Biological fate of amino acid, peptide and protein hydroperoxides

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In the course of searching for a suitable marker for studying protein oxidation, we have successfully elucidated the structures of three valine hydroperoxides, i.e. β -hydroperoxyvaline, (2S,3S)- γ -hydroperoxyvaline and (2S,3R)- γ -hydroperoxyvaline, which are novel products of protein oxidation. The corresponding valine hydroxides were obtained by sodium borohydride reduction [Fu, Hick, Sheil and Dean (1995) *Free Rad. Biol. Med.* 19, 281–292]. We hypothesized that valine hydroxides might be the major biological degradation products of valine hydroperoxides and, as such, could be useful markers for the study of protein oxidation *in vivo*. The aim of this study was to investigate the fate of valine hydroperoxide in selected biological

systems by the use of chemiluminescence detection of hydroperoxides and HPLC analysis of *O*-phthalaldehyde derivatives of amino acid residues. The degradation of hydroperoxides present on γ -radiolysed solutions of valine, Pro-Val-Gly, or BSA occurred in the presence of: (1) transition metals (Fe^{2+} , Fe^{3+} , or Cu^{2+}), (2) the detoxifying enzyme GSH peroxidase, (3) human plasma, and (4) J774 mouse monocyte macrophage cells. The major degradation product of valine hydroperoxide recovered in each case was found to be a valine hydroxide. These results suggest that valine hydroxide (derived from the hydroperoxide) may well be a useful *in vivo* marker for studying protein damage under oxidative stress.

INTRODUCTION

It has been reported that protein damage by free radical exposure (reviewed in [1]) produces reducing species such as protein-bound 3,4-dihydroxyphenylalanine (DOPA), and oxidizing species such as protein hydroperoxides, which can consume some antioxidants *in vitro* [2–4]. In the course of searching for a suitable marker for studying the latter product of protein oxidation, we have focused on the oxidation products of valine generated by free radical damage, because free valine is the amino acid on which hydroperoxides are most readily found during hydroxyl radical attack [5]. We have previously isolated, by means of HPLC, and identified, by using NMR and MS techniques, three valine hydroperoxides, i.e. β -hydroperoxyvaline (Val.OOH1), (2S,3S)- γ -hydroperoxyvaline (Val.OOH2) and (2S,3R)- γ -hydroperoxyvaline (Val.OOH3), which were generated from radiolysis of valine solution in the presence of oxygen [6]. After reduction by sodium borohydride (NaBH_4), the three valine hydroperoxides were detected in γ -radiolysed solutions of valine-containing tripeptides and BSA in the form of the corresponding hydroxides (i.e. Val.OH1, Val.OH2 and Val.OH3 respectively). We therefore proposed that valine hydroxides might be useful markers for valine hydroperoxides, provided that in biological systems valine hydroxides are the major degradation products of valine hydroperoxides [6]. The aim of this study was to investigate the degradation products of valine hydroperoxides in some biological systems by the use of a chemiluminescence assay for hydroperoxides and HPLC analysis for hydroxides after pre-column derivatization with *O*-phthalaldehyde (OPA), and thus to evaluate the potential of valine hydroxide as a biological marker. Our results show that the hydroxides are the major products of degradation of the hydroperoxides in several biochemical and cellular systems.

MATERIALS AND METHODS

Materials

Water was purified by passage through a 4-stage Milli Q system (Millipore-Waters, Australia) equipped with a 0.2 μm -pore-size final filter. Valine, Pro-Val-Gly (PVG), BSA (fatty acid free), glutathione (GSH), GSH peroxidase, OPA reagent solution (incomplete), isoluminol (6-amino-2,3-dihydro-1,4-phthalazinedione), catalase and microperoxidase (MP11) were obtained from Sigma Chemical Company (St. Louis, MO, U.S.A.). *N*-Ethylmaleimide was obtained from Calbiochem-Behring (La Jolla, CA, U.S.A.). Other chemicals, solvents and chromatographic materials were of AR or HPLC grade.

Formation of hydroperoxides by radicals generated in ^{60}Co radiolysis

Unless otherwise specified, radiolysis was performed as described below. Dilute solutions of valine (1 mM), PVG (1 mM), BSA (2 mg/ml) or human apotransferrin (2 mg/ml) in water were irradiated for 30 min in a ^{60}Co facility (dose rate 33.3 Gy/min) while gassing continuously with oxygen. In this radiolytic system, the predominant species formed are hydroxyl radicals ($\cdot\text{OH}$) and superoxide radical ions ($\cdot\text{O}_2^-$) [7]. In a limited number of experiments, human apolipoprotein-A1 (apo-A1) (10 mg/ml in 10 mM Tris Buffer, pH 7.4, containing 150 mM NaCl, 0.006% NaN_3 and 1 mM EDTA; a kind gift from Dr. K. A. Rye, University of Adelaide, Australia) was 5-fold diluted into water and similarly exposed to radiolysis; because of the presence of Tris, the radical fluxes in this experiment were more complex. In all cases, any radiolysis-generated H_2O_2 was removed after irradiation by addition of a small volume of catalase (5 $\mu\text{g}/\text{ml}$ final concentration): as we have determined previously [2] this

Abbreviations used: DOPA, 3,4-dihydroxyphenylalanine; Val.OOH1, β -hydroperoxyvaline; Val.OOH2, (2S,3S)- γ -hydroperoxyvaline; Val.OOH3, (2S,3R)- γ -hydroperoxyvaline; Val.OH1, β -hydroxyvaline; Val.OH2, (2S,3S)- γ -hydroxyvaline; Val.OH3, (2S,3R)- γ -hydroxyvaline; Val.OOH, valine hydroperoxide; PVG, Pro-Val-Gly; PVG-OOH, PVG hydroperoxide; BSA-OOH, BSA hydroperoxide; OPA, *O*-phthalaldehyde; HBSS, Hanks' balanced salt solution; MP11, microperoxidase; NBBS, new-born-calf serum; OPA-HPLC, HPLC of amino acid OPA derivatives; apo-A1, apolipoprotein A1.

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did not interfere with any of the assay systems described, and was unreactive towards amino acid hydroperoxides. As mentioned in the Figure and Table legends, the hydroperoxides in PVG hydroperoxides (PVG-OOH) consist of 55–65 % Val-OOH, with the remainder being proline hydroperoxides, which we do not as yet measure directly. On BSA, only a small portion of the hydroperoxides are formed on valine residues (see later).

Iodometric measurement of hydroperoxides

The absolute quantity of hydroperoxides generated from radiolysis was determined by using an iodometric assay [8]. The assay is based on the generation of iodine (I_2) from iodide (I^-) by peroxides in acidic solution, followed by its conversion into triiodide ion (I_3^-) in the presence of excess I^- . For this work we used the manual method [5].

Chemiluminescence detection of hydroperoxides

The post-column chemiluminescence assay [6] was modified for this study, by the omission of the HPLC-column separation. Hydroperoxide samples were delivered through an HPLC injection port to a chemiluminescence detector (CLD-110; Tohoku Electronic Industrial Co. Japan) in methanol at a flow rate of 0.5 ml/min. The chemiluminescence reagent consisted of 1 mM isoluminol and 1.5 mg/l MP11 in 0.1 M sodium borate buffer, pH 10.0, and methanol (1:1, v/v). It was mixed at a flow rate of 0.5 ml/min with samples before entering the chemiluminescence detector, which was heated to 50 °C. Hydroperoxides were quantified on the basis of peak areas.

Preparation of plasma and dialysed plasma

Human blood (20 ml) was collected from a healthy non-fasted volunteer into two EDTA-containing vacutainers (Becton Dickinson, NJ, U.S.A.). The blood was centrifuged at 2000 rev./min in a Sorvall RT6000B refrigerated centrifuge (Dupont, U.S.A.) for 15 min at 4 °C. Aliquots (5 ml) of the isolated plasma were then dialysed for 24 h against four changes of 1 litre of deoxygenated PBS containing 1.0 g/l washed Chelex 100 resin (Bio-Rad Laboratories, Sydney, Australia) in the dark at 4 °C. The Chelex was omitted in the last dialysis step, to avoid its presence in the subsequent reactions. The volume of the plasma was unchanged after dialysis. Both plasma and dialysed plasma were stored in the dark at 4 °C and used within 2 days of isolation.

Culture of J774 cells

J774A.1 mouse monocyte-macrophage cells (AT-CC:67-TIB, batch no. F-10089), were grown in RPMI 1640 (Cytosystems, Castle Hill, Australia) containing 10 % (v/v) new-born-calf serum (NBCS), penicillin G (50 units/ml), streptomycin (50 µg/ml) and 2 mM glutamine in 160 cm² tissue-culture flasks. At confluence, cells were scraped into suspension and plated in 22 mm (internal diam.) wells at 0.5×10^6 cells/well. The cells were incubated for 2 days in 1.5 ml of the above growth medium. The cell count at the time of experiments was $\sim 2 \times 10^6$ cells/well.

Gas-phase amino acid hydrolysis

The method developed to obtain the best recovery for DOPA from protein hydrolysis [3] was adopted. Aliquots of borohydride-reduced BSA (or PVG) samples were lyophilized and subjected to gas-phase hydrolysis by hydrochloric acid, in

the presence of a further reductant (mercaptoacetic acid). The hydrolysate was lyophilized to remove acid, and then reconstituted in water for HPLC analysis. Note that in spite of the reductant (mercaptoacetic acid) in this procedure, if valine hydroperoxides on peptide or protein molecules are present at the commencement of hydrolysis then they are incompletely reduced to the hydroxides; thus complete reduction by borohydride before hydrolysis is essential, to obtain quantitative conversion of hydroperoxide into hydroxide.

HPLC of OPA derivatives of amino acids (OPA-HPLC analysis)

The OPA derivatization of free valine or its oxidation products, and of pre-reduced hydrolysates prepared as above, was automated using a Shimadzu autosampler, as described previously [6]. A procedure modified from that previously published was used. The alkaline OPA reagent solution was constituted with 0.3 % (v/v) 2-mercaptoethanol just before use; the reagent always sufficed to buffer the reaction at high pH. The reaction solution (15 µl) was subjected to HPLC analysis 2 min after the onset of derivatization. The HPLC analysis was carried out on a Goldpak LC-18 reversed-phase HPLC column (25 cm $\times$ 4.6 mm, 5 µm particle size; Activon, Australia), using two different gradient elution profiles. For γ -radiolysed valine or PVG samples, elution was performed by a gradient of solvent A (methanol/tetrahydrofuran/20 mM sodium acetate, pH 5.2; 20:2.5:77.5, by vol.) and solvent B (same solvents at 80:2.5:17.5, by vol.). The gradient was generated as follows: 0 min, 0 % B; to 50 % B in 20 min; then to 100 % B in 5 min; isocratic elution for 5 min at 100 % B; then isocratic elution for 5 min at 0 % B. For γ -radiolysed BSA samples, the mobile phase used was a gradient of solvent A (methanol/40 mM sodium acetate, pH 6.5; 20:80, v/v) and solvent B (same solvents at 80:20, v/v), with the gradient as follows: 0 % B to 30 % B in 1 min; then isocratic elution for 2 min at 30 % B; then to 50 % B in 17 min; then isocratic elution for 6 min at 100 % B; then isocratic elution for 4 min at 0 % B. The flow rate was 1 ml/min and the eluent was monitored by a Hitachi F-1050 fluorescence spectrometer (excitation wavelength 340 nm, emission wavelength 440 nm). Any valine hydroperoxides present at the beginning of this procedure are completely reduced to the corresponding hydroxides by 2-mercaptoethanol in the OPA reagent as shown previously [6].

Conversion of valine hydroperoxides on γ -radiolysed solutions of valine, PVG, or BSA molecules into the hydroxides in some biological systems

In order to determine the hydroxides produced from the various hydroperoxides, the following data and calculations were required. In most cases, all hydroperoxides were consumed (as judged by chemiluminescence detection), and the levels of hydroxides were measured by OPA-HPLC analysis. The supplied hydroperoxides were measured as hydroxides after their quantitative reduction by 2-mercaptoethanol (in the case of free valine hydroperoxide) or borohydride (in the case of samples needing hydrolysis). Any difference between the initial amounts of the hydroperoxides supplied to the biological system and the amount of hydroxides recovered after a reaction indicates the formation of degradation products other than hydroxides.

Valine hydroperoxide samples were subjected to OPA-HPLC analysis after 20-fold dilution in water without any treatment by $NaBH_4$ because, as noted above, these hydroperoxides are completely reduced to their hydroxides by the 2-mercaptoethanol in the OPA reagent [6]. Samples requiring hydrolysis have to be treated with $NaBH_4$ as significant amounts of hydroperoxides are destroyed under the hydrolysis conditions, and not all the

products are hydroxides. For example, the conversion of Val.OOH1 into Val.OH1 during hydrolysis ranged from 20 % to 80 %, and this seemed to depend on several factors: notably, the nature of the molecule on which the hydroperoxide was present (amino acid versus peptide versus protein), the concentration of the hydroperoxide, and the presence of other materials (such as plasma). This problem was not investigated in detail, since it could be totally avoided by reducing the hydroperoxides prior to hydrolysis. Thus it is recommended that these hydroperoxide species on oxidized peptides or proteins are always reduced to the corresponding hydroxides by NaBH_4 prior to protein hydrolysis. After hydrolysis, the samples were lyophilized, and redissolved in water. For PVG samples, the hydrolysate was analysed directly by OPA-HPLC analysis. For BSA, the hydrolysates were first chromatographed by HPLC on a Supelcosil LC- NH_2 column (25 cm $\times$ 4.6 mm, 5 μm particle size, Supelco) with an injection volume of 20 μl . The mobile phase was 82 % acetonitrile in 10 mM sodium phosphate, pH 4.3, eluting at 1.5 ml/min. The eluent was monitored at 210 nm. Only the fraction which corresponds to the eluting window of Val.OH1 [6] was collected. After lyophilization, the fraction was dissolved in 50 μl of water. Aliquots (40 μl) were then taken for OPA-HPLC analysis.

RESULTS

1. Degradation of hydroperoxides by transition metals

Preliminary studies using HPLC with post-column chemiluminescence of the reaction of valine hydroperoxides with ferrous, ferric, or cupric ions showed that the rate of reduction was much greater for Fe^{2+} than for Fe^{3+} or Cu^{2+} (results not shown). Therefore in this study, only reactions with Fe^{2+} were investigated in detail, using chemiluminescence detection without prior HPLC column separation.

Figure 1 Degradation of hydroperoxides on γ -radiolysed solutions of valine, PVG, or BSA by ferrous ion

Into 1 ml of radiation-generated hydroperoxide samples (see the Materials and methods section) was added 5 μl of 10 mM FeCl_2 solution (50 μM Fe^{2+}) or 5 μl of water (control). In the case of γ -radiolysed solutions of BSA samples, a final concentration of 100 μM Fe^{2+} ion was also used. After reaction for 10 min, the mixture was treated by Chelex 100 resin (1 mg/ml) and then 10 μl was injected into the chemiluminescence detection system. Fe^{2+} ion at a concentration of 50 μM or 100 μM in water did not generate any signal in the system after the Chelex treatment. The levels of hydroperoxides detected by chemiluminescence were expressed as percentages of the metal-free controls. The absolute concentrations of hydroperoxides supplied were: Val-OOH, 120 μM ; PVG-OOH, 110 μM (containing approx. 75 μM Val-OOH); BSA-OOH, 80 μM (containing a small component of Val-OOH). Results are means $\pm$ S.D. from one experiment ($n = 3$), representative of several.

Figure 2 Degradation products of hydroperoxides on γ -radiolysed solutions of valine, PVG, or BSA by ferrous ion

Hydroperoxide samples, before and after reaction with Fe^{2+} ions (see Figure 1), were analysed for the formation of valine hydroxides by the OPA-HPLC method. (a) Val-OOH (120 μM) with Fe^{2+} (50 μM). (b) PVG-OOH (110 μM containing approx. 75 μM Val-OOH) with Fe^{2+} (50 μM). (c) BSA-OOH (80 μM , containing a small portion of Val-OOH) with Fe^{2+} (100 μM). For (a) and (b) the results are the means $\pm$ S.D. from three separate experiments (thus $n = 3$). For (c) the results are the means $\pm$ range for two separate experiments. Values for valine hydroxide from (b) and (c) were corrected for its recovery from hydrolysis.

Before analysis, the reaction mixture was treated with Chelex 100 resin to remove any unbound $\text{Fe}^{2+}/\text{Fe}^{3+}$ in the solution; these ions otherwise interfere with the assay by generating a large chemiluminescence signal, especially in the case of BSA samples. These signals are presumably due to redox reactions of the metals, and the effect of the BSA has not been studied further.

The reaction of Fe^{2+} with several hydroperoxides generated by γ -irradiation was monitored by chemiluminescence detection (results shown in Figure 1). At a final concentration of 50 μM , Fe^{2+} induced very rapid degradation of PVG hydroperoxides (approx. 110 μM), or valine hydroperoxides (approx. 120 μM), which was complete within the time-scale of the chemiluminescence analysis itself (i.e. less than 2 min). Complete degra-

Figure 3 Kinetics of degradation of valine- and BSA-hydroperoxides by GSH and GSH peroxidase

Valine- or BSA-hydroperoxides were generated by irradiating valine (20 mM) or BSA (10 mg/ml) in Chelex-treated 20 mM sodium phosphate buffer, pH 7.4 (1000 Gy in the presence of oxygen). After removal of radiation-generated H_2O_2 by addition of catalase (5 μ g/ml final concentration), the samples were treated with GSH (0.5 mM final concentration), or GSH as above plus GSH peroxidase (1.7 units/ml final concentration), or left as controls for spontaneous decomposition. Dilution of the samples was kept uniform by addition of phosphate buffer. Key to symbols: $\circ$, Val-OOH (control); $\triangle$, Val-OOH + GSH; $\bullet$, Val-OOH + GSH + GSH peroxidase; $\square$, BSA-OOH (control); $\blacksquare$, BSA-OOH + GSH; $\blacksquare$, BSA-OOH + GSH + GSH peroxidase. The hydroperoxides were supplied at the concentrations indicated in Figure 1. Samples were incubated at 4 °C and assayed iodometrically for hydroperoxides at measured intervals. Just before assay, *N*-ethylmaleimide (final concentration: 1 mM) was added to the samples to prevent interference from GSH. The concentration of KI in the reagent was 10% (w/v) instead of 1% [8]. Results are means $\pm$ S.D. The data for Val-OOH represent three separate experiments with single measurements for each time point ($n = 3$). The data for BSA-OOH represent four separate experiments with single measurements for each time point ($n = 4$). At 30, 60 and 90 min points, the values for BSA-OOH in the presence of GSH and enzyme are significantly different from those in the presence of GSH only, judged by Student's *t*-test ($P < 0.05$).

dation of BSA hydroperoxides (approx. 80 μ M) could only be reached if the Fe^{2+} concentration was increased to 100 μ M. The fact that more Fe^{2+} is required for BSA hydroperoxide samples may be explained by metal-binding sites on BSA which reduce their availability for some redox reactions [9,10].

OPA-HPLC analysis of these samples showed that around 60% or more of valine hydroperoxides were reduced by Fe^{2+} to the corresponding hydroxides in all cases (Figure 2). This can be judged from Figure 2 by comparing the hydroxide value determined after iron treatment with that in the relevant control, since the latter represented the starting hydroperoxide concentration. The valine hydroperoxides which did not appear as hydroxides after iron treatment (only) were probably involved in various other free radical reactions which have been studied by using ESR spin-trap techniques [11].

2. Degradation of hydroperoxides by GSH/GSH peroxidase

The degradation of valine or BSA hydroperoxides by GSH took place with or without the presence of GSH peroxidase (Figure 3). Figure 3 nevertheless also shows that valine or BSA hydroperoxides can act as substrates for GSH peroxidase at 0 °C. At higher temperatures, the effect of the enzyme is obscured by the more rapid uncatalysed reaction. In the case of valine hydroperoxide, GSH with GSH peroxidase decomposed almost all hydroperoxy groups by the time the first measurement was carried out (5 min), whereas in the reaction with GSH alone 50% of the hydroperoxy groups still remained after 90 min. In the case of BSA hydroperoxide, although the degradation rate was not as fast as that of valine hydroperoxide, the catalytic effect of GSH peroxidase was still clearly observed. Hydroperoxides on γ -radiolysed solutions of lysozyme were degraded with similar kinetics to BSA hydroperoxides (results not shown). Heat-inactivated enzyme lost the capacity to catalyse the reduction of valine hydroperoxides (results not shown).

To find out whether the major degradation products of these hydroperoxides by GSH/GSH peroxidase are the corresponding hydroxides, the enzymic reaction mixtures and corre-

Table 1 Degradation of hydroperoxides by GSH/GSH peroxidase

Hydroperoxides in γ -radiolysed solutions of valine, PVG or BSA were treated with GSH (0.5 mM final concentration), or GSH peroxidase (E) (1.7 units/ml), or GSH + E as above, or left as controls for spontaneous decomposition. Initial hydroperoxide concentrations were approx. 120 μ M for Val-OOH, and approx. 110 μ M for PVG-OOH (containing approx. 75 μ M Val-OOH), and approx. 80 μ M for BSA-OOH (containing a small portion of Val-OOH). All samples were kept on ice and aliquots were injected into the chemiluminescence system (running at 50 °C) for hydroperoxide detection shortly after mixing. The injection volume was 10 μ l, and the maximum delay before injection was 5 min. After heating for 1 min at 50 °C (to mimic the exposure to the chemiluminescence system), further aliquots of the same samples were analysed for valine hydroxides by using the OPA-HPLC method (see the Materials and methods section). Triplicate measurements were made for all samples. Results were means $\pm$ S.D. and the experiment shown is representative of several performed. Values for valine hydroxides on PVG or BSA samples were corrected for their recovery rates after hydrolysis.

Sample	Hydroperoxide (% starting)	Hydroxide (μ M)		
		Val.OH1	Val.OH2	Val.OH3
Val-OOH (control)	100.0 $\pm$ 6.4	55.3 $\pm$ 1.8	37.4 $\pm$ 1.3	30.6 $\pm$ 2.3
Val-OOH + E	98.9 $\pm$ 5.3	—	—	—
Val-OOH + GSH	17.7 $\pm$ 3.5	—	—	—
Val-OOH + GSH + E	16.3 $\pm$ 4.3	47.5 $\pm$ 1.4	30.6 $\pm$ 1.1	25.7 $\pm$ 1.5
PVG-OOH (control)	89.9 $\pm$ 6.0	32.1 $\pm$ 1.3	24.4 $\pm$ 0.8	20.1 $\pm$ 0.9
PVG-OOH + E	100.0 $\pm$ 1.8	—	—	—
PVG-OOH + GSH	10.6 $\pm$ 0.5	—	—	—
PVG-OOH + GSH + E	10.6 $\pm$ 0.9	30.8 $\pm$ 0.4	17.7 $\pm$ 0.3	15.5 $\pm$ 0.2
BSA-OOH (control)	94.3 $\pm$ 4.2	0.533 $\pm$ 0.085	—	—
BSA-OOH + E	100.0 $\pm$ 1.8	—	—	—
BSA-OOH + GSH	3.9 $\pm$ 0.9	—	—	—
BSA-OOH + GSH + E	5.3 $\pm$ 1.0	0.486 $\pm$ 0.016	—	—

Table 2 Determination of hydroxides and hydroperoxides available for reduction to hydroxides after reaction of valine- or PVG-hydroperoxides with plasma

After chemiluminescence detection for hydroperoxides, the samples described in the text [initial concentrations: Val-OOH, 60 μM ; PVG-OOH, 55 μM (containing approx. 30 μM Val-OOH)] were used for hydroxide detection by using the OPA-HPLC analysis method (see the Materials and Methods section). For Val-OOH samples, the results represented means of triplicate measurements $\pm$ S.D. For PVG-OOH samples, the standard treatment by NaBH_4 before hydrolysis was used (see the Materials and Methods section), whereas this was not necessary for Val-OOH. The values of valine hydroxides were corrected against their recovery rates from hydrolysis and represented the means together with the range. Val.OH3 in the Val-OOH + plasma sample, and Val.OH2 and Val.OH3 in the PVG-OOH + plasma samples were not recorded due to interference by amine compounds in plasma. Abbreviation: d-plasma, dialysed plasma.

Samples	Hydroxide (μM)		
	Val.OH1	Val.OH2	Val.OH3
Val-OOH (control)	24.9 $\pm$ 0.3	18.6 $\pm$ 0.5	13.5 $\pm$ 0.4
Val-OOH + d-plasma	23.8 $\pm$ 1.4	18.7 $\pm$ 0.4	15.0 $\pm$ 0.4
Val-OOH + plasma	24.8 $\pm$ 2.7	18.6 $\pm$ 1.1	—
PVG-OOH (control)	13.2 $\pm$ 0.4	10.2 $\pm$ 0.1	8.4 $\pm$ 1.2
PVG-OOH + d-plasma	9.9 $\pm$ 0.4	8.9 $\pm$ 0.5	8.1 $\pm$ 0.3
PVG-OOH + plasma	11.1 $\pm$ 0.2	—	—

sponding control mixtures without enzyme, were incubated at 50 $^{\circ}\text{C}$ for 1 min. This achieved efficient enzymic degradation (as confirmed by chemiluminescence demonstration of hydroperoxide disappearance), but allowed recovery of hydroperoxides from the control incubations. The products were then subjected to OPA-HPLC analysis. The results summarized in Table 1 showed that more than 84% of the degradation products of valine hydroperoxides were the corresponding hydroxides in all cases. The rate of degradation measured by using chemiluminescence detection was higher than that measured by using the iodometric method (results not shown), and this was probably due to the elevated temperature of the chemiluminescence detector, which was set at 50 $^{\circ}\text{C}$.

3. Degradation of hydroperoxides by human plasma

The fate of hydroperoxides in circulation is an important issue. The reaction of the peptide and amino acid hydroperoxides with fresh human plasma was therefore studied. Direct injection of plasma-containing samples into the chemiluminescence system could not be employed since protein molecules at high concentration severely suppressed the chemiluminescence signal. The iodometric assay was also unsuccessful because protein molecules at high concentration not only precipitate in the reagent, but also tend to bind I_2 released from the reaction and thus interfere with the accuracy of the assay [8]. A solution to these problems was the addition of a membrane-filtration step prior to chemiluminescence detection. The reaction mixture of valine or PVG hydroperoxides with plasma was centrifuged in a 'Microcon 3' membrane microconcentrator (Amicon, Beverly, U.S.A.) which has a molecular-mass cut-off of 3000 Da. Small molecules such as valine hydroperoxides or PVG hydroperoxides pass through the membrane and are fully recovered in the filtrate (as determined in separate control experiments). This method permits chemiluminescence detection for these hydroperoxides and OPA-HPLC analysis for their hydroxides.

When hydroperoxide degradation by plasma was monitored by chemiluminescence, it was clear that both valine

Figure 4 Degradation of hydroperoxides by J774 mouse monocyte-macrophage cells

(a) Time course of degradation. Valine hydroperoxide samples (4 ml) generated by γ -radiation were mixed with a 5-fold concentration of HBSS (1 ml), with a resulting concentration of approx. 120 μM . Aliquots (1 ml/well) were then given to cells (2×10^6 cells/well) prewashed three times with HBSS. After incubation for the specified times at 37 $^{\circ}\text{C}$, the medium was centrifuged for 2 min at 16 000 g in an Eppendorf centrifuge. Injection of 20 μl of the supernatant into the chemiluminescence system allowed detection of the hydroperoxides. Controls for this experiment included hydroperoxide samples prepared in HBSS without the presence of cells before incubation (0 min) and after incubation ($\square$), and also cells in HBSS without any hydroperoxides (results not shown). Data are means $\pm$ S.D. from a single experiment conducted in triplicate and representative of several experiments. At the end of this experiment, cell viability exceeded 95%. (b) Cell-mediated degradation of various amino acid-, peptide- and protein-hydroperoxides. This experiment was conducted as for (a), but with samples harvested either at 0 min or 180 min of incubation, as shown. Initial hydroperoxide concentrations were: Val-OOH, approx. 120 μM ; PVG-OOH, approx. 90 μM (containing approx. 50 μM Val-OOH); BSA-OOH approx. 65 μM . Results are means of duplicates together with the range, from a single experiment representative of three. Abbreviation: apoTF, apotransferrin.

hydroperoxides (approx. 60 μM) and PVG hydroperoxides (approx. 55 μM) were totally degraded by plasma within 1 h (including the 50 min of centrifugation). In samples that were incubated with dialysed plasma, hydroperoxides on γ -radiolysed solutions of valine or PVG were still largely degraded (70%). These results imply that both large and small molecules contribute to the degradation.

An OPA-HPLC study of these samples showed that the major degradation products of valine hydroperoxides or PVG valine hydroperoxides were the corresponding hydroxides (Table 2).

4. Degradation of hydroperoxides by cells

In a kinetic study on the degradation of valine or PVG hydroperoxides in Hanks' balanced salt solution (HBSS) by J774

Table 3 Determination of hydroxides and hydroperoxides available for reduction to hydroxides after incubation of valine, PVG or BSA hydroperoxides with J774 cells

After chemiluminescence detection for hydroperoxides, the same samples as described in Figure 4 [initial hydroperoxide concentration: Val-OOH, approx. 120 μM ; PVG-OOH, approx. 90 μM (containing approx. 50 μM Val-OOH); BSA-OOH, approx. 65 μM] were used for hydroxide detection by using the OPA-HPLC analysis method (see the Materials and methods section). Duplicate measurements were made for all samples. The results were means together with ranges, from a single experiment representative of three. Values for valine hydroxides on PVG or BSA samples were corrected for their recovery rates from hydrolysis.

Samples	Hydroxide (μM)		
	Val.OH1	Val.OH2	Val.OH3
Val-OOH (0 min control)	48.0 $\pm$ 0.1	39.5 $\pm$ 2.2	30.7 $\pm$ 1.7
Val-OOH (– cells)	51.7 $\pm$ 0.8	38.2 $\pm$ 0.5	28.2 $\pm$ 0.1
Val-OOH (+ cells)	50.4 $\pm$ 1.0	37.9 $\pm$ 1.5	28.8 $\pm$ 1.2
PVG-OOH (0 min control)	22.6 $\pm$ 1.8	13.5 $\pm$ 0.7	13.0 $\pm$ 1.5
PVG-OOH (– cells)	20.0 $\pm$ 1.1	12.9 $\pm$ 1.6	12.2 $\pm$ 1.2
PVG-OOH (+ cells)	21.1 $\pm$ 1.8	12.7 $\pm$ 1.8	11.2 $\pm$ 0.7
BSA-OOH (0 min control)	0.287	–	–
BSA-OOH (– cells)	0.251 $\pm$ 0.003	–	–
BSA-OOH (+ cells)	0.312 $\pm$ 0.038	–	–

mouse monocyte-macrophage cells, the level of remaining hydroperoxides in the supernatant of the HBSS medium was monitored by chemiluminescence at various incubation times. The results (Figure 4a) indicated that incubation for 3 h with cells led to almost complete degradation of hydroperoxides with virtually no degradation for the cell-free control hydroperoxide samples. The incubation time was thus kept at 3 h throughout the study. In order to expose the cells to hydroperoxides at the highest possible concentration, HBSS was prepared as a five times concentrate and diluted to the required concentration by addition of hydroperoxide samples.

Several hydroperoxides generated from γ -irradiation were studied, including valine (approx. 120 μM), PVG (approx. 90 μM), BSA (approx. 65 μM), and apotransferrin and apo-A1 hydroperoxides (absolute concentration not measured). After 3 h incubation at 37 °C with cells in HBSS, the cell viability was found to be always more than 90 % as judged by the Trypan Blue staining technique. Chemiluminescence detection showed that the hydroperoxides were degraded by cells in each case (Figure 4b). The lower degree of degradation of BSA hydroperoxides than apo-A1 hydroperoxides could imply that cell-surface binding of apo-A1 to cells through its receptor is a contributing factor for their enhanced degradation (BSA does not have such receptors on the cell surface). OPA-HPLC analysis of these samples revealed that the degradation products of valine hydroperoxides on γ -radiolysed solutions of valine, PVG, or BSA were the corresponding hydroxides (Table 3).

To rule out the possibility that the degradation was due to the presence of trace amounts of transition metals in HBSS rather than the cell itself, valine hydroperoxides were incubated with cells in normal HBSS and chelexed HBSS, as well as in 50 μM EDTA-treated HBSS. More than 95 % of the hydroperoxides were degraded by cells in all cases, indicating that it was the cells themselves that induced degradation. Results from the OPA-HPLC study also demonstrated that the apparent degradation of hydroperoxides by cells was not due to any loss of samples from the medium via endocytosis. Cells did not contain any significant portion of valine or PVG samples as judged by comparing the levels of cell and cell-free samples of valine, and valine plus

glycine residues respectively. Based on valine and lysine residues, the endocytosis of BSA samples by the cells was estimated to be less than 1 % in the 3 h incubation period, in agreement with previous findings [12].

DISCUSSION

Free radicals are an inevitable feature of cellular metabolism. The reactions of free radicals, such as peroxy and hydroxyl radicals, on biomolecules are important in physiology and pathology [13]. Free radicals are entities that contain unpaired electrons, commonly fragments of molecules. They can be generated within cells as intermediates, such as those involving redox enzymes and bioenergetic electron transfer. They may also appear under less controlled circumstances, and cause reversible or irreversible damage to macromolecules such as proteins. Previous studies have demonstrated the ready formation of protein hydroperoxides on reaction of hydroxyl radicals and other reactive radicals with a number of target proteins [2,5,6,14]. The yield of these materials is significant, with up to 0.4 peroxide groups formed on BSA per hydroxyl radical generated during the γ -irradiation [2]. This observation, together with the observation that 10–50 % of the antioxidant capacity of human plasma is thought to be due to radical-scavenging by proteins [15], suggests that reactions between proteins and free radicals and hence protein hydroperoxide formation may be of major significance *in vivo* [16]. In this study we have used γ -irradiation of aqueous samples to generate radicals, of the kinds known to occur *in vivo*, but in known quantities. The maximum dose of radicals we have used can readily be achieved *in vivo* by 1×10^7 macrophages or neutrophils in a matter of hours, as judged by *in vitro* experiments (for example [17]).

Protein hydroperoxides have been shown to be readily reactive with some important physiological reductants, such as ascorbate and glutathione [2]. The potential biological significance of such reactions is that they may cause a depletion of cellular reductants *in vivo*, and thus exert oxidative stress. These reductants, on the other hand, metabolize and reduce the peroxy group independently of proteolysis, and therefore constitute a very important aspect of biological defence.

This study provides evidence that protein hydroperoxides are degraded into less reactive products, such as hydroxides, in several biological systems, this process possibly constituting a defence mechanism. The experiments described with free valine hydroperoxides are unambiguous. However, we have pointed out already that those with the peptide- and protein-bound hydroperoxides are complicated by the need for protein hydrolysis, which can destroy some of the hydroperoxides. The devices we have used to avoid these losses, such as reduction with borohydride prior to hydrolysis, largely resolve the problem. Taking the results from free valine hydroperoxide experiments together with those for peptide and protein hydroperoxides, the only reasonable interpretation is that in all cases the major product of the reactions studied (due to metals, GSH peroxidase and cells) is the hydroxide.

Transition metals, especially ferrous ions, caused rapid decomposition of protein hydroperoxides *in vitro*. Our data indicate that around 60 % of valine hydroperoxides on γ -radiolysed valine, PVG, or BSA were degraded to valine hydroxides. Valine hydroperoxides have been shown, by ESR spectroscopy [11], to be degraded to various reactive free radicals, suggesting that protein hydroperoxides are capable of initiating further radical reactions, which may induce damage to other biomolecules or contribute to the protein oxidation chain reaction [18]. Some of

these radical reactions of hydroperoxides are probably among these which give rise to hydroxides (see reaction 2 of [11]).

Provoked by our earlier discovery that rat liver homogenate enhanced the decay of protein hydroperoxides (J. A. Simpson and R. T. Dean, unpublished work), we examined the effect of GSH peroxidase (which is found in the liver at very high concentrations) on the degradation of protein hydroperoxides. Our results showed that GSH peroxidase recognizes protein and amino acid hydroperoxides as substrates, and can accelerate their decomposition. Degradation of valine hydroperoxides by homogenates of human atherosclerotic plaque was also monitored in this study. It has been found (results not shown) that valine hydroperoxides added to plaque samples (15 mg of wet tissue per ml of chelexed PBS solution) were degraded during the subsequent homogenization. The major degradation products were found to be valine hydroxides (90 %).

As has been discussed above, human plasma, which is rich in protein, plays a very important role in scavenging free radicals generated *in vivo*. The present study revealed that human plasma also has the ability to reduce the hydroperoxides formed into hydroxides and thus eliminate the potential damage to other important biomolecules posed by the hydroperoxides.

In the course of studying the metabolism of protein hydroperoxides by cells, we observed that after incubation for 3 h J774 mouse-macrophage cells destroyed more than 95 % of exogenously supplied protein hydroperoxides (approx. 65 μ M BSA hydroperoxides). Although the mechanism of this cellular detoxification activity has not been defined, we envisage that it was via the cell surface as the endocytosis of the protein hydroperoxides was determined to be less than 1 % during the 3 h incubation. Protein hydroxides were again found to be the main degradation products during the reactions of protein hydroperoxides with cells. Protein and amino acid hydroperoxides were stable in culture conditions in the absence of cells.

Other studies demonstrated that protein damaged by radicals can accumulate within cells (where the net rate of production exceeds that of catabolic removal), and then establish a new transient steady state (when the rate of production equals that of removal [4,19–21]). On the assumption that the idea of accumulation of protein oxidation products in cells has general applicability, we predict that protein-bound hydroperoxides, and to a greater extent hydroxides, are among the species which accumulate. The present study shows that protein hydroperoxides are degradable in several biological systems and form the

hydroxides as the major stable end products. It is therefore very reasonable to choose protein hydroxides as markers for studying protein oxidation under oxidative stress. After successful detection of Val.OH1 in oxidized low-density lipoprotein and plaque samples (S. Fu and R. T. Dean, unpublished work), we believe that further investigation of this idea is worthwhile.

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