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Free radical reactions in atherosclerosis; An EPR spectrometry study

F.A. Taiwo [a,*], H.J. Powers [b], E. Nakano [b], H.R. Griffiths [c] and D.F. Nugent [d]

[a] *Leicester School of Pharmacy, De Montfort University, The Gateway Leicester LE1 9BH, UK*
[b] *Human Nutrition Unit, University of Sheffield, Coleridge House, The Northern General Hospital, Sheffield S5 7AU, UK*
[c] *Department of Life and Health Sciences, Aston University, Aston Triangle, Birmingham B4 7ET, UK*
[d] *Department of Molecular Medicine, RCSI Education and Research Centre, PO Box 9063, Beaumont Hospital, Dublin 9, Ireland*

Abstract. The copper catalysed oxidation of homocysteine has been studied by electron paramagnetic resonance (EPR) spectroscopy and spin trapping techniques to determine the nature of free radical species formed under varying experimental conditions. Three radicals; thiyl, alkyl and hydroxyl were detected with hydroxyl being predominant. A reaction mechanism is proposed involving Fenton chemistry. Inclusion of catalase to test for intermediate generation of hydrogen peroxide showed a marked reduction in amount of hydroxyl radical generated. In contrast, the addition of superoxide dismutase showed no significant effect on the level of hydroxyl radical formed. Enhanced radical formation was observed at higher levels of oxygen, an effect which has consequences for differential oxygen levels in arterial and venous systems. Implications are drawn for a higher incidence of atherosclerotic plaque formation in arteries versus veins.

Keywords: Copper, homocysteine, atherosclerosis, hydroxyl radicals, LDL, EPR

1. Introduction

Elevated plasma total homocysteine concentration has been identified as a risk factor for atherosclerosis, thrombosis and a number of other cardiovascular disorders [1–3]. In its most extreme form, homocysteinuria: as indicated by homocysteine excretion in the urine [4,5], causes patients to frequently experience premature thromboembolic events [6,7]. While the pathology associated with homocysteinuria can be quite severe, occurrences of cystathionine β-synthase deficiency are relatively rare; 1 in 200,000 individuals [8,9]. Moderate hyperhomocysteinemia however is more common, present in approximately 5–10% of individuals not receiving folic acid supplementation and whose principal cause is poor dietary intake of folate [10,11]. A wealth of information is available linking homocysteinemia with vascular disease and even incremental increases in plasma homocysteine levels with increased vascular risk, although some contention does exist regarding causal or consequential linkage [12–14]. Atherosclerosis is perhaps the one condition where a pathophysiological role for homocysteine has been implicated. A mechanism has been proposed linking homocysteine, endothelial cells and oxidation of low density lipoprotein (LDL), with the early stages of development of atherosclerosis. A more recent study suggests a role for homocysteine export by endothelial cells *in vivo*, providing a continual low level stimulus for

*Corresponding author. Tel.: +44 116 207 8111; Fax: +44 116 257 7287; E-mail: ttaiwo@dmu.ac.uk.

free radical generation, following thiol oxidation in the presence of metal ions, in particular Cu^{2+}, with the resultant oxidation of LDL [15].

Accumulating evidence suggests that either copper or iron or both may have important roles in the aetiology of neurodegenerative disorders such as Alzheimer's disease, amyotrophic lateral sclerosis, Parkinson's disease and prion diseases [16–19]. A common factor in these diseases is a measurable increase in free radical mediated oxidative stress at some stage during progression of the disease [20], with the redox reactive transition metals such as iron and copper playing significant roles [21,22]. In the present study, we investigate the nature of the free radical species formed in the course of reaction of homocysteine with Cu^{2+} ions, in an aqueous system using electron paramagnetic resonance (EPR) spectroscopy with a series of spin traps at physiological pH (7.4) and temperature 37°C.

Abbreviations DEPMPO, 5-diethoxyphosphoryl-5-methyl-1-pyrroline-N-oxide; DMPO, 5,5-dimethyl-1-pyrroline-N-oxide; DMSO, dimethyl sulfoxide; IAA, iodoacetamide; LDL, low density lipoprotein; PBN, N-tert-butyl-alpha-phenylnitrone; PBS, phosphate buffered saline; SOD, superoxide dismutase.

2. Materials and methods

2.1. Materials

Catalase, $CuSO_4$, L-homocysteine thiolactone, IAA, PBN, DMSO, PBS tablets and SOD were purchased from Sigma, UK. DMPO was purchased from Sigma-Aldrich, UK. DEPMPO was purchased from Calbiochem.

2.2. Preparation of L-homocysteine from L-homocysteine thiolactone

L-homocysteine was prepared from L-homocysteine thiolactone by the method of Duerre and Miller [23]. L-homocysteine thiolactone was dissolved in a small aliquot of 5 M NaOH and incubated for 5 min at room temperature, to lyse the lactone ring. The resultant solution was neutralised with a larger volume of 1 M KH_2PO_4 and finally diluted to 10 ml with distilled water. Aliquots of L-homocysteine were immediately frozen and stored at −20°C until required.

2.3. Spin-trapping

Reactive oxygen species were analysed as spin adducts of DMPO, PBN and DEPMPO. Solution of 5,5-dimethyl-1-pyrrolidine-N-oxide (DMPO) in water was purified by filtration through activated carbon and Whatman syringe filter pore size 0.45 μm and stored frozen as 250 μl aliquots until required. Concentration of DMPO solution was routinely determined using a molar extinction coefficient of $\varepsilon_{234} = 7700\ M^{-1}\,cm^{-1}$ in water. Solutions of PBN and DEPMPO were similarly passed through activated carbon until found to be 'spectrally clean' and then used without further purification. The reactants were added in quick succession and in the following order: L-homocysteine, the spin trap (DMPO, 1 mM; PBN, 50 mM; or DEPMPO, 20 mM), and $CuSO_4$ to a calculated volume of PBS (pH 7.4, 1.700 cm^3, and final volume of 1.831 cm^3). The reaction mixture was incubated at 37°C and for the time course studies, at regular interval, aliquots of reaction mixture were transferred to a quartz flat cell for EPR detection. For single time point assays, the PBS volume and total reaction volume were reduced to 327 μl and 352 μl respectively, as sampling "out" at multiple time points was not done. EPR signals of DMPO, PBN and DEPMPO spin adducts of hydroxyl radicals were identified by their characteristic hyperfine coupling constants [24].

2.4. Electron Paramagnetic Resonance (EPR) spectrometry

First derivative EPR spectra were recorded on a Bruker EMX 6/1 EPR spectrometer operating at X-band frequency range (9.7 GHz) and a modulation frequency of 100 kHz. Instrument settings were as follows: field set 3488 G, scan width 100 G, microwave power 5 mW, modulation amplitude 1.5 G, time constant 10.24 ms, scan time 83.89 s. For the purpose of positive identification the hydroxyl radical was independently generated by the Fe^{II}/H_2O_2 reaction, and the superoxide radical anion $O_2^{\cdot-}$ by the xanthine oxidase enzyme catalysed oxidation of hypoxanthine to uric acid in the presence of oxygen and DMPO. Spectral simulations were performed using the SimFonia program from Bruker Biospin.

2.5. Oxidation state of copper by EPR spectroscopy

EPR measurements of frozen samples were made at 77 K. Aliquots of a 1.25 mM Cu^{2+} and 0.50 mM homocysteine reaction in an assay volume of 2 ml (without spin trap added) were prepared for EPR spectroscopy for the purpose of measuring the redox state of copper. Constant dimension cylindrical pellets of the frozen solution were made in liquid nitrogen (77 K) as described previously [25]. The pellets were positioned in a finger dewar filled with liquid nitrogen and set in a rectangular cavity type TE_{102}. Instrument settings were as follows: Instrument settings were as follows: field set 3488 G, scan width 100 G, microwave power 10 mW, modulation amplitude 1.5 G, time constant 10.24 ms, scan time 83.89 s. Characteristic EPR signals of Cu^{2+} were identified by the quartet of parallel features and a sharp perpendicular line in the free spin region [26].

2.6. Measurement of oxygen levels

Oxygen levels in superoxygenated, ambient and deoxygenated PBS (pH 7.4) solutions were determined using a Clarke oxygen electrode (Rank Brothers, Cambridge, England) connected to a meter. The meter was calibrated with oxygen saturated PBS (240 μM O_2) and the zero percent oxygen level was achieved by addition of a few grains of sodium dithionite to PBS. Oxygen content was measured in experimental solutions set-up in parallel for this purpose only and without inclusion of DMPO. Enhancement of hydroxyl radical intensity by increasing oxygen content was recorded. To determine oxygen content of reaction media, it was deemed sufficient to use only the buffer solution as this constituted the bulk of the assay volume in the Cu^{2+}/homocysteine reactions. To obtain a deoxygenated solution, PBS was warmed to boiling and allowed to cool under a continuous flush of nitrogen gas. Superoxygenated PBS was obtained by continuous flushing of oxygen gas through 3–4 ml of the buffer solution for 20 min. The PBS was then used immediately in the assay. $CuSO_4$ and L-homocysteine, but not the DMPO stock solutions were gently purged of oxygen by passing oxygen-free nitrogen gas through them for 20 min. All procedures for deoxygenated mixtures were performed in a nitrogen filled glove box.

3. Results

3.1. Reactivity of copper and homocysteine

Incubation of copper sulphate and homocysteine resulted in the formation of free radicals observed by spin trapping and EPR spectrometry. Fitting of experimental spectra and simulation Fig. 1, gave nitrogen and hydrogen splittings; $a^{\mathrm{N}} = 1.491$ mT and $a^{\mathrm{H}} = 1.491$ mT characteristic of DMPO-OH [24]. Features

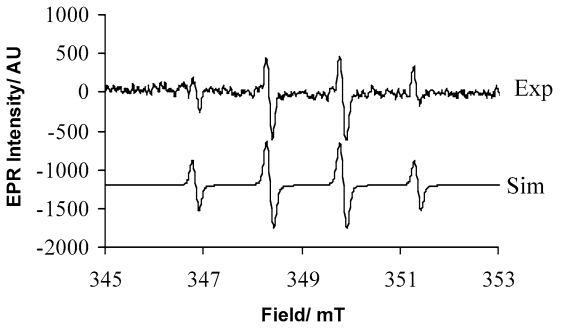

Fig. 1. First derivative EPR spectrum of radical adduct formed on incubation of 125 μM Cu^{2+} with 50 μM L-homocysteine in the presence of 1 mM DMPO (top). Spin Hamiltonians of spectrum confirmed by simulation (bottom) are: $a^{N} = 1.49$ mT, and $a^{N} = 1.49$ mT which are typical of hydroxyl radical.

in the low field wings and dissymmetry observed in the second and fourth lines of DMPO-OH appear to correspond to $a^{N} = 1.491$ mT and $a^{N} = 1.6$ mT which is typical of DMPO-SG adduct [27,28]. No other free radical types were observed in this system, using DMPO as spin trap. On application of DEPMPO as spin trap, a composite spectrum shown in the series presented in Fig. 2(a) was obtained. The '0 min' spectrum (top) is deemed to contain all species detectable while the last in the series, '120 min' is a neat terminal spectrum comprising the most stable constituent. The gradation of spectra for reaction times of 0 minutes up to 120 minutes is thus a display of timely compositions of radical species. The two arrows show lines in the wings that diminish with time. The brace in the middle shows a doublet of lines whose separation increases with time. The terminal spectrum (at 120 min) was analysed by simulation to be DEPMPO-OH with parameters similar to literature values (see Table 1). On deconvolution of the '0 minute' spectrum starting with the '120 minute' spectrum as DEPMPO-OH, two other spectra were obtained, shown in Fig. 2(b). Parameters derived from simulations, shown in Table 1, indicate formation of three radicals; alkyl, thiyl and hydroxyl. The large hydrogen splitting of 2.125 mT is typical of alkyl radical adducts.

A profile of variation of relative amounts of the radicals with respect to time is shown in Fig. 2(c).

3.2. Effect of oxygenation

The hydroxyl radical is the predominant and most persistent free radical generated by Cu^{2+} induced oxidation of L-homocysteine. A series of deoxygenation and superoxygenation experiments were run to determine whether oxygen dissolved in the various reaction media (buffers, $CuSO_4$ and homocysteine) was required for the generation of the hydroxyl free radical. Deoxygenation of solutions used in the experiments, by prolonged purging of each solution with nitrogen gas, showed diminished rate and final amount of hydroxyl free radical generated over time (although in some experiments only the initial rate was affected, with the final amount of free radical generated being equivalent under both conditions, at the final incubation time points tested). Figure 3 shows the variation in hydroxyl radical generation under conditions of ambient oxygen versus superoxygenated conditions. It was found difficult to obtain an absolute oxygen-free environment, as some of the dissolved oxygen appeared to remain in solution, as determined by oxygen probe measurements, with slightly higher dissolved oxygen levels being found in the PBS deoxygenated by nitrogen flush than recorded for sodium dithionate treatment, suggesting the deoxygenation process was incomplete. It was preferable to use the nitrogen flush in order to avoid chemical effects of dithionite on our primary reaction.

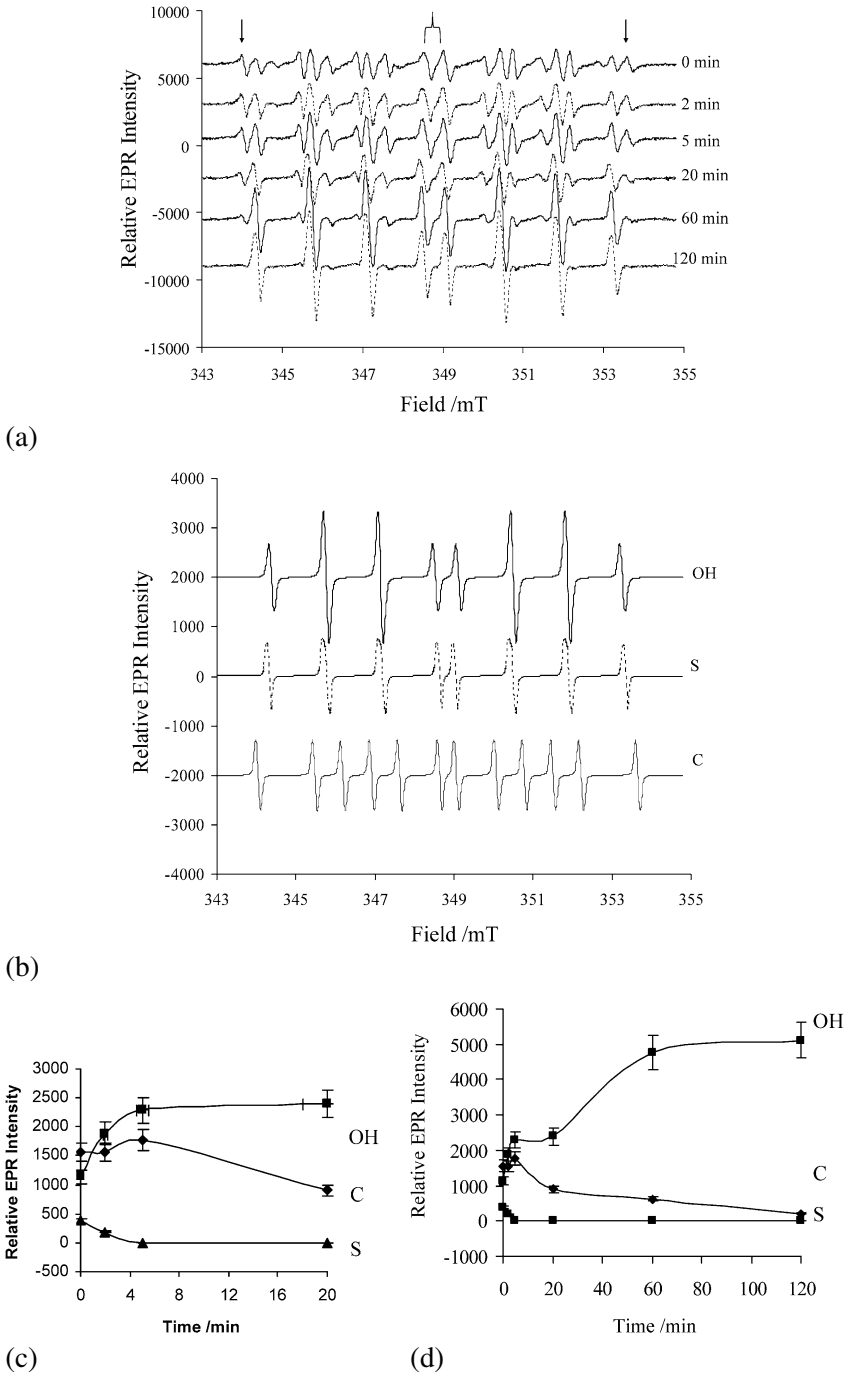

Fig. 2. (a) EPR spectra of DEPMPO-trapped radicals from Cu^{2+}/homocysteine reaction at time intervals showing a gradation of features starting from 0 min to 120 min. (b) Constituent spectra obtained from DEPMPO-trapped radicals showing three major fractions identified as $^{\cdot}OH$, C-centred and S-centred radicals. (c) and (d) Time-dependent composition of DEPMPO spectra with respect to constituent fractions.

Table 1

Reaction	Spin trap	a^N/mT	a^H/mT	A^P/mT	Source
$FeSO_4 + H_2O_2$	DMPO	1.491	1.491	–	This study
Cu/homocysteine	DMPO	1.484	1.484	–	This study
Cu/homocysteine	PBN	1.511	0.333	–	This study
Cu/homocysteine/DMSO	PBN	1.536	0.361	–	This study
Cu/homocysteine	DEPMPO	1.427	2.147	4.60	This study
		1.41	1.49	4.71	This study
		1.40	1.32	4.73	This study
GSNO + $h\nu$	DEPMPO	1.41	1.49	4.58	Ref. [30]
$FeSO_4 + H_2O_2$	DEPMPO	1.40	1.32	4.73	Ref. [30]
Homocysteine + $h\nu$	DMPO	1.528	1.680	–	Ref. [47]
Autooxidation of cysteine	DMPO	1.530	1.720	–	Ref. [48]

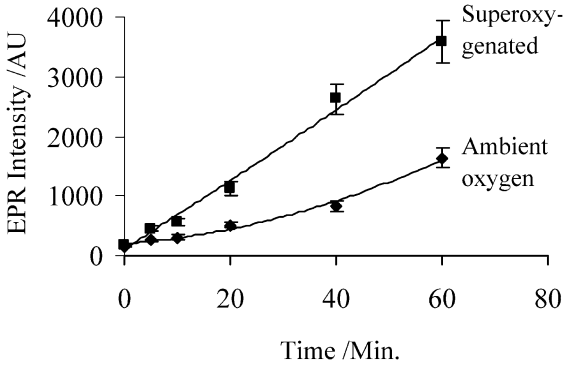

Fig. 3. Effect of oxygenation on intensity of hydroxyl radical formed with time in PBS (pH 7.4) in the presence of 125 μM Cu^{2+} + 50 μM L-homocysteine, showing higher levels of hydroxyl radical formed under superoxygenated versus ambient oxygen levels. The data points are the mean ± S.D. of three separate experiments ($n = 3$).

3.3. Effect of catalase

Further studies were carried out to determine the effect of catalase on hydroxyl radical formation in Cu^{2+} ion induced oxidation of L-homocysteine. Initially, a number of single time point assays were performed (125 μM Cu^{2+}/50 μM) following a 30 minute incubation at 37°C, under varying levels of catalase activity (0–8000 units of activity per assay), as shown in Fig. 4. This was to determine the optimum concentration of catalase for the assay system. A relationship between increasing enzyme activity levels and decreasing hydroxyl radical formation was found, with high levels of enzyme activity resulting in little or no EPR signal intensity. Figure 5 shows clearly a reduction in hydroxyl radical generation by catalase in a dose dependent manner over a prolonged incubation time of 120 min. Significantly lower levels of hydroxyl free radical were formed in the presence of catalase, with even lower levels of the free radical formed under conditions of high catalase activity, with no evidence of the EPR signal intensity reaching "normal": levels following prolonged incubation times (>2 hrs, data not shown), unlike the superoxygenation data. This suggests that hydrogen peroxide may be an intermediate formed in the production of hydroxyl free radicals in this reaction.

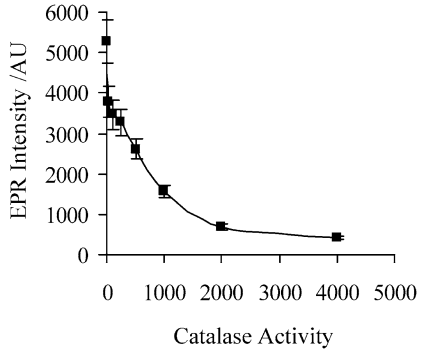

Fig. 4. Variation in intensity of hydroxyl radical formed with varying catalase enzyme activity, following a 30 min reaction of 125 μM Cu^{2+} + 50 μM L-homocysteine in PBS (pH 7.4). The data points are the mean ± S.D. of three separate experiments ($n = 3$).

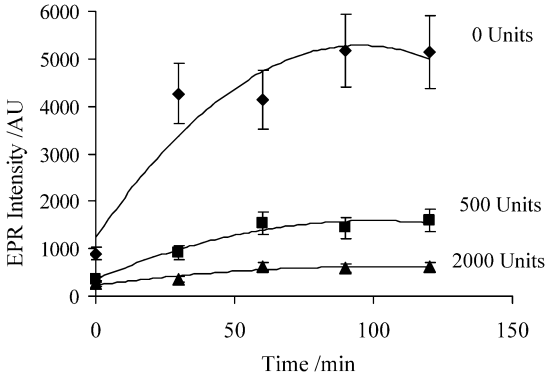

Fig. 5. A time course showing hydroxyl radical formation under varying conditions of catalase enzyme activity, in the 125 μM Cu^{2+} + 50 μM L-homocysteine reaction system. The data points are the mean ± S.D. of three separate experiments ($n = 3$).

3.4. Effect of superoxide dismutase

Superoxide dismutase (SOD) was examined for its ability to affect copper mediated oxidation of homocysteine. SOD catalyses the protonation of superoxide free radicals to generate hydrogen peroxide and molecular oxygen: $2O_2^{\cdot-} + 2H^+ \rightarrow H_2O_2 + O_2$, and could constitute a potential mechanism for the generation of the hydrogen peroxide intermediate. SOD at 100 and 1000 units of activity (per assay volume) appeared to have no effect on the rate of hydroxyl free radical formed in the Cu^{2+}/homocysteine reaction, at any of the time points tested; Fig. 6. While it cannot be discounted that the superoxide free radical was not being generated in the Cu^{2+}/homocysteine reaction system and indeed no such radical was detected with DMPO, it is possible that the natural rate of dismutation of superoxide, $K = 5.4 \times 10^5\ M^{-1}\ s^{-1}$ [29] is so rapid, that any further enhancement of the rate by SOD ($K = 2.3 \times 10^9\ M^{-1}\ s^{-1}$) [30] is not detectable in the time scale of our EPR experiments, due to rapid completion in both cases.

The SOD experiments were repeated at a more alkaline pH (9.0 vs 7.4), in an attempt to slow the natural protonation rate of superoxide radical. Under these conditions, SOD had little effect on the overall EPR signal intensity generated by the oxidation of homocysteine by copper ions. This is not unexpected as the enzyme activity is independent of pH in the range 5.3 to 9.5; the second order rate constant being of the order of $1.8 \times 10^9\ M^{-1}\ s^{-1}$ [30].

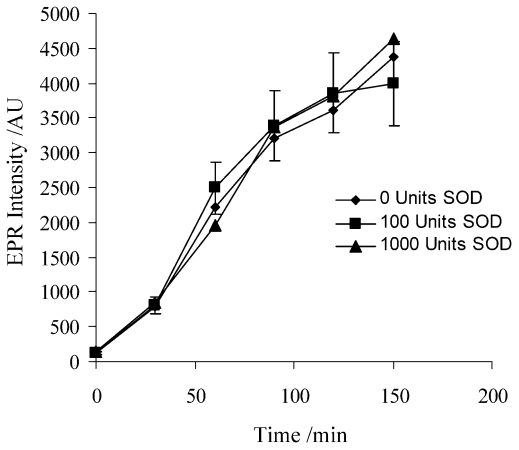

Fig. 6. A time course showing hydroxyl radical formation under varying conditions of SOD enzyme activity in the 125 μM Cu^{2+} + 50 μM L-homocysteine reaction system. The data points are the mean ± S.D. of three separate experiments ($n = 3$).

3.5. Oxidation state of copper by EPR spectroscopy

To determine any changes in the oxidation state of copper, EPR measurements were made at 77 K as described under materials and methods. Cu^{2+}, (d^{10}; s^1) has an EPR spectrum which disappears as it is converted to Cu^{+}, (d^{10}; s^0) [31]. In a reaction system consisting of PBS, homocysteine and $CuSO_4$, with no DMPO added, copper appears initially in the 2+ state showing a characteristic spectrum, which diminishes with time as the 500 μM L-homocysteine + 1250 μM Cu^{2+} assay proceeds Fig. 7. Loss of the copper signal must be due to conversion of Cu^{2+} to Cu^{+}. Similar results have been obtained in the direct oxidation of lipid by copper [32].

3.6. Selectivity of spin traps

Owing to limitations of DMPO as spin trap for superoxide radicals, other spin traps; N-tert-butyl-α-phenylnitrone (PBN) and 5-diethoxyphosphoryl-5-methyl-1-pyrroline-N-oxide (DEPMPO) were used. In particular, the ability of DEPMPO to form a stable spin trap adduct with superoxide radicals was of interest, although it traps hydroxyl and sulphur-centred radicals, as well as the sulfite radical anion [27] all having overlapping features.

Overall, the comparison of EPR spectra obtained in the classic Cu^{2+}/homocysteine reaction, using either PBN or DEPMPO as spin traps versus DMPO, suggested hydroxyl free radicals being a major and most persistent radical formed. The carbon-centred radical which was initially formed slowly diminished. A very small amount of sulphur-centred radical formed also decayed rapidly as shown in Fig. 2(c,d). Table 1 shows EPR parameters for a series of spin trapped adducts obtained in this study.

Spin trapping with PBN showed two radicals with EPR parameters of $a^{\mathrm{N}} = 1.511$ mT, $a^{\mathrm{H}} = 0.333$ and $a^{\mathrm{N}} = 1.677$ $a^{\mathrm{H}} = 0.333$ mT with the former in relatively higher proportion. Close similarity of PBN-adduct parameters makes it rather difficult to use them as distinguishing means of identification. Comparison with test results from the Fenton reaction ($Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + {}^{\bullet}OH + OH^{-}$) EPR parameters of a^{N} 1.600 mT and a^{H} 0.333 mT would suggest one of our radicals is hydroxyl. Thus, all three spin traps: DMPO, PBN and DEPMPO point towards hydroxyl free radical being the main free radical detectable following oxidation of L-homocysteine by Cu^{2+} ions. There appeared no evidence of

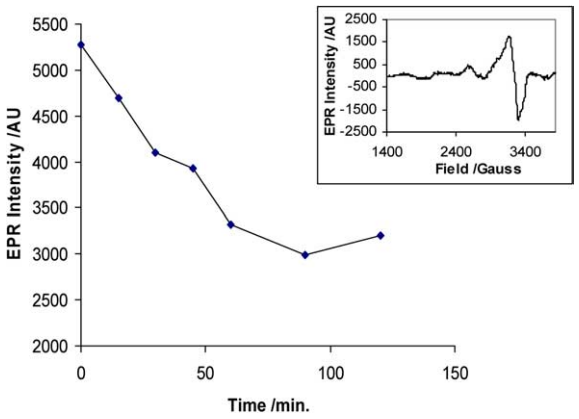

Fig. 7. Plot showing reduction in amount of Cu^{2+} with time during oxidation of homocysteine, following the incubation of 1.25 mM Cu^{2+} + 500 μM L-homocysteine in PBS (pH 7.4). Inset shows the EPR spectrum of Cu^{2+}; the large signal is the perpendicular feature ($g_x g_y$) measured peak-to-peak over time to get the main plot.

superoxide radical formation in the Cu^{2+}/homocysteine reaction system, as the DEPMPO-superoxide radical adduct should be a distinctive EPR spectrum, which was not apparent from the spectra obtained. On the matter of the disappearing peaks, of which there are a number and often closely positioned (to the left and to the right) of the peaks that constitute the DEPMPO-hydroxyl radical adduct, it has not been possible so far to identify a species of free radical that may account for these peaks. If the signal is due to a free radical, it must be one where the free radical-spin trap adduct is very unstable, so that by the final incubation times (of 120–150 min) the signal has practically fully disappeared. Such disappearing peaks were also noted in the Fenton reaction spectra, yet they were not as numerous and those that were there have been ascribed to a methanol effect and these peaks do not correspond with the disappearing peaks of the Cu^{2+}/homocysteine reaction system. It is possible that contamination in the DEPMPO preparation, may be contributing to the signal, such as hydroxylamines [33], but oxidation of these compounds may not yield peaks at these positions and would not be absent from the Fenton system. It should be noted that it is only with the DEPMPO spin trap that an EPR signal separate from the hydroxyl radical, was observed, pointing to sensitivities of the DEPMPO binding kinetics absent from the other spin traps tested.

In order to determine the effective reactivity of the sulphydryl group in the homocysteine molecule, the thiol-blocking reagent iodoacetamide was included in the reaction scheme. Homocysteine (500 μM) was pretreated with 50 μM of IAA for 30 min at 37°C before addition of Cu^{2+} ions in the typical Cu^{2+}/homocysteine reaction (125 μM Cu^{2+}/50 μM L-homocysteine). No success however was achieved in preventing hydroxyl radical formation following Cu^{2+} ion induced oxidation of thiol blocked L-homocysteine and ultimately it was determined (by omitting homocysteine from the reaction system) that both thiol blockers tested were themselves subject to Cu^{2+} ion mediated oxidation, with the resultant production of hydroxyl free radicals, possibly possessing one or more chemical moieties that are sensitive to such oxidation reactions.

4. Discussion

Previous studies have shown that copper inhibits the alkylation of sulphydryl groups by iodoacetamide in an oxidative process [34]. It is thus clear that to be an effective thiol blocker, the agent not only must

directly react with the thiol groups in question and thereby eliminate their participation in metal ion mediated oxidation reactions, but also not provide any chemical groups that are readily oxidisable by metal ions. In relation to the question of which chemical group in homocysteine is responsible for free radical generation in the presence of Cu^{2+} ions, involvement of the homocysteine thiol group must be well considered, as this is one of the more readily oxidisable chemical groups present in amino acids.

Of interest is a study by Berman and Martin [35], where endothelial barrier function was assessed using an *in vitro* model, whereby albumin transfer across monolayers of bovine aortic endothelial cells grown on polycarbonate membranes was measured. An increase in albumin transfer across the monolayer was noted in the presence of a combination of homocysteine (1.5 mM) and copper sulfate (50 μM) and this increase was abolished by catalase and unaffected by SOD. These enzymatic observations are consistent with those of the present study and with the conclusion that hydrogen peroxide, not the superoxide anion was responsible for endothelial cell damage caused by copper catalysed oxidation of homocysteine. Though our study has used non-physiological high amounts of copper and homocysteine, the model is intended to be a chemical representation amenable to detection levels of EPR spectrometry.

Aspects of mechanism:

$$Cu^{+} + RSH \rightarrow Cu^{+} + RS^{\cdot} + H^{+}, \tag{1}$$

$$RS^{\cdot} + RS^{\cdot} \rightarrow RSSR, \tag{2}$$

$$Cu^{+} + O_2 \rightarrow Cu^{2+} + O_2^{\cdot -}, \tag{3}$$

$$O_2^{\cdot -} + 2H^{+} \rightarrow H_2O_2, \tag{4}$$

$$Cu^{+} + H_2O_2 \rightarrow Cu^{2+} + OH^{-} + OH^{\cdot}. \tag{5}$$

On the occurrence of a carbon centred radical, there are two possible explanations. Formation of a carbon centred radical could arise from abstraction of hydrogen by the thiyl radical from its α-carbon position [36]. An overlap of sulphur p- and the sp3 hybrid orbitals of its α-carbon results in spin distribution onto the carbon hence simultaneous formation of both carbon as thiyl radicals. It is observed that the decay of the carbon and thiyl radicals is also simultaneous and rapid, Fig. 2(c).

Earlier studies by Lodge et al. [37] on the oxidation of thiols by copper showed the formation of only hydroxyl radicals using DMPO spin trapping. This is similar to our results in which hydroxyl radicals was a major product. Dithiothreitol has also been shown to produce hydrogen peroxide following oxidation by Cu^{2+} ions. The peroxide will ultimately in a metal catalyzed reaction produce hydroxyl radicals. Our use of DEPMPO facilitated further detection of both an alkyl and a thiyl radical. The simultaneous growth of the hydroxyl radical with decay of both alkyl and the suspected thiyl radical is an interesting observation in these reactions.

It has been established that though cysteine and homocysteine are both aminothiols it is the latter that is implicated as a potential marker of peripheral vascular disease and atherosclerosis [38]. No evidence is available to implicate cysteine in the pathophysiology of these diseases. One potential explanation for this may be that under physiological conditions (pH 7.4), the rate of autooxidation for homocysteine is significantly greater than for cysteine [39]. This may be explained by homocysteine being a stronger nucleophile than cysteine and hence more reactive in this system. A rise in pH to more alkaline conditions (pH 8.9) drastically increases the autooxidation rate of homocysteine, but does not modify the rate of oxidation of cysteine and this may be due to the lower pK_a of the cysteine thiol group [40]. It has to be said however that pH variation does have more far reaching consequences than presently envisaged.

There are a number of properties of homocysteine and copper chemistry that favour the role of homocysteine over cysteine in the pathophysiology of a number of disease processes. Homocysteine is a far better reducer of Cu^{2+} than either cysteine or methionine, the former having only between 20–25% of homocysteine's copper reducing effect [41]. It has long been thought that reduction of copper would potentiate the cell damaging properties of copper ions to endothelial cells [42], where homocysteine has been shown to induce in a dose and time dependent manner, the lysis of endothelial cells in the presence of Cu^{2+} ions in a manner indicative of the generation of H_2O_2. Copper toxicity has been demonstrated in mouse neuronal cultures, in which homocysteine potentiates Cu^{2+} neurotoxicity far more than cysteine does at the same concentration. Bathocuprione, the copper complexing agent was found to significantly reduce the neurotoxic effects of homocysteine and copper, suggesting an important role for copper in mediating cell damage [43]. Homocysteine plus Cu^{2+} appears to generate significantly greater amounts of H_2O_2 than cysteine and copper, pointing to a greater pathophysiological role for homocysteine over cysteine.

In protein structures, homocysteine shows greater capacity in forming disulphide bonds with protein thiol groups, both intracellularly and extracellularly, in comparison with cysteine and glutathione [44] and as protein cysteine thiol groups are involved in the function of many enzymes, structural proteins and receptors, it suggests a greater role for homocysteine's ability to disrupt protein function in this manner than is the case with other aminothiols. Formation of hydroxyl radicals from hydrogen peroxide through a Fenton reaction, involving Cu^{2+} ions was shown to be compromised by deoxygenation of the buffer and by the addition of catalase. These results closely mirror our own, where we have shown the rate of hydroxyl radical formation, following the oxidation of homocysteine by Cu^{2+} ions, is influenced by the amount of dissolved O_2 in the buffer.

Starkebaum and Harlan [42] showed that Cu^{2+} stimulated oxidation of homocysteine in an aqueous cell free balanced salt solution is an oxygen dependent reaction, with the reaction kinetics of oxygen consumption in relation to increasing [Cu^{2+}] closely mirroring that of homocysteine oxidation. The authors also monitored oxidation of 500 μM homocysteine by 50 μM Cu^{2+} ions under nitrogen and found less than 1% of the homocysteine was oxidized under these conditions. Catalase was also found to reduce the rate of O_2 consumption, pointing to the production of H_2O_2 measured by the horseradish peroxidase-mediated oxidation of fluorescent scopoletin and in the presence of 50–500 μM homocysteine. Scopoletin was oxidized in a dose dependent manner, with both catalase and EDTA inhibiting the rate of oxidation. The molar ratio of homocysteine oxidized to O_2 consumed was found to be 4.0, suggesting that very little H_2O_2 would accumulate during the copper catalysed oxidation of homocysteine. The authors found no H_2O_2 accumulation following the oxidation of 50–500 μM homocysteine by Cu^{2+} ions after 3 min incubation. These results mirror our own on several points. Firstly, we have found the oxidation of homocysteine by Cu^{2+} ions to be influenced by the level of O_2 in the physiological buffer solutions, with superoxygenated buffer solutions promoting enhanced rates of hydroxyl radical formation versus buffer solutions with normal O_2 levels (Fig. 3). Slower rates of hydroxyl radical formation were noted under N_2, although we were not successful in establishing an absolute oxygen-free environment. Catalase also significantly reduced the rate and amount of hydroxyl free radical formed following homocysteine oxidation by Cu^{2+} ions, suggesting that H_2O_2 too may be formed in our reaction system. The non-detection of H_2O_2 in their scheme may be due to the rapid breakdown of H_2O_2 catalysed by Cu^{2+}. As in our reactions, H_2O_2 may be the intermediate formed in the copper catalysed oxidation of homocysteine, to ultimately yield the detectable final product, hydroxyl radicals.

An interesting study by Lodge et al. [45] showed that oxygen consumption is an important component of Cu^{2+}-induced peroxidation of LDL [45], with a typical lag phase, followed by an increased in O_2

consumption. The O_2 consumption appeared to increase with increasing LDL or Cu^{2+} ion concentration, although increase with the latter was less significant. The O_2 consumption was also sensitive to conditions of low versus high starting O_2 concentrations. The lag phase of O_2 consumption appeared to decrease, with increasing starting concentrations of Cu^{2+} ions and O_2, but not LDL and the rate of O_2 consumption increased with increased starting concentrations of LDL, Cu^{2+} and O_2, although with Cu^{2+} the increases were modest. Therefore, it appears that both Cu^{2+}-mediated oxidation of homocysteine and Cu^{2+}-mediated LDL peroxidation are O_2 sensitive processes and a link thus exists between the two, as hydroxyl radicals generated by Cu^{2+}-mediated oxidation of homocysteine may be responsible for initiating abstraction reactions, whereby the hydroxyl radical oxidises the organic substrate/LDL forming water and an organic radical ($^{\bullet}OH + RH \rightarrow R^{\bullet} + H_2O$).

Of relevance to the observation that SOD failed to enhance the rate of hydroxyl free radical formed in the Cu^{2+}/homocysteine reaction, is the observation that bovine erythrocyte SOD is slowly and irreversibly inactivated by hydrogen peroxide, the rate of which is dependent on concentrations of enzyme and hydrogen peroxide. It is possible therefore that the hydrogen peroxide generated following oxidation of homocysteine by Cu^{2+} ions inhibits SOD activity, thus preventing it from enhancing protonation of superoxide radical to form hydrogen peroxide and ultimately increased hydroxyl radical formation. It does seem probable that the hydroxyl radicals generated following the oxidation of homocysteine by Cu^{2+} ions, are ultimately generated by a Fenton type reaction ($H_2O_2 + Cu^{+} \rightarrow Cu^{2+} + OH^{-} + HO^{\bullet}$).

As shown in this study, increased oxygen tension has been reported to lead to higher levels of hydrogen peroxide [46]. Conversely though, a regime of hyperoxia over several weeks reduces atherosclerosis in rats [47]. While we propose that hyperoxia leads to more hydrogen peroxide and therefore more hydroxyl radicals for oxidation of LDL, the reported *in vivo* effects on rats may be a typical case of adaptive response over several weeks in which endogenous antioxidants like catalase and SOD have been up-regulated during the time of continuous exposure to hyperbaric conditions [48].

Conclusion

Detection and positive identification of hydroxyl, alkyl and sulphydryl radicals by spin trapping and EPR spectroscopy present incontrovertible evidence for the identity and participation of radicals in the copper–homocysteine reaction. The major formation and persistence of hydroxyl radical is noted as important in the pathophysiology of atherosclerosis. Although concentrations of reagents, copper and homocysteine in particular were much higher than physiological, nevertheless the methodology constitutes an *in vitro* model for important aspects of atherosclerosis. The physiological significance of this reaction to atherogenesis is well known but the mechanism is only now being explained as radical mediated.

The effect of oxygen on the rate of generation and amount of hydroxyl radical is found to be important in the comparative biochemistry of this reaction in arterial and venous systems. This study offers a simple explanation for the observed higher incidence of plaque formation in arteries than veins in terms of the higher level of oxygen in arteries and the associated enhanced generation of the hydroxyl radical.

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