Peroxidation of proteins before lipids in U937 cells exposed to peroxyl radicals

Steven GIESEG*[1], Sean DUGGAN* and Janusz M. GEBICKI†

*Free Radical Biochemistry Laboratory, Department of Zoology, University of Canterbury, Private Bag 4800, Christchurch, New Zealand, and †Department of Biological Sciences, Macquarie University, Sydney, NSW 2109, Australia

This study provides the first report of the formation of protein hydroperoxides in cells attacked by reactive oxygen species. U937 cells exposed to peroxyl radicals generated by the thermal decomposition of a water-soluble azo compound gradually accumulated hydroperoxide (-OOH) groups. In an incubation for 22 h, 1.2 mM peroxyl radicals was generated and each cell acquired 1.5×10^8 -OOH groups. These groups were located on the cell proteins; no lipid peroxidation was detected. The extent of protein peroxidation was proportional to the rate of generation of the peroxyl radicals. There was no lag period before the onset of peroxidation, indicating that cell antioxidants could not protect the proteins. The half-life of protein hydroperoxides in cell suspensions was approx. 4 h at 37 °C. Our results suggest that protein hydroperoxides might have a significant role as intermediates in the development of biological damage initiated by reactive oxygen species.

Key words: free radical, lipid oxidation, protein hydroperoxide, protein oxidation.

INTRODUCTION

The identification of the initial cellular targets of reactive oxygen species (ROS) is of crucial importance to the rational prevention of their damaging effects. Most of the physiologically significant ROS can oxidize or otherwise damage a wide range of biomolecules, generating active secondary products able to propagate the damage. The final stage is irreversible injury or death of the cell.

Of all the components of the cell, proteins are the most likely first targets of the ROS. After the inert water, they are by far the most abundant cell components. They react readily with the hydroxyl, peroxyl and alkoxyl free radicals, as well as with oxidants such as hypochlorite, nitric oxide, peroxynitrite and singlet oxygen [1]. In addition, many proteins can stabilize metals in forms able to redox cycle, allowing the site-specific formation of very reactive products from the relatively inert H_2O_2 and $O_2^{-\cdot}$ (superoxide) radical [2]. Such interactions inevitably lead to the formation of damaged proteins. It was held for some years that the damaged molecules did not constitute a hazard to living organisms, because they were recognized by the digestive enzymes, removed, and replaced in due course [3]. However, recent observations have shown that proteins damaged by ROS acquire chemically reactive moieties, which can act as new sources of damage to cell components [4]. Our studies have focused on the formation of reactive hydroperoxide (-OOH) groups on a wide range of proteins exposed to a wide range of ROS. We have shown that these groups can form in high yields and that the peroxidized proteins can initiate some potentially damaging processes [5]. Thus protein hydroperoxides can act as important links in the chain of reactions connecting the formation of ROS with biological damage, and should themselves be regarded as reactive oxygen species. In the present study we set out to test whether protein hydroperoxides are an early product of the interaction of the physiologically significant peroxyl radicals with living cultured cells.

MATERIALS AND METHODS

All reagents were of AR grade or better and were obtained from BDH Chemicals New Zealand Ltd or from Sigma Chemical Company (St Louis, MO, U.S.A.). 2,2′-Azobis(amidinopropane) dihydrochloride (AAPH) was supplied by Aldrich Chemical Company (Sydney, Australia). Tissue culture media and plasticware were supplied by Life Technologies Ltd (Auckland, New Zealand).

U937 cells were cultured in RPMI 1640 medium with 5% (v/v) heat-inactivated fetal calf serum, 100 i.u./ml penicillin and 100 μg/ml streptomycin. Cells were prepared for experiments by being washed twice in PBS to remove the culture medium and being suspended in Earle's balanced salt solution (EBSS). Cell incubations were performed in six-well plates with 6 ml of cells per well at a final concentration of 5×10^6 cells/ml. AAPH stock solutions were also made up in EBSS and added to the wells to give the final concentration required. The cells were incubated at 37 °C in an air/CO_2 (19:1) atmosphere. At the end of the incubation, cold trichloroacetic acid (TCA) (final concentration 10%, w/v) was added and the precipitate was collected by centrifugation. The pellets were washed three times with 5 ml of cold 10% (w/v) TCA and resuspended in 900 μl of 25 mM sulphuric acid by vortex-mixing for the determination of hydroperoxide. This followed a published procedure in which ferrous ions in acid solution are oxidized by the hydroperoxide; the resultant ferric ions are measured as the Xylenol Orange complex [6]. In brief, 50 μl each of 5 mM ferrous ammonium sulphate and Xylenol Orange in 25 mM H_2SO_4 were added to 900 μl of the washed TCA precipitate suspended in 25 mM H_2SO_4; the mixture was incubated at room temperature for 30 min in the dark, then centrifuged to remove cellular debris. A_{560} was measured; water was used to zero the spectrophotometer. Protein concentrations were determined by the bicinchoninic acid method (kit by Pierce, Rockford, IL, U.S.A.) with BSA as a standard, and lipid hydroperoxides by the thiobarbituric assay with detection by HPLC [7]. Results shown were obtained from single experiments,

Abbreviations used: AAPH, 2,2′-azobis(amidinopropane) dihydrochloride; EBSS, Earle's balanced salt solution; -OOH, hydroperoxide group; ROS, reactive oxygen species; TCA, trichloroacetic acid.

[1] To whom correspondence should be addressed (e-mail s.gieseg@zool.canterbury.ac.nz).

representative of three. The data points are shown as means ± S.E.M. for triplicate treatments.

RESULTS AND DISCUSSION

Treatment of the cells with TCA allowed a rapid and simple removal of the AAPH and low-molecular-mass cell components, which could interfere with the ferric-Xylenol Orange hydroperoxide assay. However, the ability of the assay to measure hydroperoxides in the TCA precipitates had to be checked because the assay is known to be sensitive to metal chelators, which could be present in the cell debris, and pH, which could be altered by any residual TCA. For this, preparations containing 5×10^6 cells/ml were treated and washed with TCA; the precipitate was resuspended in 25 mM H_2SO_4. The A_{560} value of the supernatant was measured after the addition of 50 μM Xylenol Orange and centrifugation. A known amount of Fe^{3+} was then added and the absorbance was measured again after a few minutes. The calculated value of the molar absorption coefficient of the Fe^{3+} in the cell extract solution was 19400 $M^{-1} \cdot cm^{-1}$, close to the 20100 determined previously [8]. There was no evidence of the presence of hydroperoxides on any of the cell preparations, unless they had previously been incubated with the peroxyl radical donor.

The ferric-Xylenol Orange hydroperoxide assay produced low positive A_{560} readings in all TCA precipitates of cells. Part of this was caused by the small residual amount of the 10% (w/v) TCA solution, which contained 123 μM Fe^{3+} and could not be removed without the loss of material, and part by the 0.01% Fe^{3+} contamination of the ferrous salt used in the assay. The concentrations of hydroperoxides in oxidized cells were corrected for this by subtracting the absorbance readings produced by control cells that had not been exposed to AAPH.

The thermal decomposition of AAPH generates peroxyl radicals at known rates [9], which induce the formation of hydroperoxides in lipids and proteins [10,11]. Exposure of U937 cells to increasing concentrations of AAPH for 22 h followed by the assay of TCA precipitates for the presence of hydroperoxides resulted in an increase in A_{560} proportional to the initial AAPH concentration (Figure 1). Although Trypan Blue exclusion tests

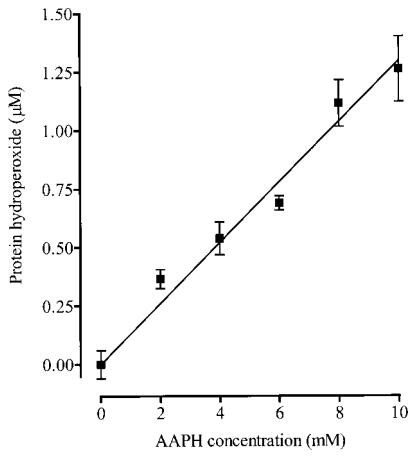

Figure 1 Effect of increasing AAPH concentration on protein hydroperoxide formation in U937 cells

U937 cells (5×10^6/ml) were exposed to various concentrations of AAPH for 22 h. The cellular proteins were concentrated by precipitation with TCA before assaying for hydroperoxides as described in the Materials and methods section.

indicated that many of the cells were dying at this stage, the increase in A_{560} was not caused by the release of Fe^{3+} from the oxidized or control cells; the addition of Xylenol Orange to the TCA cell precipitates suspended in 25 mM H_2SO_4 gave no increase in A_{560} in the absence of Fe^{2+}. The possibility that residual AAPH could be responsible was eliminated by a comparison of the effectiveness of dialysis and TCA precipitation in removing the AAPH. In dialysis, cells were oxidized by treatment for 12 h with 10 mM AAPH; suspensions containing 5×10^6 cells/ml in dialysis sacs were stirred overnight at 4 °C with three changes of 350 vol. of PBS. The cells were then collected and their hydroperoxide content was measured as usual. In parallel experiments, the cells were treated with TCA and also tested for hydroperoxides. The results showed only a 9% decrease in A_{560} in the TCA preparation in comparison with the dialysed samples. The absorbance measurements were converted to concentrations when subsequent experiments showed that the -OOH groups were located on proteins. Use of the molar absorption coefficient measured for BSA-OOH [6] showed that the dialysed cells contained 3.4 μM protein hydroperoxide, and the TCA-precipitated cells 3.1 μM protein hydroperoxide, per 3×10^7 cells. In most subsequent experiments the TCA procedure was used.

Protein hydroperoxides have been shown to be reduced by various reducing agents [4]. Treatment of TCA-precipitated AAPH oxidized cellular protein (suspended in phosphate buffer, pH 7.4) with 20 mM $NaBH_4$ or 0.5 mM ascorbate at room temperature decreased the measured peroxide content by 67 ± 8% and 83 ± 1% respectively after 60 min. Additional treatments decreased the hydroperoxide content further to a variable extent. Previous studies [11] had demonstrated that none of the five reducing agents employed was able to remove -OOH groups completely from oxidized BSA or lysozyme, probably because some of them were not accessible.

The only TCA-precipitable cell constituents that can be peroxidized to significant extent by the peroxyl radicals are lipids and proteins. The possibility that lipids were responsible for the positive ferric-Xylenol Orange hydroperoxide test was eliminated by two series of tests. In the first, measurements of the thiobarbituric-acid-reactive substances gave values of 575 ± 46 nM malonyl dialdehyde equivalent in control cells, and 532 ± 12 nM in cells incubated for 12 h at 37 °C with 10 mM AAPH. Thus no lipid hydroperoxides, easily detectable by this method, were generated. In the second test the cells were incubated for 20 h at 37 °C in the presence or absence of 10 mM AAPH, harvested, washed in PBS and treated with 10% (w/v) TCA; the precipitates were washed twice with TCA. Some of the precipitates were tested directly for the presence of hydroperoxides, whereas others were first washed twice with acetone to remove lipids. The acetone wash resulted in the loss of 16.4 ± 4% of the material absorbing at 560 nm from the controls and 14.5 ± 2% from the oxidized cell precipitates. The absorbance readings were measured against a water blank, so that positive values were recorded in all samples. The loss of absorbance after the acetone wash was probably due to the removal of some of the protein but this could not be measured because of insolubility of the TCA precipitates.

In additional experiments, the TCA cell pellets were washed with methanol followed by hexane. This treatment removes over 99% of the lipids [12] but no loss of peroxide activity was observed. There seemed to be a better retention of protein with the methanol/hexane wash. The protein pellets were more dense than those observed with the acetone wash. This might explain the higher recovery of hydroperoxide activity. Taken together, these results confirmed the absence of lipid hydroperoxides,

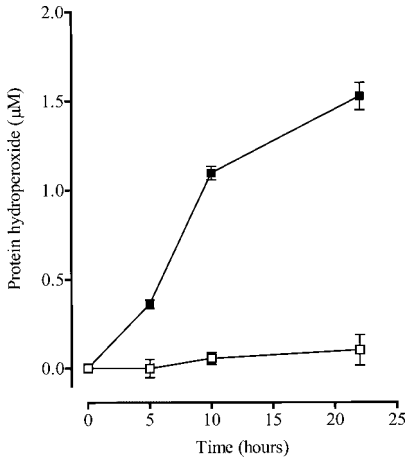

Figure 2 Formation of protein hydroperoxides in U937 cells over time

U937 cells (5×10^6/ml) were incubated in EBSS in the absence (□) or presence (■) of 10 mM AAPH at 37 °C. At indicated time points, cells were collected and the cellular proteins were concentrated by precipitation with TCA before being assayed for hydroperoxides as described in the Materials and methods section.

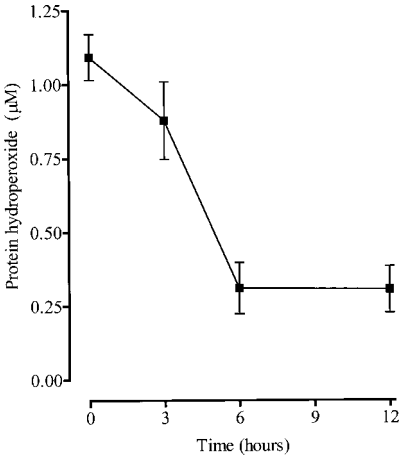

Figure 3 Decay of cellular protein hydroperoxides

Cellular hydroperoxides were formed in U937 cells by incubating 5×10^6 cells/ml in EBSS containing 10 mM AAPH. After 12 h the cells were washed twice in PBS and suspended in RPMI 1640 medium. Aliquots (6 ml per well) of the oxidized cells were applied to plates. At the indicated time points the cells were harvested and assayed for the amount of hydroperoxide present.

leaving protein hydroperoxides as the species accounting for the total peroxides found in the oxidized cells. Measurements of the cell protein content showed that treatment with AAPH resulting in the generation of 1 μM hydroperoxide corresponds to 770 nmol -OOH residues/mg of cellular protein.

The time course of the generation of protein hydroperoxides in U937 cells incubated at 37 °C with 10 mM AAPH is shown in Figure 2. There was no lag period and only a small decrease in the rate of peroxidation with time. Control cells, incubated in EBSS only, showed no significant formation of hydroperoxides. Because of the length of the experiment, the hydroperoxide levels detected at any time represent the balance between their rates of formation and decay. We investigated the stability of the cellular hydroperoxides by incubating the AAPH-oxidized cells for 12 h in RPMI 1640 medium in the absence of AAPH and then measuring their persistence (Figure 3). There was a fairly rapid loss of -OOH groups in the first 6 h, followed by a steady level at approx. 0.3 μM peroxide.

The mechanism of formation of the protein hydroperoxides in these experiments is likely to be similar to that proposed for peroxidation by hydroxyl radicals [11]. Here, the oxidation is initiated by peroxyl radicals (ROO$^\bullet$) derived from AAPH (R-R):

$$\text{R-R} + \text{O}_2 \rightarrow 2\ \text{ROO}^\bullet$$

$$\text{ROO}^\bullet + \text{PrH} \rightarrow \text{ROOH} + \text{Pr}^\bullet$$

$$\text{Pr}^\bullet + \text{O}_2 \rightarrow \text{PrOO}^\bullet$$

$$\text{PrOO}^\bullet + \text{RH} \rightarrow \text{PrOOH} + \text{R}^\bullet$$

The carbon-centred protein radical (Pr$^\bullet$) reacts rapidly with oxygen and is finally reduced to hydroperoxide by RH, which might be a protein or a low-molecular-mass compound but is unlikely to be lipid because no lipid hydroperoxides were detected. Calculation of the extent of peroxidation of cell proteins shows a low efficiency of formation of -OOH groups in comparison with the quantities of peroxyl radicals generated, but a still significant number of -OOH groups per cell. If we assume that at 37 °C the rates of formation of peroxyl radicals and protein -OOH groups were 3.19×10^{-7} [AAPH] M/s [9] and 1.6×10^{-11} M/s (Figure 1) respectively, one -OOH group was generated per 200 peroxyl radicals. Clearly, most of the radicals disappeared in reactions not leading to the formation of hydroperoxides, either on protein or on lipids. This is not unexpected. Many radicals would recombine in the cell suspensions before reaching the small number of target cells, and many would react with small molecules in the medium, with products removed in the precipitation steps with TCA. Those able to attack the cells would oxidize a range of accessible molecules, primarily on the cell surface. How these reactions might affect the functions of the cells is not known but it is likely that many of them will be of little if any consequence. In contrast, a simple calculation shows that, after exposure for 22 h to 10 mM AAPH, each cell acquired 1.5×10^8 -OOH groups. This could constitute a significant hazard to the cells, especially as some of these residues persist for hours.

Unfortunately the significance of this hazard cannot yet be estimated. Microscopic observations and experiments with Trypan Blue exclusion suggested that increasing numbers of cells exposed for several hours to peroxyl radicals lost their viability, while simultaneously the whole population acquired protein peroxide groups (Figure 3). However, techniques currently available cannot establish what connection might exist between these phenomena. For this it is necessary to compare the viability of cells exposed to peroxyl radicals under conditions allowing the formation of protein peroxides (as in this study) with parallel experiments in which protein peroxidation is prevented. Such comparisons are not possible because no agents or conditions able to prevent oxidant-induced protein peroxidation without affecting cell viability are known.

Previous studies have shown that protein hydroperoxides can be the source of new free radicals, react with cellular antioxidants, inactivate glutathione reductase, and cross-link to DNA [4,13]. It seems likely that the deleterious effects of AAPH on animals [14] are due at least in part to the formation and further reactions of protein hydroperoxides. In this context the most significant biological aspect of the generation of protein hydroperoxides by ROS might be the inability of the natural antioxidants present in cells (Figure 2) to inhibit their formation.

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