Chemiluminescence determination of hydroperoxides following radiolysis and photolysis of free amino acids

Stephen Robinson[a], Ruth Bevan[a], Joseph Lunec[a], Helen Griffiths[b,*]

[a] *Division of Chemical Pathology, Centre for Mechamisms of Human Toxicity, MRC Toxicology Unit, University of Leicester, PO Box 138, Lancaster Road, Leicester, LE1 9HN, UK*
[b] *Pharmaceutical Sciences, Aston Triangle, Aston University, Birmingham, B4 7ET, UK*

Received 8 May 1998; revised version received 26 May 1998

Abstract Hydroperoxides were determined in selected amino acids using three free radical generating systems by a sensitive (50 pmol limit of detection) and specific high performance liquid chromatography (HPLC)/chemiluminescence method. UVB and gamma radiation produced significant hydroperoxide formation, particularly in the aromatic amino acids tyrosine and tryptophan. Hydroperoxide yield was found to be dependent on both amino acid and irradiation source. Generation of hydrogen peroxide as a by-product of irradiation caused interference with chemiluminescence detection demonstrating the need for catalase addition. Hydroperoxides were not detectable following metal-catalysed H_2O_2 breakdown. We suggest that metal ions could interfere with the detection of hydroperoxides by causing preferential decomposition.

Key words: Free radical; Hydroperoxide; Chemiluminescence; Amino acid; Gamma radiolysis; UVB radiation

1. Introduction

Free radicals are generated in vivo as by-products of normal metabolism [1], with the primary radicals in biological systems being of an oxygen origin, such as singlet oxygen and peroxy, hydroxyl and superoxide radicals [2]. As well as metabolic production of free radicals, environmental sources of radiation such as UVB generate free radicals by direct photoactivation of proteins and DNA [3]. These radicals have long been established to cause oxidative damage to biological molecules and as such are proposed to be involved in the physiology of ageing [4] and in the development of many disease states. These include atherosclerosis [5], cataract formation [6], autoimmunity [7] and altered lymphocyte trafficking within UV exposed skin [8], where oxidation of the proteins apolipoprotein B, lens crystallins and immunoglobulin, respectively, have been suggested to play a causal role.

In recent years, attention has been focused on the effect of free radicals on specific, critical amino acids present in proteins and the ultimate effects of their oxidation. Free radical attack has been known to cause amino acid oxidation and modification [9]. Alteration of the primary structure with subsequent conformational alterations underlies the inactivation and/or increased susceptibility to proteolytic degradation [10]. Examples of specific amino acid oxidation leading to functional change in proteins have been described for tryptophan oxidation by hydroxyl radicals inactivating lysozyme [11], and cysteine oxidation inactivating α-1 anti-trypsin [12].

Protein damage by free radical exposure produces oxidising species such as protein and amino acid hydroperoxides [13], whose formation has been described for a wide variety of biochemical and biological systems [14]. As it has been shown that proteins and amino acids are sites of hydroperoxide formation [15], interest in these, with relevance to biological systems, has led to the development of a number of methods for their determination and quantitation such as the iodometric technique [16] and high-performance liquid chromatography (HPLC) with chemiluminescence detection.

The iodometric technique has been the method of choice to quantify total hydroperoxides formed on amino acids, proteins and more commonly lipids but is complicated by the requirement for the samples to be tested anaerobically. Such problems are avoided by the use of a simple chemiluminescence detection system as described in this manuscript.

Amino acids are proposed to have differing susceptibilities to various free radical species. In this study we determine the susceptibility of selected amino acids to hydroperoxide formation from oxygen free radicals using three separate radical generating systems.

2. Materials and methods

2.1. Materials

The water used throughout was purified by a Milli-Q system (Millipore Waters, UK). All amino acids, hydrogen peroxide, *tert*-butyl hydroperoxide, catalase, luminol and microperoxidase were from the Sigma Chemical Company (Poole, Dorset, UK). All other chemicals, solvents and chromatographic materials were of HPLC grade.

2.2. Methods

2.2.1. Exposure of amino acids to gamma-radiolysis generated free radicals. Dilute solutions of amino acids (1 mM in Milli-Q water) were irradiated for increasing lengths of time using a Vickrad Cobalt 60 source, to obtain set radiation doses of 0, 63, 313, 625, 1250 and 2500 Grays (Gy). After irradiation, a small volume of catalase (5 μg/ml final concentration) was added to the samples to remove radiation-generated hydrogen peroxide. The catalase did not interfere with the assay systems described (unpublished data).

2.2.2. Exposure of amino acids to UVB generated free radicals. Dilute solutions of amino acids (1 mM in Milli-Q water) were irradiated using a UVM-57 Chromato-vue UVB lamp (Knight Optical Technologies, Surrey, UK, with a spectral range from 272–352 nm) for various periods of time to achieve doses of 0, 2.8, 5.6, 11.2, 22.3 and 44.6 kJ/m^2. After irradiation the samples were split into equal volumes with one half receiving a small volume of catalase (1500 U/ml final concentration).

2.2.3. Exposure of amino acids to metal catalysed reactions. To dilute solutions of amino acids (1 mM in Milli-Q water) copper sulfate solution (20 μM final concentration) was added and vortex-mixed. To this solution hydrogen peroxide was added to achieve final concentrations of 0, 10, 100 and 1000 μM of hydrogen peroxide. These

*Corresponding author. Fax: (44) (121) 359 0733.
E-mail: h.r.griffiths@aston.ac.uk

PII: S0014-5793(98)00679-6

Table 1
Hydroperoxide formation in selected amino acids on UVB irradiation

	UVB dose (kJ/m^2)									
	2.8		5.6		11.2		22.3		44.6	
Catalase	−	+	−	+	−	+	−	+	−	+
Tyrosine	11.53	8.03	37.34	24.02	60.65	41.42	48.06	26.80	125.13	53.90
(S.D.)	(1.64)	(2.24)	(11.24)	(10.2)	(28.0)	(17.0)	(5.83)	(1.49)	(11.1)	(13.0)
Tryptophan	15.23	5.98	43.29	10.53	76.53	18.84	114.93	10.84	178.94	22.82
(S.D.)	(4.87)	(0.34)	(8.39)	(2.99)	(26.1)	(9.07)	(13.9)	(2.39)	(13.5)	(5.27)
Valine	3.01	0.00	4.43	0.00	8.78	0.00	17.08	0.00	41.96	3.14
(S.D.)	(0.29)	(NA)	(0.92)	(NA)	(3.48)	(NA)	(5.87)	(NA)	(16.1)	(0.41)
Lysine	3.48	0.00	5.73	0.00	16.31	0.00	18.49	0.00	43.99	3.00
(S.D.)	(0.19)	(NA)	(2.16)	(NA)	(14.4)	(NA)	(7.32)	(NA)	(11.6)	(0.78)
Histidine	9.88	0.00	13.56	0.00	26.96	0.00	20.48	5.00	59.79	4.79
(S.D.)	(3.77)	(NA)	(8.02)	(NA)	(17.5)	(NA)	(8.68)	(1.42)	(29.2)	(0.97)

Hydroperoxide concentration (μM) of amino acids with increasing doses of UVB radiation (kJ/m^2). Samples measured as described in Section 2.2 both with (+) and without (−) the addition of catalase (1500 U/ml) (mean ± S.D., $n = 3$). NA: not applicable.

samples were incubated for 1 h before catalase (1500 U/ml final concentration) was added to remove any hydrogen peroxide present.

2.2.4. Hydrogen peroxide determination. Using a 96-well plate, to 100 μl of dilute solutions of amino acids (1 mM in Milli-Q water), post-irradiation, 100 μl of 'Phenol red assay solution' was added. The mixture was vortex-mixed and incubated at 37°C for 5 min. To stop the reaction, 10 μl of sodium hydroxide (1 M) was added, shaken on a plate shaker and the final absorbance read at 620 nm using an Anthos 2001 plate reader (Anthos Labtec Instruments, Salzburg, Austria). A standard curve of known hydrogen peroxide concentrations was run in parallel to determine the hydrogen peroxide concentration in the samples. The specificity of the assay for hydrogen peroxide was ascertained using an equivalent standard curve of *tert*-butyl hydroperoxide.

The 'Phenol red assay solution' consisted of phosphate buffered saline (10 mM final concentration, pH 7.2), dextrose (5.5 mM final concentration), horseradish peroxidase (8 U/ml final concentration) and 0.1% Phenol red.

2.2.5. Chemiluminescence determination of amino acid hydroperoxides. The assay involved the sample being injected and mixed with a chemiluminescence reagent containing microperoxidase and luminol. This method was an adaptation of work carried out by Fu et al. [4].

The experimental conditions were as follows: Amino acid samples (post-irradiation; 50-μl sample volume for each injection) were injected onto a mobile phase of 100% methanol being pumped at 1 ml/min and mixed via a 3-way mixing tee (Anachem, UK) with chemiluminescence reagent (70:30 (v/v) methanol/sodium borate buffer (50 mM, pH 10) containing 1 mM luminol and 10 μg/ml microperoxidase (MP11)) pumped at 1 ml/min. The light emission was detected by a Soma Chemi Lumi detector/S-3400 (supplied by Applied Chromatography Systems Ltd, UK). Limit of detection was 50 pmol.

2.3. Statistics

All amino acid treatments and the subsequent analysis of hydroperoxides were undertaken in triplicate and results analysed using single test ANOVA (Microsoft Excel statistical package) for statistical significance.

All graphs show standard error of means (S.E.M.) or standard deviation (S.D.).

3. Results

A sensitive, specific and reproducible chemiluminescence technique was developed for the determination of amino acid hydroperoxide formation in order to show the effect of differing free radical generating systems on selected amino acids.

The results obtained from the reaction of all five amino acids with a copper catalysed system showed no detectable hydroperoxide formation up to and including the highest dose of hydrogen peroxide (1000 μM) (data not shown). However, readings of both tyrosine and tryptophan native fluorescence showed a dose dependent decrease in their fluorescence (Ex. 282 nm, Em. 303 nm and Ex. 297 nm, Em. 352 nm, respectively), suggesting oxidation was occurring (data not shown).

The effect of UVB on the amino acids at increasing doses with and without the addition of catalase (see Table 1) shows that the addition of catalase post-irradiation for all amino acids causes a marked decrease in the concentration of hydroperoxides detected. This is due to the quenching of excess hydrogen peroxide by the catalase addition. Thus samples were analysed after catalase was added post-irradiation, to reflect the true production of hydroperoxides due to free radical oxidation.

When observing the data recorded for the amino acids subjected to increasing UVB dose (see Fig. 1), tyrosine and tryptophan show the largest response. Both amino acids exhibit a dose dependent increase up to and including 11.2 kJ/m^2 with the 5.6-kJ/m^2 dose showing a significant increase ($P < 0.05$) from the lowest UVB dose (2.8 kJ/m^2). Conversion to hydroperoxide after 44.6 kJ/m^2 was 5.4% for tyrosine and 2.4% for tryptophan.

At a dose level of 22.3 kJ/m^2 both tyrosine and tryptophan show a decrease, followed by a subsequent increase in hydroperoxide concentration, to the maximum irradiation dose (44.6 kJ/m^2). Valine and lysine show similar results to each other; hydroperoxides are not detected until the largest dose of UVB is given (44.6 kJ/m^2). Histidine shows the same pattern except both 22.3-kJ/m^2 and 44.6-kJ/m^2 doses give concentrations of hydroperoxides akin to each other, with no

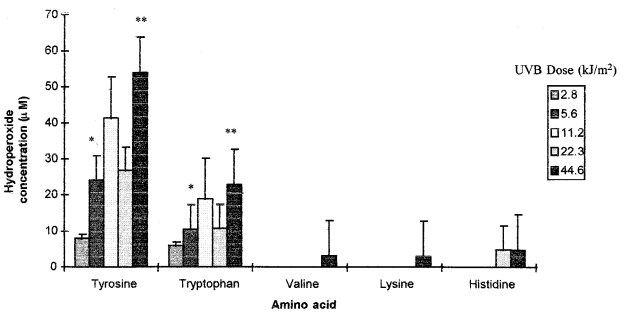

Fig. 1. Hydroperoxide determination (μM) of amino acids following increasing doses of UVB radiation (kJ/m^2). Values are mean ± S.E. ($n = 3$). Where results were statistically compared with the previous dose * indicates $P < 0.05$, ** indicates $P < 0.01$.

evidence of a linear dose response. Results of the phenol red assay (see Fig. 2A) demonstrate the specificity of the assay for hydrogen peroxide rather than hydroperoxide. Fig. 2B shows that all five amino acids exhibit a dose dependent increase in hydrogen peroxide concentration with increasing UVB dose. Tryptophan shows the largest increase giving a maximum concentration of 73 μM hydrogen peroxide following a UVB dose of 22.3 kJ/m^2. Tyrosine, valine, lysine and histidine mimic each other very closely in the increase of hydrogen peroxide production with increasing UVB dose yielding ~30 μM following a UVB dose of 22.3 kJ/m^2. In all cases hydrogen peroxide was not detected in non-irradiated native amino acid samples.

The effect of gamma radiation on the amino acids can be seen in Fig. 3. Tyrosine showed a very strong dose dependent response to increasing gamma radiation dose, with 3.6% conversion at the highest dose. Valine also showed a dose dependent increase in hydroperoxide yield. The concentration of valine hydroperoxide always exceeded hydroperoxide formation on the other amino acids tested, where 52 μM hydroperoxide was detected following 2500 Gy (5.2% conversion). Tryptophan and histidine gave similar results to each other, showing a dose response up to and including 625 Gy. Any increase in dose above this level showed no significant increase in hydroperoxide production which reached a plateau at around 22–25 μM for both amino acids between 625 and 2500 Gy ($P > 0.05$). Lysine also showed a dose related increase up to and including 625 Gy but then a reduction in hydroperoxide yield was noted as gamma radiation dosage increased to 2500 Gy.

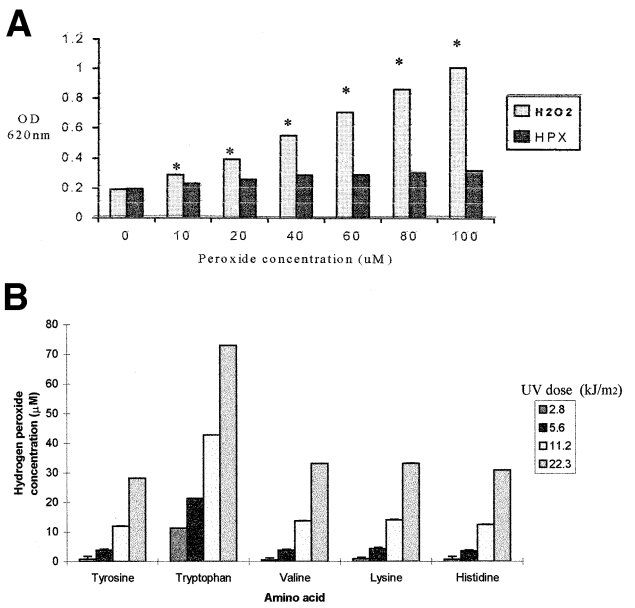

Fig. 2. A: Standard curve for the phenol red assay to measure hydrogen peroxide, demonstrating non-interference using *tert*-butyl hydroperoxide (HPX). Results are expressed as OD values at 620 nm, and are the means of triplicate analyses, where the standard deviation was less than 1% and $*P < 0.002$. B: Hydrogen peroxide formation (μM) measured by the phenol red assay from selected amino acids following increasing doses of UVB radiation (kJ/m^2). Values are mean ± S.E. ($n = 3$). Error bars presented for all gamma doses but some too small to be able to view on figure.

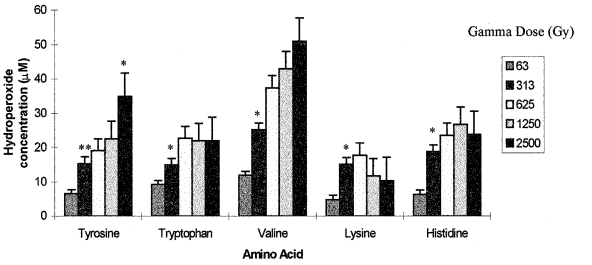

Fig. 3. Hydroperoxide determination (μM) of selected amino acids following increasing doses of gamma radiolysis (Gy). Values are mean ± S.E. ($n = 3$). Where results were statistically compared with the previous dose * indicates $P < 0.05$, ** indicates $P < 0.01$.

4. Discussion

It is thought that 10–50% of the antioxidant capacity of human plasma is due to radical scavenging by proteins [17]. This suggests that reactions between proteins/amino acids and free radicals, and the subsequent protein/amino acid hydroperoxide formation, may be of major significance in vivo.

In this study we have used three oxygen free radical generating systems to produce free radicals in aqueous samples in known pathophysiologically occurring quantities. Metal catalysed degradation of hydrogen peroxide produces primarily hydroxyl radicals. However, our results show that hydrogen peroxide in the presence of Cu(II) did not induce amino acid hydroperoxide formation with a system limit of detection of 50 pmol, despite a decrease in UV fluorescence (indicative of amino acid oxidative attack; data not shown). Fu et al. [18] have shown that transition metals (especially iron), cause rapid decomposition of protein hydroperoxides in vitro, and the relatively large concentrations of copper being used in this generating system may explain the absence of hydroperoxide production, through metal catalysed degradation.

The literature states that hydroperoxides are produced by hydroxyl radical mediated damage when using gamma radiation [4]. The results obtained herein, using gamma radiolysis, show that tyrosine and valine give a dose response of hydroperoxide formation, with valine showing the greatest sensitivity to gamma radiolysis. This type of free radical generating system produces equal quantities of both hydroxyl and superoxide radicals.

Several studies [4] have shown that hydroperoxide formation is hydroxyl radical mediated and that valine is the most sensitive to this type of attack. We support these observations and in addition demonstrate that with tryptophan and histidine hydroperoxide formation plateaus at a concentration of 25 μM using a dose of 625 Gy. This could be explained by degradation of products at high doses of gamma radiation. If degradation occurs at a similar rate to hydroperoxide production, then the net outcome is no significant change in concentration. Alternatively the sites for hydroperoxide formation may be saturated above the 625-Gy dose.

Previous studies have stated that tryptophan is the most sensitive amino acid to UV radiation due to the indol ring structure which can act as a photosensitiser. From the results obtained from the hydrogen peroxide production assay this appears to be the case; much higher concentrations of hydrogen peroxide were detected from tryptophan with increasing UVB irradiation when compared to the other amino acids

tested. However, it is unlikely that the lamp used would photolyse H_2O_2 to make $OH^{\cdot}$. Indeed, in our study, tyrosine showed the greatest sensitivity to UVB-induced hydroperoxide formation, suggesting that this reaction is not exclusively hydrogen peroxide concentration dependent. Tyrosine hydroperoxide formation from UVB possibly arises from singlet oxygen attack on the double bond found in the ring of tyrosine, with subsequent endoperoxide formation. The apparent drop in hydroperoxide yield between 11.2 and 22.3 kJ/m^2, whilst being highly reproducible, was not statistically significant, and may reflect maximal conversion above 11.2 kJ/m^2. For tryptophan photolysis [19], an electron is ejected leading to a tryptophan radical which then reacts with molecular oxygen to form a tryptophan hydroperoxide. By a means of re-arrangement, *N*-formylkynurenine (NFK) is subsequently produced. The presence of hydrogen peroxide then facilitates the formation of kynurenine. This sequence is thought to be the mechanism of copper catalysed formation of NFK and kynurenine in apolipoprotein B [20].

If this mechanism is applied to our observations for tryptophan in the metal catalysed peroxide degradation reactions, the presence of copper ions could explain the lack of hydroperoxides, as they may be re-arranged to NFK products before detection takes place with further breakdown to kynurenine in the presence of hydrogen peroxide. Tyrosine produced little hydrogen peroxide, and this amino acid hydroperoxide may therefore undergo less degradation. Again, the trace levels of metal ions found in the diluent could be influential in causing the degradation of hydroperoxides. We suggest that the degradation products of hydroperoxides to hydroxides, and tryptophan to kynurenines should be evaluated after free radical attack in the three systems outlined in this publication, to determine the true part that metal ions play in hydroperoxide yield.

Acknowledgements: This work was supported by the Ministry for Agriculture, Fisheries and Food (MAFF) under the Antioxidants and Nutrition Program ANO435.

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