long-lived oxidants generated by human neutrophils: characterization and bioactivity. Science **222**, 625–628
56 Davies, M. J. and Dean, R. T. (1997) Radical-Mediated Protein Oxidation: from Chemistry to Medicine, Oxford University Press, Oxford
57 Hawkins, C. L. and Davies, M. J. (2001) Generation and propagation of radical reactions on proteins. Biochim. Biophys. Acta **1504**, 196–219
58 Morin, B., Davies, M. J. and Dean, R. T. (1998) The protein oxidation product 3,4-dihydroxyphenylalanine (DOPA) mediates oxidative DNA damage. Biochem. J. **330**, 1059–1067
59 Pattison, D. I., Dean, R. T. and Davies, M. J. (2002) Oxidation of DNA, proteins and lipids by DOPA, protein-bound DOPA, and related catechols and catecholamines. Toxicology **177**, 23–37

Received 1 November 2002; accepted 28 November 2002
Published as BJ Immediate Publication 28 November 2002, DOI 10.1042/BJ20021710

© 2003 Biochemical Society