Protein Hydroperoxides are a Major Product of Low Density Lipoprotein Oxidation During Copper, Peroxyl Radical and Macrophage-mediated Oxidation

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Damage to apoB100 on low density lipoprotein (LDL) has usually been described in terms of lipid aldehyde derivatisation or fragmentation. Using a modified FOX assay, protein hydroperoxides were found to form at relatively high concentrations on apoB100 during copper, 2,2'-azobis(amidinopropane) dihydrochloride (AAPH) generated peroxyl radical and cell-mediated LDL oxidation. Protein hydroperoxide formation was tightly coupled to lipid oxidation during both copper and AAPH-mediated oxidation. The protein hydroperoxide formation was inhibited by lipid soluble α-tocopherol and the water soluble antioxidant, 7,8-dihydronopterin. Kinetic analysis of the inhibition strongly suggests protein hydroperoxides are formed by a lipid-derived radical generated in the lipid phase of the LDL particle during both copper and AAPH-mediated oxidation. The protein hydroperoxide formation was inhibited by lipid soluble α-tocopherol and the water soluble antioxidant, 7,8-dihydronopterin. Kinetic analysis of the inhibition strongly suggests protein hydroperoxides may form in vivo within atherosclerotic plaques. In contrast to protein hydroperoxide formation, the oxidation of tyrosine to protein bound 3,4-dihydroxyphenylalanine (PB-DOPA) or dityrosine was found to be a relatively minor reaction. Dityrosine formation was only observed on LDL in the presence of both copper and hydrogen peroxide. The PB-DOPA formation appeared to be independent of lipid peroxidation during copper oxidation but tightly associated during AAPH-mediated LDL oxidation.

Keywords: Low-density-lipoprotein; Protein-hydroperoxide; Protein-bound-DOPA; Free-radical; Lipid-peroxidation; Macrophage; Neopterin

Abbreviations: AAPH, 2,2'-azobis(amidinopropane) dihydrochloride; EDTA, Ethylenediamine-tetraacetic acid disodium salt; HPLC, high performance liquid chromatography; LD, low density lipoprotein; PBS, Phosphate buffered saline; PMA, phorbol 12-myristate 13-acetate; PB-DOPA, protein-bound-3,4-dihydroxyphenylalanine; TCA, trichloroacetic acid

INTRODUCTION

The oxidation of low density lipoprotein is considered to be a key process in the development and progression of atherosclerosis. Oxidised LDL is rapidly taken up by macrophages via the scavenger receptor, leading to the transformation of the macrophages to lipid loaded foam cells. In vitro, LDL oxidation begins with the consumption of various endogenous antioxidants and is followed by initiation of the peroxyl radical-mediated chain reaction, leading to peroxidation of polyunsaturated fatty acid esters within the LDL particle. Concomitantly, the structural integrity of the apoB100 protein is lost. Oxidation of the LDL protein moiety results in the loss of select amino acids, carbonyl formation, derivatisation with lipid aldehydes, protein fragmentation and aggregation through protein cross-linking.

Free radical-mediated protein oxidation also results in the formation of two types of reactive amino acid residues; protein hydroperoxides and protein bound 3,4-dihydroxyphenylalanine (PB-DOPA). Both reactive groups are known to form on apoB100 during LDL oxidation but, unlike protein carbonyls, protein hydroperoxides and PB-DOPA can initiate further oxidative...
damage to the protein or surrounding biomolecules.  

Protein hydroperoxides are formed when a hydroxyl or peroxy radical removes a hydrogen atom from an amino acid side chain. The resulting carbon-centred radical may then react with oxygen and hydrogen ions to form a hydroperoxide on the amino acid residue. Though relatively stable, protein hydroperoxides readily react with DNA, oxidising nucleotides and forming protein–DNA cross-links. Protein hydroperoxides also oxidise cellular thiols and consume the key cellular antioxidants ascorbate and glutathione. Protein hydroperoxides have been shown to form on various proteins, plasma proteins, lipoproteins and cellular proteins in living cells.

PB-DOPA is formed from tyrosine residues by hydroxyl radial addition to the phenol ring hydrogen atom, followed by hydroxylation of the resulting tyrosyl radical. As a reducing agent, PB-DOPA can stimulate further Fenton-mediated hydroxy radical production by reducing transition metal ions. This mechanism appears to be responsible for PB-DOPA's ability to oxidise DNA. PB-DOPA has been extensively studied using pure proteins, and identified in cataract lens and oxidised lipoproteins during copper-mediated oxidation.

Both PB-DOPA and the breakdown products of protein hydroperoxides have been identified in atherosclerotic plaques and on ApoB in plasma from healthy donors. However, no study has addressed the relative significance of PB-DOPA or protein hydroperoxides during LDL oxidation, nor demonstrated the formation of either protein oxidation product during cell-mediated LDL oxidation. In this study we show that protein hydroperoxides are a major product of copper ion, peroxy radical and cell-mediated LDL oxidation. The lipid soluble antioxidant, α-tocopherol (vitamin E), and the water soluble antioxidant, 7,8-dihydronicotinamide, have been used to examine the relationship between lipid oxidation and protein hydroperoxide formation during LDL oxidation. 7,8-Dihydronicotinamide is a macrophage-synthesised antioxidant, which has previously been shown to inhibit lipid oxidation on LDL and protein hydroperoxide formation on albumin exposed to peroxy or hydroxyl radicals.

MATERIALS AND METHODS

All chemicals and reagents were of AR grade or better and obtained from the Sigma Chemical Company (USA) or BDH Chemicals New Zealand Limited. 7,8-dihydronicotinamide was obtained from Schirck’s Laboratories, Switzerland. 2,2′-azobis(aminopropyl) dihydrochloride (AAPH) was supplied by Aldrich Chemical Company Inc. Ethylenediaminetetraacetic acid disodium salt (EDTA) was supplied by BDH chemicals.

All solutions were prepared with high purity water from a NANOpure ultrapure water system from Barnstead/Thermolyne (Iowa, USA). Phosphate buffered saline (PBS) (160 mM sodium chloride, 10 mM sodium phosphate buffer pH 7.4), was treated with chel-ex-100 resin (BioRad, Richmond, USA) to remove trace amounts of redox active metal ions.

LDL-plasma was prepared from blood taken by venipuncture from healthy female and male donors (age 20–45 years). The LDL-plasma from groups of five donors was pooled and frozen at −80°C in 0.6% sucrose for up to 3 months. LDL was prepared by a single 22 h ultracentrifugation of the pooled EDTA-plasma using a buoyant density four step discontinuous gradient in a Beckman SW41 rotor. For oxidation experiments, the LDL was desalted by 24 h of dialysis with four changes of nitrogen-gassed PBS containing 0.5 g/l of chel-ex-100 resin. The LDL molar concentration was determined by enzymatic cholesterol determination using the “Chol MPR 2” kit supplied by Roche Chemicals (New Zealand), assuming an LDL molecular weight of 2.5 MDa and a cholesterol content of 31.6%. The α-tocopherol concentration in LDL was increased by incubating 4 ml of plasma with 40 μl of 100 mM α-tocopherol in ethanol under argon gas for 4 h at 37°C with gentle shaking. The LDL was then purified as described above.

LDL (100 nM) in PBS was oxidised in a glass flask in the dark at 37°C in a Bioliner shaker (Edwards Instruments Co. Australia), which gently swirled the flask at 80 rpm.

Protein hydroperoxides were isolated by acid precipitation and solvent lipid extraction, before analysis using a modified FOX assay. The assay was performed in triplicate, by adding 140 μl of 72% w/v trichloroacetic acid (TCA) to 1 ml samples of LDL (100 nM) and incubating on ice for 10 min. The protein was pelleted by a 5 min centrifugation at 23,000g, 4°C. The pellet was washed by vortexing in 1 ml of 1:1 methanol/chloroform before centrifugation. The resulting pellet was allowed to dry in air at room temperature for 5 min, before being suspended in 900 μl of ice cold 50% v/v glacial acetic acid. Fifty microlitre each of 5 mM ferrous ammonium sulphate and xylenol orange in 25 mM H2SO4 was added to the protein suspension. The mixture was incubated at room temperature for 30 min, during which time ferrous ions were oxidised to ferric ions by the hydroperoxides. Ferric ions are detected by the formation of a coloured complex with xylenol orange, which is measured by absorbance at 560 nm against a water blank. An extinction coefficient of 48,000 M cm was
used to calculate the concentration of protein hydroperoxide.\[33\]

For cell-mediated oxidation experiments, 3 ml of the LDL-containing media was centrifuged at 400 g to eliminate any cellular material. The supernatant was then incubated with 420 μl of 72% w/v TCA on ice for 10 min before centrifugation at 4100 g for 15 min at 4°C in thick walled glass centrifuge tubes. The pellet was washed with chloroform/methanol and assayed for protein hydroperoxides as described above.

Lipid oxidation was monitored by removing aliquots of the reaction mixture and measuring the formation of conjugated dienes by the increase in absorbance at 234 nm.\[35\] As the breakdown product of AAPH absorbs at 234 nm, the absorbance of an incubated AAPH solution was subtracted from the LDL oxidation mixture.

PB-DOPA and dityrosine were measured by acid hydrolysis and HPLC analysis, as previously described.\[31,36\] The samples were prepared in triplicate by placing 900 μl of LDL solution into glass “Durham” tubes, 50 × 7.5 mm (Samco, USA), containing 10 μl of 20 mg/ml butylated hydroxytoluene (BHT) in methanol, 10 μl of 100 mg/ml EDTA and 140 μl of 72% TCA. Samples were incubated on ice for 10 min before centrifugation at 10,000 g for 15 min at 4°C. The supernatant was removed and the pellet washed by vortexing with 500 μl of 20°C acetone, then 500 μl of diethyl ether, with centrifugation between each wash to pellet the protein. After drying in air, the sample tubes were placed in a “picotag hydrolysis vial” (Waters, USA) and subjected to gas phase acid hydrolysis over 16 h at 110°C.\[11\] Concentration of DOPA and dityrosine in the hydrolysate was determined by reverse phase HPLC, with fluorescence detection as previously described.\[36,37\] The dityrosine standard was prepared by oxidation of tyrosine with horseradish peroxidase and purification on DEAE-sephacel (Sigma).\[38\] The concentration of the purified dityrosine was determined by measuring the absorbance at 315 nm, using an extinction coefficient of $E_{315} = 5080$ M$^{-1}$ cm$^{-1}$.\[39\]

Suspension cultures of THP-1 cells were maintained in RPMI 1640 media, supplemented with 5% heat inactivated fetal calf serum, 100 U/ml penicillin and 100 μg/ml streptomycin, in a humidified incubator with 5% CO$_2$ at 37°C. The cells were transformed to adherent macrophage-like THP-1 cells, by treatment for 7 days with 100 ng/ml phorbol 12-myristate 13-acetate (PMA), to give a cell density of $1 \times 10^5$ cells/ml in a standard 6 well plate.\[40\]

Oxidation experiments were conducted in triplicate, using Hams F10 media, supplemented with 3 μM FeSO$_4$$_2$ after first washing the cells three times in warm PBS. Filter sterilized LDL was added to the media to give a final concentration of 40 nM before incubation for various times at 37°C in 5% CO$_2$.

The results shown are from single experiments, representative of a minimum of three separate experiments. The data points shown in the figures are the means and standard errors of triplicate analysis taken from a single flask.

RESULTS

The oxidation of 100 nM LDL by 5 μM copper ions at 37°C over 2 h resulted in the formation of $311 \pm 20$ moles of total hydroperoxides/mole of LDL using the standard protein FOX assay with acid precipitation\[33\] (data not shown). Removing lipid from the sample by acid precipitation and solvent washing, as described in the methods, reduced the FOX-measured hydroperoxides to 52 protein hydroperoxides per LDL particle. This equates to 16% of the combined protein and lipid hydroperoxide value. Unoxidised LDL gave a background reading of 0.01 absorbance units, which corresponds to a reading of 2 mole per mole LDL. Analysis of delipidated and native unoxidised LDL showed the delipidation procedure did not generate compounds which reacted in the FOX assay. Treatment of the oxidised LDL with sodium borohydride removed all of the FOX-assayed reactivity generated by the copper oxidation. The susceptibility to sodium borohydride, and the effect of acid and solvent washing, confirm the presence and formation of significantly large levels of protein hydroperoxides during LDL oxidation.\[21,41\] The change from sulphuric acid\[33\] to 50% glacial acetic acid to partially solubilise the precipitated apoB100 protein is a critical modification of the FOX assay, which enabled LDL protein hydroperoxides to be measured. It was also found to be important to keep the glacial acetic acid on ice before conducting the FOX assay at room temperature.

Kinetic analysis of the oxidation showed that protein hydroperoxide formation closely paralleled the formation of conjugated dienes with both copper and AAPH-mediated oxidation (Figs. 1 and 2). No protein or lipid oxidation was observed in the oxidant-free controls over the same time period. Like the conjugated dienes formed during lipid peroxidation, the protein hydroperoxide kinetics show a lag period followed by a rapid formation phase. In lipid peroxidation this rapid oxidation phase is usually termed the propagation phase as it is during this part of the reaction that a lipid radical chain reaction forms.\[33\] The maximum protein hydroperoxide formation rate with copper ions was calculated at 1 nM/s while the lipid conjugated diene formation was calculated at 4.3 nM/s. Following the copper-mediated propagation phase, both protein and lipid oxidation reached a plateau (Fig. 1A). With AAPH oxidation, the maximum protein hydroperoxide and
diene formation rates were 0.3 and 2 nM/s, respectively (Fig. 1B).

To determine the possible linkage between the oxidation of LDL's protein and lipid components, the effect of a lipid soluble and a water soluble antioxidant on the oxidation process was investigated. Increasing the \(\alpha\)-tocopherol content of LDL, from 8.5 to 54 \(\alpha\)-tocopherols per LDL particle, doubled the length of the lag phase during copper-mediated LDL oxidation (Fig. 2A). The additional \(\alpha\)-tocopherol had no significant effect on the propagation rates of lipid and protein oxidation and did not disrupt the linkage observed between protein hydroperoxide and diene formation. During AAPH-mediated oxidation, the increase in \(\alpha\)-tocopherol caused a dramatic reduction in the concentration of both dienes and protein hydroperoxides. This inhibition was particularly apparent for the protein hydroperoxides from the 30 min time point onwards (Fig. 2B).

Like \(\alpha\)-tocopherol, the presence of the water soluble antioxidant 7,8-dihydrosiphenopterin lengthened the lag phase but had little impact on the propagation phase kinetics of dienes and protein hydroperoxides during copper-mediated oxidation (Fig. 3A). In the particular example shown, diene values did peak much earlier than the hydroperoxides during oxidation of the native LDL but this was not consistently observed. During AAPH oxidation, the presence of 7,8-dihydrosiphenopterin caused a lengthening of the lag phase (Fig. 3B). At first glance, lipid peroxidation appears the most affected by the 7,8-dihydrosiphenopterin in the propagation phase but close inspection of the data shows that the presence of 7,8-dihydrosiphenopterin virtually blocked the formation of dienes during the lag phase. In the absence of 7,8-dihydrosiphenopterin, 40 dienes per...
LDL particle had formed at the end of the lag phase (60 min time point) compared to only 12 dienes per LDL particle at the end of the lag phase in the presence of 7,8-dihydroneopterin. Protein hydroperoxide formation was also reduced by 7,8-dihydroneopterin during this lag period.

PB-DOPA and dityrosine formation were also observed during LDL oxidation (Table I). Only PB-DOPA was observed after a 3 h LDL oxidation with copper alone, while the addition of hydrogen peroxide resulted in both the detection of dityrosine and a doubling of PB-DOPA. Hydrogen peroxide alone was unable to cause the formation of either tyrosine oxidation product.

The kinetics of PB-DOPA formation during copper oxidation were significantly different to the rate of diene formation (Fig. 4A). The PB-DOPA continued to increase well after the peak in diene formation. In contrast, PB-DOPA formation appeared tightly coupled to the lipid diene formation during AAPH-mediated oxidation (Fig. 4B). AAPH-mediated oxidation of LDL (100 nM) was incubated in PBS in the presence and absence of 5 μM copper chloride or 5 mM hydrogen peroxide at 37°C for 3 h. The zero time point was subtracted from the value at 3 h of each treatment as there is a small but significant background level of both oxidation products in LDL purified from human plasma. The data shown is the mean ± standard error of three replicates.

**TABLE I** Fenton formation of PB-DOPA and PB-Dityrosine on ApoB100

<table>
<thead>
<tr>
<th>Treatment</th>
<th>DOPA mmoles/mole LDL</th>
<th>Dityrosine mmoles/mole LDL</th>
</tr>
</thead>
<tbody>
<tr>
<td>LDL</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>LDL + H₂O₂</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>LDL + Cu²⁺</td>
<td>157 ± 20</td>
<td>0</td>
</tr>
<tr>
<td>LDL + Cu²⁺ + H₂O₂</td>
<td>348 ± 1</td>
<td>70 ± 0.1</td>
</tr>
</tbody>
</table>

LDL (100 nM) was incubated in PBS in the presence and absence of 5 μM copper chloride or 5 mM hydrogen peroxide at 37°C for 3 h. The zero time point was subtracted from the value at 3 h of each treatment as there is a small but significant background level of both oxidation products in LDL purified from human plasma. The data shown is the mean ± standard error of three replicates.
DOPA formation on LDL was unexpected because hydroxyl but not peroxyl radicals were believed to generate PB-DOPA. A Fenton-type mechanism, using hydrogen peroxide generated from AAPH, was unlikely because the addition of catalase failed to inhibit PB-DOPA formation. Unlike PB-DOPA, no dityrosine was generated during either the copper or AAPH-mediated LDL oxidation. Control experiments confirmed that, in the absence of copper or AAPH, no significant PB-DOPA or dienes were detectable (data not shown).

In comparison to the protein hydroperoxide, PB-DOPA and dityrosine were relatively minor reaction products. Only one DOPA was detected per 380 protein hydroperoxides (comparing data in Fig. 1 with Table I) and we therefore did not examine the reaction kinetics of tyrosine oxidation further.

Incubation of LDL with macrophage-like THP-1 cells resulted in the oxidation of both protein and lipid (Fig. 5). Protein hydroperoxides and dienes peaked at 6 h, at concentrations of 27 moles per mole of LDL and 225 moles per mole of LDL, respectively. A second rise of 234 nm absorbing material was observed at 24 h, though this is most likely due to advanced lipid oxidation products which also absorb at 234 nm. Protein hydroperoxide and diene formation both occurred at slower rates in the absence of cells due to auto-oxidation in the presence of iron and air.

DISCUSSION

It has long been recognised that damage to the apoB100 moiety of LDL is an important consequence of LDL oxidation. In addition to the more classical types of damage to LDL, we have now clearly shown that the formation of protein hydroperoxides on the LDL apoB100 protein is a significant form of LDL modification. The maximum amount of protein hydroperoxide recorded during copper oxidation was 79 protein hydroperoxides per LDL particle, compared with 309 conjugated dienes per particle (Fig. 2). This protein hydroperoxide concentration is six times greater than the reported level of protein carbonyls formed during copper-mediated LDL oxidation.

Modifying the sample preparation procedure to include protein precipitation and solvent lipid extraction enabled the FOX assay to specifically measure protein hydroperoxides. Degradation of the FOX-measured protein hydroperoxides by reduction with sodium borohydride confirmed early work, which detected protein hydroperoxide in oxidised LDL using an iodometric assay.

Protein hydroperoxide and conjugated diene formation paralleled each other during both copper and AAPH-mediated LDL oxidation (Fig. 1). Copper mediated LDL oxidation appears to involve a Fenton-like process occurring at specific copper binding sites on the surface of the LDL particle, possibly on the apoB100. In contrast, AAPH is a water soluble peroxyl radical generator, capable of forming protein hydroperoxides in pure protein solutions and oxidising LDL via the formation of lipid peroxyl radicals. Despite the differences in these two radical generating systems, a high level of similarity was observed in the kinetics of protein hydroperoxide and diene formation. This suggests that the oxidation of both moieties was initiated by a common radical intermediate.

The inability of α-tocopherol and 7,8-NP to uncouple the oxidation of the lipid and protein components of LDL during copper and AAPH-mediated oxidation suggests that radical intermediates of the lipid peroxidation are responsible for protein hydroperoxide formation in LDL (Fig. 2). α-Tocopherol’s location within the lipid component of LDL supports a lipid origin for the radicals responsible for initiating protein hydroperoxide formation. 7,8-dihydroneopterin and α-tocopherol inhibit LDL oxidation by competing for the lipid peroxyl radical during both copper and AAPH-mediated LDL oxidation. Lipid alkoxyl radicals are unlikely to be involved in protein hydroperoxide formation as these radicals predominate in the latter stages of lipid oxidation, after protein hydroperoxide formation has peaked. The most likely lipid radical responsible for protein hydroperoxide formation on LDL is therefore the chain-propagating lipid peroxyl radical. The finding that α-tocopherol can prevent protein hydroperoxide formation on apoB100 (Fig. 2), potentially broadens its role to protection of the entire LDL particle from oxidative insult.
PROTEIN HYDROPEROXIDE FORMATION ON apoB100

The lipid soluble antioxidant probucol has also been shown to protect apoB100 by inhibiting lipid peroxidation, carbonyl formation and protein fragmentation.\[^{17,46}\] It is possible that a significant part of this apoB100 damage results from the breakdown of protein hydroperoxides. Degradation of hydroperoxide on the \(\alpha\)-carbon of model peptides has been shown to cause fragmentation,\[^{47}\] while the decomposition of hydroperoxide on the amino acid residue side chain generates carbonyl groups.\[^{48}\] The amino acids lysine, histidine and proline have been identified as highly labile in apoB100 during copper-mediated LDL oxidation.\[^{5}\] This loss has previously been attributed to protein fragmentation or carbonyl formation. These three amino acids are now known to readily form amino acid hydroperoxides during exposure to hydroxyl radicals.\[^{43}\] This suggests that some of the previously described amino acid loss during LDL oxidation is probably due to protein hydroperoxide formation. It is interesting to note that \(\alpha\)-tocopherol has also been shown to inhibit protein fragmentation of apoB100 during copper oxidation.\[^{46}\]

It is generally assumed that, because AAPH is water soluble, AAPH-derived peroxyl radicals will form in the aqueous phase of a reaction mixture. As a result, LDL protein hydroperoxides would be expected to form via direct attack by AAPH peroxyl radicals in the aqueous phase, as is observed with albumin.\[^{29}\] The lipid soluble \(\alpha\)-tocopherol should have had little or no affect on the LDL protein hydroperoxide formation during our experiments. Though water soluble, 95% of AAPH can partition in the lipid phase of SDS-stabilized linoleic acid micelles.\[^{49}\] This suggests the majority of the AAPH peroxyl radicals in our experiments are forming in the lipid fraction of the LDL particle, not the aqueous phase. If the AAPH derived radicals were forming in the aqueous phase, they would be able to directly attack the ApoB 100 to form protein hydroperoxide, with out \(\alpha\)-tocopherol having any affect. This model of AAPH-mediated oxidation is further supported by the finding that the length of the LDL lag phase is directly proportional to the concentration of \(\alpha\)-tocopherol, but only during AAPH-mediated oxidation.\[^{50}\]

Macrophage cells are considered to play a key part in the modification of LDL within the arterial wall. The novel finding that cell-mediated LDL oxidation also generates significant amounts of protein hydroperoxides suggests that protein hydroperoxides could form in atherosclerotic plaques (Fig. 5). Analysis of atherosclerotic plaque has shown the presence of hydroxylcine and hydroxyvaline, known breakdown products of protein hydroperoxides.\[^{26}\] Considering the known reactivity of protein hydroperoxides with thiols and ascorbate,\[^{10}\] it is possible that they play a role in plaque formation.

In comparison to protein hydroperoxide formation, oxidative modification of tyrosine does not appear to constitute a major reaction during the oxidation of LDL. The concentration observed during copper-mediated oxidation after 8 h was only one PB-DOPA per five LDL particles (Fig. 4A). PB-DOPA concentrations have been reported as high as eight DOPA per LDL particle, but this was only after 8 days of oxidation.\[^{14}\] Measurement of amino acid loss during copper-mediated LDL oxidation also confirmed tyrosine to be a minor reaction target during copper-mediated LDL oxidation.\[^{5}\]

The appearance of PB-DOPA during AAPH-mediated LDL oxidation was unexpected (Fig. 4B), as previous studies had shown that only hydroxyl radicals were capable of initiating DOPA formation from tyrosine.\[^{19}\] The strong coupling observed between the diene and DOPA formation strongly suggests that the lipid peroxy radical is involved in the mechanism. Spin trap studies have shown peroxy and alkoxyl radicals can undergo a range of fragmentation reactions releasing superoxide radicals, which can dismutate to hydrogen peroxide.\[^{51}\] The lack of effect by catalase suggests that either there is another mechanism leading to DOPA formation or the reaction site where the hydrogen peroxide is reacting to form hydroxyl radicals is not accessible to the catalase enzyme.

In contrast, during copper-mediated LDL oxidation, the PB-DOPA formation appeared to be relatively independent of the diene formation (Fig. 4A). A direct copper/tyrosine interaction rather than a lipid radical intermediate appears to be involved, though this has not been observed in our studies with albumin and copper.\[^{42}\]

Significant dityrosine formation was only observed under the most oxidative of conditions, when hydrogen peroxide was added to the copper/LDL reaction mixture. After 3 h of incubation only one dityrosine per 14 LDL particles was detected (Table I). This may reflect the requirement of two tyrosyl radicals to occur in close proximity to each other to form dityrosine.\[^{52}\] Studies on lens proteins have also found that hydrogen peroxide has to be present with the copper ions for dityrosine to be generated by the Fenton reaction.\[^{53}\]

This study clearly demonstrates that protein hydroperoxides are a significant product of LDL oxidation under a variety of oxidation systems, including cell-mediated oxidation. Others have clearly shown that protein hydroperoxides are found in atherosclerotic plaques\[^{56}\] and will react to consume thiols and ascorbate.\[^{12}\] This raises the possibility that protein hydroperoxides may
contribute to some of the pathological behaviour of oxidised LDL in vivo.

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References


