Measurements of Human Plasma Oxidation

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2. **Materials and Methods**

3. Results

FIGURES

α – TOH	α-tocopherol
α-$TO^{\bullet}$	α-tocopheroxyl radical
μM	micro mole per cubic meter
AAPH	2,2-azobis (2-amidinopropane) dihydrochoride
BHT	butylated hydroxytoluene
BSA	bovine serum albumin
CL	cholesterol
CL-OOH	cholesterol hydroperoxide
Cu^{+}	cuprous ion
Cu^{2+}	cupric ion
$CuCl_2$	copper chloride
$CuSO_4$	copper sulphate
DOPA	3,4-dihydroxyphenylalanine
e	electron
e^{-}_{aq}	aqueous electron
DHA	dehydroascorbate
ED	ethylenediamine
EDTA	ethylenediamine-tetraacetic acid disodium salt
Fe^{2+}	ferrous ion
Fe^{3+}	ferric ion
Fe- XO	Fe^{3+} xylenol orange complex
FOX	ferric-xylenol orange
$FeSO_4$	ferrous sulfate
GSH	glutathione
H^{+}	proton

H_2	hydrogen
HCl	hydrochloric acid
H_2O	water
H_2O_2	hydrogen peroxide
HOCl	hypochlorous acid
HPLC	high performance liquid chromatography
H_2SO_4	sulfuric acid
$L^{\bullet}$	fatty acid radical
LDL	low-density lipoprotein
$LOO^{\bullet}$	fatty acid peroxyl radical
LOOH	lipid hydroperoxide
M	moles per cubic meter
MeOH	methanol
mg	milli gram
ml	milli litre
mM	milli mole per cubic meter
N_2	nitrogen gas
$NaBH_4$	sodium borohydride
NaCl	sodium chloride
Na_2EDTA	sodium ethylenediaminetetraacetic acid
NaH_2PO_4	sodium dihydrogen orthophosphate
NaOH	sodium hydroxide
O_2	oxygen
$O_2^{\bullet -}$	superoxide radical
$OH^{\bullet}$	hydroxyl radical

OH^-	hydroxide ion
PB-DOPA	protein-bound L-3,4-dihydroxyphenylalanine
PBS	phosphate buffered saline
$Pr^{\bullet -}$	protein radical
PrH	protein
$PrO^{\bullet -}$	protein alkoxyl radical
PrOH	protein hydroxide
$PrOO^{\bullet -}$	protein peroxyl radical
PrOOH	protein hydroperoxide
PrOOHs	protein hydroperoxides
PUFA	polyunsaturated fatty acid
PUFA's	polyunsaturated fatty acid's
$R^{\bullet}$	radical
$ROO^{\cdot}$	peroxyl radical
ROS	reactive oxygen species
Rpm	revolutions per minute
SOD	superoxide dismutase
TCA	tricloroacetic acid
TFA	trifluoroacetic acid
XO	xylenol orange

Abstract

The oxidation of lipids and antioxidants has been extensively studied in human plasma but little attention has been given to how plasma proteins are oxidised. Proteins make up the majority of biomolecules in cells and plasma and therefore are the most likely reactants with oxidants and free radicals. Previous studies in the laboratory had shown that peroxyl radicals generated by the thermolytic decay of 2-azobis (2-amdinopropane) dihydrochloride (AAPH) generated significant amounts of protein hydroperoxides, but only after a six hour lag period. In this study the existence of the six hour lag period was confirmed and shown by dialysis of the plasma to be due to the presence of low molecular weight antioxidants. The addition of both uric acid and ascorbic acid to the dialysed plasma restored the lag phase suggesting that *in vivo* these antioxidants act to prevent protein hydroperoxide formation. Lipid oxidation was also observed in the plasma but only after a two hour lag phase. This was the first time lipid oxidation has been observed in the absence of protein oxidation. The lipid lag phase was also abolished by dialysis of the plasma and restored by the addition of ascorbic acid and uric acid. The kinetics of tocopherol loss suggests that the tocopherol radicals act to inhibit lipid oxidation by transferring the electrons to the water-soluble ascorbate. The loss of ascorbate appears to cause the formation of a tocopherol radical mediate the lipid peroxidation process. Overall the data shows ascorbic acid scavenging the peroxyl radicals while uric acid acts to reduce the overall AAPH generated radical flux.

In a separate investigation, the production of protein-bound DOPA (PB-DOPA) on albumin during X-ray radiolysis and copper mediate Fenton oxidation was investigated using a fluorescence based derivatisation method (ED-DOPA), which was compared with the more specific acid hydrolysis and HPLC analysis method. The ED-DOPA method consistently gave a much higher reading that the HPLC based methods, suggesting that the ED-DOPA method was measuring DOPA plus DOPA oxidation products. This was confirmed by oxidising X-ray radiolysis generated PB-DOPA with Cu^{++} to cause DOPA oxidation. The Cu^{++} treatment drastically increased the level of signal given by the ED-DOPA assay while HPLC analysis showed all the DOPA had been oxidised.

INTRODUCTION

1.1 Overview

When there is an over production of free radicals, the balance of oxidants and reductants is disrupted. This causes the level of free radicals to exceed the capacity of the antioxidants, which leads to oxidative stress. Once formed, free radicals can induce the oxidative damage to a range of biomolecules, with the most important targets being lipids, proteins and DNA. It has only recently been determined that proteins are most likely the primary target molecule of free radicals, with the reactive protein peroxyl radicals and hydroperoxide formation resulting from this reaction. These products are able to cause damage to other proteins, cross-link DNA, consume antioxidants, and damage lipids (Gebicki *et al*., 2000a; Gebicki, 1997). Oxidative stress is thought to be the initiating factor in the pathologies of various diseases including cancer, atherosclerosis and diabetes (Granot and Cohen, 2004; Karten, 2000; Du and Gebicki, 2004; Yeum *et al*., 2003; Salvi *et al*., 2001; Ames *et al*., 1981; Suh *et al*., 2003; Gebicki *et al*., 2000a).

Previous studies investigating the kinetics of oxidation have focussed on protein oxidation generated by irradiation-induced hydroxyl radicals ($OH^{\bullet}$) and superoxide radicals ($O_2^{\bullet -}$), and lipid peroxidation using isolated LDL. Research in this laboratory by Ling, (2004) and Yang, (2005) has shown protein oxidation by water soluble peroxyl radicals in human blood plasma begins with a six hour lag phase where very few protein hydroperoxides (PrOOHs) form, followed by a rapid increase in formation. The six hour lag phase is not present when dialysed plasma is exposed to peroxyl radicals, suggesting one or a number of small molecular weight antioxidants are involved in this protection.

This study will examine the role of plasma antioxidants in controlling both lipid and protein oxidation in undiluted plasma exposed to peroxyl radicals, along with the activity of α-tocopherol under these same conditions. Antioxidants such as uric acid, ascorbic acid, and suggested sacrificial antioxidants like amino acids, will be investigated for their role in the protection of plasma proteins and lipids exposed to peroxyl radicals. This study will also measure the formation of 3,4-

dihydroxyphenylalanine (DOPA) on pure proteins exposed to radiation and Fenton radicals. It will also investigate the effectiveness of the specific fluorescence assay in detecting PB-DOPA compared to the old but more reliable HPLC method.

1.2 Free radicals

A 'free radical' is described as a molecular chemical species containing one or more unpaired electrons. These unpaired electrons make the molecule very unstable, therefore short lived and extremely reactive, with a tendency to extract an electron from a neighbouring molecule in order to attain a lower energy level and gain stability (Cheeseman *et al*., 1993; Morgan *et al*., 2004).

The most significant free radicals in biological systems are derived from oxygen, assigning oxygen and its derivatives (superoxide and hydroxyl radical) as the most important reactants in the biological system. Oxygen is the most abundant molecule in the biological system and exists as a di-radical that can react rapidly with other radicals (Karten, 2000). With free radicals oxygen reacts rapidly, but the reaction with non-radical species is slow (Cheeseman *et al.,* 1993; Davies and Delsignore, 1987). When oxygen reacts with free radicals, it is reduced by the addition of a single electron producing a superoxide free radical anion ($O_2^{-\bullet}$), which is the radical that is generated under the widest range of conditions (Equation 1).

(1) $O_2 + e \rightarrow O_2^{-\bullet}$

$O_2^{-\bullet}$ is relatively stable but can dismutate to produce hydrogen peroxide (H_2O_2) and molecular oxygen, either spontaneously or by the catalysis of superoxide dismutase (SOD) (reaction 2). In most healthy situations, this superoxide anion undergoes dismutation by SOD to produce hydrogen peroxide, which is broken down to produce water. However, the hydrogen peroxide can be reduced to form highly reactive and potent hydroxyl radicals ($OH^{\bullet}$) via the metal catalysed Fenton reaction (reaction 3) (Byrne *et al*., 2003).

(2) $O_2^{-\bullet} + 2H^+ \rightarrow H_2O_2 + O_2$

(3) $Fe^{2+}/ Cu^{+} + H_2O_2 \rightarrow Fe^{3+}/ Cu^{2+} + OH^{\bullet} + OH^{-}$

The hydroxyl radical has a short half-life of approximately 10^{-9} s and is only able to react with molecules within a short distance of 6 nm from the site of production (Gebicki, 1997; Dean *et al*., 1997; Du and Gebicki, 2004). Despite this short lifetime and limited reaction area, $OH^{\bullet}$ is capable of oxidizing nearly all cell constituents causing substantial damage (Gebicki, 1997; Cheeseman *et al*., 1993; Morgan *et al*., 2004; Chang *et al*., 2000; Blakeman *et al*., 1998; Nordberg *et al*., 2001; Granot and Cohen, 2004).

1.2.1 Free radicals *in vivo*

The human body is continuously exposed to radicals generated in biological systems as a result of normal cellular processes (enzymes, mitochondrial respiration and neutrophils) and also as a result of exposure to external agents (air pollutants, natural gasses, irradiation, chemicals and toxins) (Granot and Cohen, 2004; Frei *et al*., 1989; Luxford *et al.,* 1999). These oxidants all produce reactive oxygen species (ROS) that are formed and degraded by all aerobic organisms on a regular basis to generate the required concentrations for important processes like phagocytosis. In unfavourable situations, over production can produce excessive quantities of ROS as by products of metabolism (Nordberg *et al*., 2001; Frei *et al*., 1989; Rodgers *et al*., 2004; Cheeseman *et al*., 1993; Davies *et al*., 1995; Yeum *et al*., 2003). The overproduction of free radicals disrupts the balance of oxidants and reductants causing the ROS to exceed the capacity of antioxidants. This increases the risk of biomolecules, including proteins, lipids and DNA, being attacked and leading to oxidative stress (Yeum *et al*., 2003; Du *et al*., 2004; Granot *et al*., 2004; Karten, 2000). This reaction may cause structural modifications and damage to the cellular membranes and the cellular organization of the molecule (Nordberg *et al*., 2001; Davies *et al*., 1995; Ames *et al*., 1981). In-depth studies have identified that oxidative stress caused by increased free radical formation may be responsible for the initiation and development of a number of pathological and debilitating conditions, including atherosclerosis, diabetes, Wilson's disease, cancer, aging and hemochromatosis (Du and Gebicki, 2004; Yeum *et al*., 2003; Salvi *et al*., 2001; Ames *et al*., 1981; Suh *et al*., 2003, Gebicki *et al*., 2000a).

1.3 Radical generation

Investigating free radical-mediated reactions of physiological relevance requires a source of free radicals that is similar to those occurring in the body. 2,2-azobis (2-amidinopropane) dihydrochloride (AAPH), X-ray radiolysis and the Fenton reaction are three well-known and popular radical generators.

1.3.1 AAPH

AAPH is an azo compound, which decomposes unimolecularly without enzymes or biotransformation to yield nitrogen and two carbon centred radicals (reaction 15). The carbon radicals are formed in pairs in close proximity, with some joining to form a stable product. However, most diffuse and react with oxygen molecules forming peroxyl radicals in the aqueous region (reaction 16). When incubated at 37˚C the half-life is around 175 hours, so for the first few hours the rate of radical generation is relatively constant at 3.19×10^{-7} Ms^{-1} (Niki, 1990). This constant rate makes AAPH a useful tool in studying the damage caused by peroxyl radicals.

AAPH has been used to generate oxidation products on both proteins and lipids, with protein hydroperoxides observed on pure proteins (Gebicki and Gebicki, 1993; Platt and Gieseg, 2003), plasma (Ling, 2004; Yang, 2005), U937 cells (Gieseg *et al*., 2000) and low density lipoprotein (LDL) exposed to peroxyl radicals generated by AAPH (Gieseg *et al*., 2003).

(15) $\text{R-C-N=N-C-R} \rightarrow \text{R-C}^{\bullet} + N_2 + {}^{\bullet}\text{C-R}$

(16) $\text{R-C}^{\bullet} + O_2 \rightarrow \text{R-COO}^{\bullet}$

1.3.2 X-ray radiolysis

X-ray radiolysis is another means of radical production used in the investigation of protein oxidation. Many researchers have irradiated proteins in an attempt to study the products formed on proteins under such conditions. X-ray radiolysis generates high numbers of free radicals by exciting water molecules (Halliwell and Gutteridge, 1990). This excitation causes the water molecules to split into a number of primary

species which, under aerobic conditions, go on to react with oxygen producing further radicals (reaction 17).

(17) $H_2O \rightarrow {}^{\bullet}OH + e^-_{aq} + H^{\bullet}$

$\downarrow \quad \downarrow \quad \downarrow$

$H_2O_2 \quad O_2^{\bullet -} \quad HO_2^{\bullet -}$

$\downarrow \quad \downarrow$

$H_2O_2 \quad H^+ + O_2^{\bullet -}$

X-ray radiation of pure proteins in the presence of constant oxygenation is capable of oxidizing tyrosine residues to DOPA residues (Cudina and Josimovic, 1987; Parkes, 2005). X-ray radiation also produces PrOOHs on proteins exposed to irradiation.

1.3.3 Fenton reaction

Oxidative damage by free radicals in biological systems is often linked to the Fenton reaction. The Fenton reaction is the one electron reduction of hydrogen peroxide (H_2O_2) by the transition metal ions, iron and copper (reaction 3). Both iron and copper have been used as the transition metals when investigating the Fenton reaction however, it has been thought that the concentration of copper *in vivo* is too low for significant involvement. It can also be rendered inert by its ligation to thiols, often making iron the more favourable transition metal. (Kehrer, 2000; Koppenol, 2001). The Fenton reaction is another way of producing free radicals in experimental settings but not at a known rate.

1.4 Protein oxidation

Early investigations identified the main free radical targets as lipids and DNA. Proteins were simply described as antioxidants, protecting against oxidative stress by scavenging ROS and binding metal ions to inhibit the formation of $HO^{\bullet}$ from H_2O_2 (Gebicki *et al*., 2000a). The major reaction occurring from free radical attack is now thought to be between proteins and free radicals. This is due to their abundance in the cell, their ability to bind transition metal ions, and their high rate constants for

reactions as they readily react with biologically significant ROS undergoing extensive oxidation (Davies, 2003; Gebicki, 1997; Gebicki *et* al., 2000a &b; Gieseg *et al*., 2000; Morgan *et al*., 2004; Luxford *et al*., 1999; Du *et al*., 2004).

The interaction of free radicals with proteins results in the alteration of the protein structure. This alteration occurs from the modification of amino acid side chains, protein fragmentation and DNA cross linking (Gebicki and Gebicki, 1999), which can lead to a loss of biological function, as well as increased susceptibility to proteolysis and heat denaturation (Rodgers *et al*., 2004). It has been suggested that ROS species first damage a target molecule and, if the molecule is important in the survival of the cell, it undergoes apoptosis or is transformed. The more probable event is that the first molecule attacked is converted to a secondary free radical or reactive intermediate, which goes on to generate more reactive species (Gebicki, 1997). The oxidation of proteins by free radicals and ROS can generate a range of stable and reactive products, with the two major reactive products being protein hydroperoxides and protein bound DOPA (PB-DOPA) (Simpson *et al*., 1992; Dean *et al*., 1997; Fu *et al*., 1998, 1995; Luxford *et al*., 1999). The former is an oxidising spcies while the latter is areducing species. These reactive products have been associated with diseases such as Alzeimer's, diabetes, atherosclerosis and aging (Du and Gebicki, 2004; Yeum *et al*., 2003; Salvi *et al*., 2001; Ames *et al*., 1981; Suh *et al*., 2003, Gebicki *et al*., 2000a).

1.4.1 Protein hydroperoxides

One of the reactive but non-radical moieties produced as a consequence of the reaction between ROS and proteins is hydroperoxides. Hydroperoxide groups form mainly on amino acids that possess secondary or tertiary carbons, giving the amino acid radical sufficient stability to form hydroperoxide groups in the presence of oxygen. Hydroperoxide groups can alternatively form on the α-carbon backbone (Gebicki, 1997; Simpson *et* al., 1992; Gebicki and Gebicki, 1993). PrOOHs are relatively stable and have a long lifetime, enabling them to diffuse large distances within cells and tissues. Although relatively stable, hydroperoxides are still able to cause considerable damage by consuming antioxidants, inactivating enzymes, and causing DNA crosslinking. In the presence of transition metal ions PrOOHs can be decomposed to produce secondary radical intermediates, which go on to produce

more radicals and further damage (Dean *et al*., 1997; Gebicki, 1997; Dean *et al*., 1997; Gieseg *et al*., 2000; Du and Gebicki, 2004; Gieseg *et al*., 2003).

PrOOH formation starts with a free radical ($R^{\bullet}$) extracting a hydrogen atom to produce a carbon-centred radical ($Pr^{\bullet}$) (reaction 4), which in the presence of oxygen, is converted to a protein peroxyl radical ($PrOO^{\bullet}$) (reaction 5, 6). This $PrOO^{\bullet}$ is stabilized to PrOOH under reducing conditions (reaction 7). The $PrOO^{\bullet}$ could alternatively extract a hydrogen atom and electron from the side chain of another protein to form PrOOH and a new carbon-centred radical (reaction 8) that is thought to start the reaction over again (Neuzil *et al*., 1993), although reaction 8 has not been fully demonstrated.

(4) $PrH + R^{\bullet} \rightarrow Pr^{\bullet} + RH$

(5) $Pr^{\bullet} + O_2 \rightarrow PrOO^{\bullet}$

(6) $Pr^{\bullet} + e \rightarrow PrOO^{-}$

(7) $PrOO^{\bullet} + H^{+} \rightarrow PrOOH$

(8) $PrOO^{\bullet} + PrH \rightarrow PrOOH + Pr^{\bullet}$

Protein hydroperoxide formation on proteins can be measured by the Ferric-Xylenol Orange (FOX) assay. The original assay was developed by S.P Wolff for lipid hydroperoxides (Wolff, 1994) and later modified by Gebicki for the measurement of protein hydroperoxides on proteins. This modification was achieved by the removal of AAPH and the isolation of protein by precipitation with acid before measuring the absorbance of PrOOHs (Gay *et* al., 1999a, b and c). The adapted FOX assay measures the amount of PrOOHs formed on proteins based on the ability of PrOOHs to oxidize ferrous ions (Fe^{2+}) to ferric ions (Fe^{3+}) (reaction 9). The resulting ferric ion reacts with xylenol orange, forming a coloured complex (reaction 10) with an absorbance that can be measured at 560 nm on a spectrophotometer (Gay *et al*., 1999 a and b).

(9) $PrOOH + Fe^{2+} \rightarrow PrO^{\bullet} + OH^{-} + Fe^{3+}$

(10) $Fe^{3+} + XO \rightarrow Fe - XO_{complex}$

1.5 Plasma oxidation

The kinetics of protein and lipid oxidation has in the past, been investigated on pure proteins and isolated LDL. This has shown that protein hydroperoxides are produced on pure proteins while both protein and lipid hydroperoxides form on isolated LDL (Esterbauer *et al*., 1989). Gieseg *et al*., (2003) identified PrOOHs on LDL exposed to copper, and THP-1 macrophages exposed to AAPH. Pure protein oxidation was investigated using BSA solutions exposed to X-ray radiolysis and demonstrated that ROS formed from radiation could generate PrOOHs in a linear fashion, but only in the presence of oxygen (Simpson *et al*., 1992; Neuzil *et al*., 1993; Platt and Gieseg, 2003). While investigating the formation of PrOOHs in human blood serum exposed to hydroxyl radicals, Gebicki *et al*., 2000a showed that PrOOHs could be detected in human plasma proteins with no detectable lag phase. Furthermore ascorbate did not protect against $OH^{\bullet}$ attack in the plasma. It was suggested that the attack of $OH^{\bullet}$ on proteins could result in the formation of reactive protein peroxyl radicals and hydroperoxides that are able to damage other proteins, DNA, antioxidants, and lipids. Also, proteins may constitute an early and vital step in the transmission of ROS-mediated biological damage induced by acquiring chemically reactive moieties, which can act as new sources of damage to all components. Ling, (2004) and Yang, (2005) further investigated the formation of PrOOHs in human blood plasma by exposure to peroxyl radicals. In this system, a six hour lag phase was observed where very little PrOOHs were formed, followed by a rapid increase in formation. Dialysed plasma exposed to peroxyl radicals removed this lag phase, indicating that a small molecular weight antioxidant is protecting the plasma proteins during the first six hours of oxidation. Lipid peroxidation in human blood plasma exposed to peroxyl radicals begins with a lag phase where no or very few lipid hydroperoxides formed during the first 50-60 minutes, followed by a linear increase in formation post-lag phase (Thomas *et al*., 1997; Neuzil *et al*., 1993; Frei *et al*., 1988; Esterbauer *et al*., 1989). The lag phase coincides with ascorbic acid depletion; once ascorbic acid is consumed lipid oxidation occurs. The fact that the AAPH-induced kinetics of both lipid and

protein oxidation begin with lag phases suggests the processes are connected, with an antioxidant causing the lag phases in both processes.

1.6 Lipid oxidation

Lipid oxidation is a process occurring when the double bond in polyunsaturated fatty acids (PUFAs) is attacked by free radicals. The reaction proceeds by the removal of a hydrogen atom from the PUFA (LH) by a radical ($R^{\bullet}$), generating a fatty acid radical ($L^{\bullet}$) (reaction 11). The resulting fatty acid radical can readily, and rapidly, react with oxygen to form a fatty acid peroxyl radical ($LOO^{\bullet}$) (reaction 12). Adjacent unsaturated fatty acids (L'H) will be attacked by this $LOO^{\bullet}$, promoting further oxidative damage to lipids. Oxidation is, therefore, a self-propagating chain reaction that can lead to the formation of lipid hydroperoxides (LOOHs). The termination of this reaction occurs when fatty acid radicals react with each other, or with endogenous antioxidants, producing non-radical products (reaction 13) (Kehrer, 2000). Further oxidation of the lipid hydroperoxide causes the production of a variety of highly reactive secondary products, mainly short-chained aldehydes (reaction 14). These aldehydes can react with apolipoproteins so that the lipoprotein recognition by receptors for native lipoproteins is hindered. The lipoprotein oxidation can be inhibited by several antioxidants, which scavenge free radicals and form more stable products (Karten *et al*., 1997).

(11) Initiation $LH + R^{\bullet} \longrightarrow L^{\bullet} + RH$

(12) Propagation $L^{\bullet} + O_2 \longrightarrow LOO^{\bullet}$

$LOO^{\bullet} + L'H \longrightarrow LOOH + L'^{\bullet}$

(13) Termination $LOO^{\bullet} + LOO^{\bullet} \longrightarrow$ Non-radical product

(14) $LOOH$ ----------> $LO^{\bullet}$, $LOO^{\bullet}$, aldehydes

Carbon-centred radicals such as the lipid radical ($L^{\bullet}$) can be stabilised by molecular rearrangement to form a conjugated diene. The formation of conjugated dienes is an indicator of lipid oxidation and can be observed by measuring the absorbance at 234

nm (Esterbauer *et al*., 1989; Kritharides *et al*., 1993; Halliwell and Gutteridge, 1999). Lipid oxidation occurs in three distinct steps, starting with a lag phase where little or no lipid oxidation occurs and followed by a propagation phase of increasing lipid oxidation. The third and final phase shows a decrease in lipid oxidation products and is caused by more products being degraded than formed. Lipid oxidation leads to the modification of LDL, causing the modified LDL to be susceptible to increased uptake by the scavenger receptor of macrophage cells (Esterbauer *et al*., 1989).

1.7 The antioxidant defence system

The human body has adapted itself to living with a constant efflux of ROS. The most important mechanism in plasma is the antioxidant defence system, with antioxidants either synthesized in our bodies or consumed through our diet (Granot and Cohen, 2004). However, oxygen-based reactants that escape detoxification by cellular antioxidant systems are responsible for an estimated 10,000 DNA base modifications per cell per day. This clearly illustrates that the mechanisms for protecting the body from harmful oxidants is not perfect (Mayo *et al*., 2003).

An antioxidant is described as 'a substance that when present in low concentrations compared to that of an oxidizable substrate, prevents or delays the oxidation of the substrate' (Halliwell, 1988; Santos *et al*., 1999). The antioxidants synthesized in our bodies include all antioxidant proteins and various small molecules (Frei *et al*., 1989). Due to the existence of a wide variety of reactive oxygen species that produce damage to target molecules at different locations, there are many different antioxidants and they can provide protection in a range of ways. Preventative and chain-breaking antioxidant activities are just two examples. To provide optimal protection from oxidants, antioxidants often work together as co-antioxidants, recycling each other or working in unison. The most important plasma antioxidants are thought to be ascorbate, urate, α-tocopherol, bilirubin and albumin (Frei *et al*., 1988).

1.7.1 α-Tocopherol (Vitamin E)

α-Tocopherol (α-TOH) is the major lipid soluble antioxidant found in LDL present at a quantity of 6 molecules per lipoprotein (Terentis *et al*., 2002; Esterbauer *et al*., 1992). Its phyl side chain is required for incorporation and retainment in membranes

and lipoproteins (Upston *et al*., 2003). α-Tocopherol has predominantly been described as a classical chain breaking antioxidant that protects LDL against lipid peroxidation by directly scavenging free radicals and reacting rapidly with the chain carrying lipid peroxyl radical (LOO$^{\cdot}$) to break propagation (Kontush *et al*., 1996; Niki *et al*., 2005; Thomas and Stocker, 1999; Bowry *et al*., 1992). This occurs by α-tocopherol donating its phenolic hydrogen to LOO$^{\cdot}$, creating a less reactive α–tocopheroxyl radical (α-TO$^{\cdot}$) and LOOH. It can, alternatively, react directly with the initiating radical oxidant to prevent LOO$^{\cdot}$ formation, producing an inactive oxidant and an α-TO$^{\cdot}$ radical (reaction 18). The α-TO$^{\cdot}$ radical that is formed can then be eliminated by a radical-radical reaction with another LOO$^{\cdot}$, generating non-radical products (reaction 19) (Upston and Stocker, 1994; Thomas and Stocker, 2000; Karten *et al*., 1997). As α-TO$^{\cdot}$ is free to diffuse in homogenous solutions and rapidly reacts with other radicals, these reactions lead to both the termination of the chain reaction and the consumption of α-tocopherol. Furthermore, as α-TOH and α-TO$^{\cdot}$ both scavenge radicals, each molecule of α-TOH can terminate two potential chain reactions (Thomas and Stocker, 2000).

(18) Inhibition α-TOH + LOO$^{\cdot}$ → α-TO$^{\cdot}$ + LOOH

α-TOH + Radical oxidant → inactive oxidant + α-TO$^{\cdot}$

(19) Termination α-TO$^{\cdot}$ + LOO$^{\cdot}$ → LOOH + non-radical products

The classical chain breaking antioxidant mechanism is proposed to occur when the radical flux is high. Under high radical flux the concentration of LOO$^{\cdot}$ is sufficiently high for the termination reaction to predominate, resulting in both the prevention of lipid peroxidation and rapid consumption of α-tocopherol (reaction 19). By contrast, a low radical flux reduces the concentration of LOO$^{\cdot}$, causing termination reactions to become infrequent. This switch to a pro-oxidant state is described by the tocopherol-mediated peroxidation (TMP) model (reaction 20).

(20) α-TO$^{\cdot}$ + LH → L$^{\cdot}$ + α-TOH

The TMP model describes α-TOH acting as a phase transfer agent that reacts with aqueous radical oxidants ($R^{\cdot}$). This results in the formation of α-$TO^{\cdot}$ and therefore, the import of radicals from the aqueous into the lipid phase. Additionally, α-$TO^{\cdot}$ becomes trapped within the LDL and cannot undergo the radical-radical termination. Termination can now only occur when a second $R^{\cdot}$ enters the oxidizing particle and reacts with the radical, stopping the reaction. α-$TO^{\cdot}$ can initiate lipid peroxidation by abstracting a hydrogen atom either from the surface or core lipids that contain bisallic hydrogens. The resulting $LOO^{\cdot}$ is scavenged rapidly by α-TOH to produce LOOH and a new α-$TO^{\cdot}$. During AAPH-mediated oxidation, the albumin appears to scavenge so many $ROO^{\cdot}$ that the concentration of $LOO^{\cdot}$ is low and TMP predominates (Upston *et al*., 2003; Esterbauer *et al*., 1992; Upston *et al*., 1999). Although the TMP reaction occurs in the absence of other antioxidants, it can be inhibited by the presence of suitable co-antioxidants that can react with α-$TO^{\cdot}$ (Upston *et al*., 2003; Stocker 1999; Upston *et al*., 1999; Thomas and Stocker, 2000). This enables the regeneration of α-TOH and forms a co-antioxidant derived radical that exports the radical back into the aqueous phase to produce a non-radical product and stopping the reaction. Ascorbate is the most well-known co-antioxidant for α-tocopherol, while urate and β-carotene are not thought to act as co-antioxidants (Neuzil and Stocker, 1994).

1.7.2 Ascorbic acid (Vitamin C)

Ascorbic acid or, as it also known, vitamin C, is a potent water-soluble antioxidant in plasma. It is a strong reducing agent with multiple antioxidant properties, ranging from the scavenging of various types of reactive oxygen species (superoxide and hydroxyl radicals) to the reduction of protein hydroperoxides that are formed. It is also known to act as a co-antioxidant recycling the α-tocopheroxyl radical back to α-tocopherol via donation of an electron (Mendiratta *et al*., 1997; Schafer *et al*., 2003; Retsky *et al*., 1993; Suh *et al*., 2003; Nieto *et al*., 2000; Gebicki *et al*., 2000b). This proceeds by ascorbate undergoing two consecutive reversible one-electron oxidation reactions to form the ascorbate radical, dehydroascorbate (DHA). The unpaired electron of this ascorbate radical is in a highly delocalized system, making the radical unreactive (Mendiratta *et al*., 1997; Schafer *et al*., 2003; Halliwell, 1990; Retsky *et al*., 1993; Stait *et al*., 1994).

The pattern of lipid oxidation has also been linked to the presence of ascorbic acid. Plasma lipids have been found to be oxidised by peroxyl radicals only after a lag phase during which ascorbic acid is depleted. It has been suggested that ascorbic acid reacts with the initial product of protein oxidation product ($PrOO^{\bullet}$) from the hydroxyl radical attack. Once the ascorbic acid has been oxidized, and is no longer present in the plasma, the lipids are able to react with the protein radicals leading to lipid peroxidation (Gebicki *et al*., 2000a).

Although ascorbic acid is an excellent antioxidant, it is also known to act as a pro-oxidant in the presence of transition metal ions due to its reducing properties. The process of ascorbate-mediated metal reduction further generates hydrogen peroxide, which is essential in the oxidation of many single state organic molecules (Retsky *et al*., 1993; Stait *et al*., 1994; Suh *et al*., 2003).

1.7.3 Uric acid

Uric acid is a water-soluble antioxidant present in the plasma as the monoanion urate (Nieto *et al*., 2000). It is found at much higher levels in humans than other primates due to the absence of an enzyme, urate oxidase, in human tissues. In other primates, uric acid is broken down to allantoin and this along with urea is excreted as the major nitrogen containing products of purine degradation (Becker, 1993). The absence of urate oxidase in human tissues has lead to the suggestion that uric acid is an important antioxidant for humans (Santos *et al*., 1999; Becker, 1993). In support of this, the concentration of approximately 300-500 μM uric acid in plasma is higher than other non-enzymatic antioxidants. A suggested ability to absorb 30-65% of the peroxyl radical scavenging capacity of plasma also suggests uric acid is a major radical scavenger in human plasma (Becker, 1993).

The antioxidant property of uric acid arises from its ability to directly scavenge free radicals and also by binding to transition metal ions in forms that do not accelerate free radical reactions (Schlott *et al*., 1998). The ability of uric acid to chelate metal ions and react with potent biological oxidants, such as the hydroxyl radical and

hypochlorous acid, produces relatively stable products including allantoin and oxonic acid (Santos *et al*., 1999).

Like ascorbic acid, uric acid has also been suggested to possess pro-oxidant properties due to reactions with hydroxyl or peroxyl radicals, thereby generating uric acid radicals that can cause damage. The one electron oxidation of urate produces a urate radical anion, which is relatively stable and does not react with oxygen (reaction 20). The anion can, however, react with a weaker oxidant like ascorbate, regenerating urate and producing an ascorbate radical (reaction 21), this is known as ascorbate-urate redox cycling. Conversely, when ascorbic acid is not present, urate is oxidized to allantoin (Halliwell, 1990; Becker, 1993).

(20) $OH^{\bullet} + U^{-} \rightarrow OH^{-} + U^{\bullet}$ Urate oxidation

(21) $U^{\bullet} + Asc^{-} \rightarrow U^{-} + Asc^{\bullet}$ Ascorbate oxidation

1.7.4. Amino acids

Some molecules while not recognized as classical antioxidants, are able to intercept the reaction between target molecules and oxidants. These molecules are known as sacrificial antioxidants, acting as antioxidants only in these situations. Free amino acids are theorized to serve as sacrificial antioxidants in the protection of plasma proteins (Halliwell, 1989).

Free amino acids in human plasma are highly susceptible to oxidative attack by ROS formed as by-products of metabolism, pollutants in the atmosphere, or radiation (Stadtman and Levine, 2003). Valine, leucine, isoleucine, proline, and glutamine are the amino acids found to produce the highest levels of peroxides, with valine being the amino acid on which hydroperoxides are formed most favourably under radical attack (Fu *et al*., 1995; Soszynski *et al*., 1995; Davies *et al*., 1995; Gebicki *et al*., 1995). These peroxides are known to be relatively stable, but in the presence of biological reductants or metal ions, they are decomposed to reactive free radicals, causing further damage (Soszynski *et al*., 1995; Luxford *et al*., 1999). The ability of amino acids to react with $OH^{\bullet}$ or $ROO^{\bullet}$ and form peroxides could potentially influence protein synthesis because a change in protein metabolism results in

modifications to the plasma free amino acid profile. This indicates that free amino acid levels are good indicators of protein metabolism and nutritional status (Wu *et al*., 1998; Muscaritoli *et al*., 1998; Chuang *et al*., 2006). Significant differences in free amino acid levels have been found between untreated arthritis patients and normal individuals; in workers in stressful environments compared to workers in less stressful environments (Borden *et al*., 1952; Wu *et al* 1998).

1.8 PB-DOPA

Two classes of protein bound reactive oxygen species exist when proteins are exposed to hydroxyl radicals and metal-dependent Fenton systems. These classes are PrOOHs and PB-DOPA (Fu *et al*., 1998; Gieseg *et al*., 1993). PB-DOPA is the major reducing species produced by hydroxyl radical attack on tyrosine residues, and it is one of the major oxidized species elevated in pathological tissues (Rodgers *et al*., 2004; Gieseg *et al*., 1993; Luxford *et al*., 1999; Jain *et al*., 1997).

DOPA formation from tyrosine can occur via two non-enzymatic pathways, one in the presence of oxygen (O_2), and the other in the absence of O_2. When O_2 is absent, the pathway occurs via the elimination of two $OH^{\bullet}$ radicals in a disproportion reaction. This requires two tyrosine molecules and forms both tyrosine and DOPA. By contrast, the presence of O_2 promotes a higher yield of PB-DOPA. A third pathway for PB-DOPA formation is enzymatic and involves tyrosine catalysing the conversion of tyrosine residues to DOPA (Ito *et al*., 1984). PB-DOPA may also form when the free amino acid DOPA becomes incorporated into proteins (Rodgers *et* al, 2002). It is a stable molecule, allowing it to cause damage away from the site of formation (Rodgers *et al*., 2004; Gieseg *et al*., 1993; Luxford *et al*., 1999).

By using the acid hydrolysis of proteins followed by the separation of the hydrolysates via high performance liquid chromatography (HPLC), Gieseg et al., (1993) originally described the detection of PB-DOPA formation. The method is specific for measuring PB-DOPA but very time consuming. A new method was later proposed by Armstrong and Dean, (1995) using ethylenediamine (ED) to derivatise free catecholamines. The reaction involves condensation between ED and the two carbonyls of *o*-benzoquinones that are formed upon oxidation. After incubation with

ethylenediamine, the fluorescence of the ED-DOPA derivative is measured on a spectrophotometer. Parkes, (2005) adapted the method proposed by Armstrong and Dean, (1995), noting the fluorescence intensity of PB-DOPA reached a maximum at an emission of 430 nm and an excitation of 545 nm. Further, an incubation period of 60 minutes was shown to be optimal.

1.9 Objectives of study

Most investigations have examined PrOOHs only on pure proteins (Simpson *et al*., 1992; Gebicki *et al*., 2000a and b; Gay and Gebicki, 2003; Gieseg *et al*., 2000; Gebicki and Gebicki, 1993). More recently, however, Gebicki *et al*., (2000a) investigated the hydroxyl radical-induced formation of PrOOHs in human blood serum and noted their immediate production. The lack of an observable lag phase contrasted with the existence of a lag phase for lipid oxidation. A similar PrOOH trend was observed by Gieseg *et al*., (2000) when exposing U937 cells to AAPH-derived peroxyl radicals. Radical-induced lipid oxidation has also been predominantly investigated using only pure lipid substrates. Very little or no lipid hydroperoxides are formed over the first 60 minutes, which is followed by a rapid linear increase in formation (Thomas *et al*., 1997; Neuzil *et al*., 1993; Frei *et al*., 1988).

Although the human body possesses an exceptional defence system, it is unable to completely protect plasma from protein and lipid oxidation. PrOOHs and lipid hydroperoxides are known to form when exposed to hydroxyl and peroxyl radicals, with lipid hydroperoxide formation occurring only after the complete consumption of the antioxidant ascorbate. α-Tocopherol and uric acid also act as antioxidants in this system, lowering the rate of lipid peroxidation by scavenging chain-propagating peroxyl radicals (Gebicki *et al*., 2000a; Frei *et al*., 1989; Suarna *et al*., 1995; Stocker *et al*., 1991). Previous research in the laboratory has demonstrated a six hour lag phase during peroxyl radical-mediated PrOOH formation in whole human plasma. The inhibition does not appear to be caused by α-tocopherol, thiols or glutathione (Ling, 2004; Yang, 2005). Although, uric and ascorbic acid contributed to the inhibition, they could not separately account for the entire lag phase. This indicates that uric and ascorbic acid, combined may be responsible for the six hour lag phase.

The initiation of lipid oxidation has been found to coincide with the depletion of ascorbic acid; once ascorbic acid is consumed, lipid oxidation occurs. The lag phases and the contribution of ascorbic acid to the inhibition of both lipid and protein oxidation suggests the processes are connected.

As a continuation of Ling, (2004) and Yang, (2005's) research, this study will examine the kinetics of PrOOH formation in human plasma during exposure to the peroxyl radical generator AAPH. To identify the antioxidant responsible for the six hour lag phase during AAPH-induced human blood plasma oxidation, plasma will be dialysed to remove low molecular weight molecules. Such molecules include ascorbic acid, uric acid and free amino acids. These low molecular weight antioxidants will subsequently be added to the dialysed plasma in controlled quantities, and exposed to AAPH. PrOOH formation in each treatment will then be measured in an attempt to isolate the source of the lag phase.

This research will also examine the kinetics of cholesterol diene formation in human blood plasma when exposed to AAPH-derived peroxyl radicals. To understand the kinetics of CL diene formation further, plasma will be dialysed and supplemented with ascorbic and uric acid. These are the antioxidants postulated to be involved in the inhibition of protein oxidation (Yang, 2005). Supplemented plasma will be exposed to peroxyl radicals by incubation with AAPH. α-Tocopherol loss will also be monitored to determine the significance of this antioxidant in protecting plasma from AAPH-induced oxidation.

A final part of this thesis is distinct from the above research but aims to complete the work of Parkes, (2005) a former Master's student in the Free Radical Biochemistry Laboratory. As such, the concentration of irradiation-induced PB-DOPA will be measured on pure proteins by both the ED-DOPA assay and HPLC analysis. BSA will also be exposed to a system simulating the Fenton reaction to examine whether PB-DOPA can be produced on a pure protein under these conditions. The two PB-DOPA assays will ultimately be compared to determine which one is best and whether the ED-DOPA assay is specific for PB-DOPA or also detects additional products.

MATERIALS AND METHODS

2.1 Materials

2.1.1 Chemicals

All reagents used in the research were of analytical grade or better. All water used was de-ionised and filtered by a nanopure ultra-filtration system supplied by Millipore, USA.

α–Tocopherol	Sigma Chemical Co., St. Louis, MO, USA
2,2'-azobis (2-amidinopropane) dihydrochloride (AAPH)	Arcos, NJ, USA
L-3,4-dihydroxyphenylalanine (L-DOPA)	Sigma Chemical Co.
Acetic acid (glacial)	Merck Ltd, Poole, England
Acetonitrile	Merck Ltd
Alanine	Sigma Chemical Co.
Ammonium ferrous sulfate	Hopkin and Williams Ltd, Essex
Anhydrous sodium acetate	BDH Chemicals Ltd
Arginine	Sigma Chemical Co.
Argon gas	BOC Gasses, New Zealand
Ascorbate oxidase	Sigma Chemical Co.
Ascorbic acid	Sigma Chemical Co.
Bovine serum albumin (BSA)	Sigma Chemical Co.
Butylated hydroxytoluene (BHT)	Sigma Chemical Co.
Catalase	Sigma Chemical Co.
Chelex-100 resin	Bio-Rad Laboratories, CA, USA
Chloroform	Merck Ltd and Univar
Cholesteryl linoleate ester	Sigma Chemical Co.
Copper chloride	BDH Chemicals Ltd
Copper sulfate	BDH Chemicals Ltd
Diethyl ether	BDH Chemicals Ltd
Dodecyltrimethyl ammonium chloride	Fluka
Ethanol	BDH Chemicals Ltd
Ethylenediamine	Sigma Chemical Co.
Ethylenediamine dihydrochloride	Sigma Chemical Co.
Ethylenediamine tetraacetic acid (EDTA)	BDH Chemicals Ltd
Ethylenediaminetetraacetic acid disodium salt (Na_2EDTA)	BDH Chemicals Ltd
Glutamine	Sigma Chemical Co.

Glycine	Sigma Chemical Co.
n-Hexane	BDH Chemicals Ltd
Histidine	Sigma Chemical Co.
Hydrochloric acid (HCl)	BDH Chemicals Ltd
Hydrogen peroxide	BDH Chemicals Ltd
Isoleucine	Sigma Chemical Co.
Isopropanol	Merck Ltd
Leucine	Sigma Chemical Co.
Lysine	Sigma Chemical Co.
Methanol	Merck Ltd
Mercaptoacetic acid (Thioglycolic acid)	Sigma Chemical Co.
Nitrogen gas	BOC gasses
Oxygen gas	BOC gasses
Phenol	Sigma Chemical Co.
Proline	Sigma Chemical Co.
Sodium chloride (NaCl)	BDH Chemicals Ltd
Sodium dihydrogen orthophosphate (NaH_2PO_4)	BDH Chemicals Ltd
Sodium hydroxide (NaOH)	BDH Chemicals Ltd
Sucrose	Chelsea sugar refinery: Auckland, NZ
Sulfuric acid (H_2SO_4)	BDH Chemicals Ltd
T-butyl methyl ether	BDH Chemicals Ltd
Toluene	BDH Chemicals Ltd
Trichloroacetic acid (TCA)	Merck Ltd
Trifluoroacetic acid (TFA)	Sigma Aldrech Chemical Co.
Tyrosine	Sigma Chemical Co.
Uric acid	Sigma Chemical Co.
Uricase	Sigma Chemical Co.
Valine	Sigma Chemical Co.
Xylenol orange	Sigma Aldrech Chemical Co.

2.1.2 Specialised solutions and buffers preparation

A) Protein oxidation

Phosphate buffered saline (PBS)

PBS composed of 150 mM sodium chloride (NaCl) and 10mM sodium dihydrogen orthophosphate (NaH_2PO_4), at pH 7.4, was prepared from stock solutions. Washed chelex-100 was added to the solution and left stirring overnight to remove any transition metals in the solution. The chelex was removed the next day by filtration through a 0.22 μM filter. The PBS was then sonicated in order to remove air bubbles and stored at 4˚C. Prior to use, the PBS was warmed to room temperature.

2,2'-azobis (2-methyl-propanamidine) dihydrochloride (AAPH)

A stock solution of 250 mM was prepared on the day of the experiment by dissolving AAPH (MW 271.19 g/mol) in ice-cold PBS. The stock solution was stored on ice until required.

Bovine serum albumin (BSA) solutions

A stock solution of 10 mg/ml was prepared on the day of the experiment by dissolving BSA in PBS. Diluting the 10 mg/ml stock with PBS created various concentrations of BSA.

B) Solutions for FOX assay

Xylenol orange solution

A 5 mM solution was composed by dissolving xylenol orange (MW 760.6 g/mol) in 25 mM sulfuric acid (H_2SO_4). The solution was stored at room temperature for up to one month.

Ammonium ferrous sulfate

A 5 mM solution was composed by dissolving ferrous ammonium sulfate (MW 392.16) in 25 mM sulfuric acid (H_2SO_4). The solution was stored at room temperature and used within one week.

Sulfuric acid

A 1 mM stock solution of H_2SO_4 was composed by adding 5.4 ml of H_2SO_4 to 94.6 ml of water. A 25 mM solution was made by dilution of 0.5 ml stock solution with 19.5ml of water.

TCA

TCA was prepared at concentrations of 72% and 5% w/v in water.

C) Antioxidants and amino acids

Amino acids

10 mM stock solutions of tyrosine, proline, leucine, isoleucine, arginine, histidine, glutamine, alanine, glycine, valine and lysine were prepared by dissolving the amino acid in 50 ml PBS.

D) HPLC cholesterol diene analysis

Butylated hydroxytoluene (BHT)

A 20 mg/ml solution was prepared by dissolving BHT in methanol. The solution was stored at 4˚C.

Ethylenediamine-tetraacetc acid disodium salt (EDTA)

A 100 mg/ml solution was prepared by dissolving EDTA in nanopure water. The pH was adjusted to 7.4 by adding drops of 10 mM NaOH and then stored at 4˚C.

Cholesteryl linoleate hydroperoxide standard

20 mg of cholesterol linoleate ester was dissolved in 1 ml of toluene. 2 ml of 100 mM AAPH was added to the 15 ml black-top glass tube and incubated at 37 ˚C under constant mixing at 110 rpm for 24 hours. The top toluene layer was transferred into a tapered test tube using a Pasteur pipette. The layer was dried down under nitrogen in an oxygen-free water bath, reconstituted in 5 ml of hexane and then run through an aluminium oxide column. 10 ml of T-butyl methyl ether was run through the column and the cholesterol hydroperoxide (CL-OOH) was collected in a black-top glass centrifuge tube. This was dried down again and reconstituted in 8 ml of acetonitrile/isopropanol (30:70). The concentration was determined by its absorbance

at 234 nm using the extinction coefficient 29, 500 $M^{-1}cm^{-1}$ (Sattler *et al*., 1994). After layering the tube with argon, it was kept in a -80 ˚C freezer until required.

E) HPLC α-Tocopherol analysis

α-Tocopherol standard

A stock solution of 100 mM α-tocopherol was stored in the -20˚C freezer. The stock solution was diluted to 10 μM with cold methanol and injected into the HPLC for α-tocopherol analysis.

F) ED-DOPA assay

Ethylenediamine dihydrochloride

A 2 M ethylenediamine dihydrochloride solution was prepared by dissolving ethylenediamine dihydrochloride in nanaopure water. Sonication ensured all the ethylenediamine dihydrochloride was dissolved.

Ethanol/Diethyl ether solution

Ethanol and diethyl ether were mixed together in a ration of 3:1. The solution was inverted to ensure the solutions had mixed.

DOPA stock solutions

A 1 mM solution of DOPA was prepared on the day of analysis by dissolving DOPA in nanopure water. The solution was sonicated to allow the DOPA to dissolve and the solution was stored at 4˚C in the dark until required. This stock solution was diluted to 50 μM with nanopure water. DOPA standards, to concentrations of 0, 5, 10, 25, and 50 μM, were prepared from DOPA stock solutions and nanopure water. Standards were made up to a final volume of 500 μl.

G) HPLC DOPA solutions and buffers

6M HCl with 1% w/v phenol solution

In a 50 ml solution of 6 M HCl, phenol was dissolved to a final concentration of 1% w/v.

Mobile phase

A 1 litre 0.1% TFA solution was made up by adding 1ml TFA to 900 ml of nanopure water. The pH was adjusted to pH 2.5 with of 10 mM HCl. The volume was then made up to a final volume of 1 litre with nanopure water. The solution was filtered through a 0.22 μM filter and sonicated for ten minutes to degas.

H) HPLC determination of ascorbate and urate

Mobile phase

A 1 litre solution was made up by dissolving 3.28 g of sodium acetate anhydrous, 0.2 g of Na_2EDTA and 0.130 g of dodecyltrimethylammonium in 800 ml of nanopure water. 75 ml of methanol was added and the pH of the solution adjusted to 4.8 with glacial acetic acid. Nanopure water was added to make a final volume of 1 litre. The solution was filtered through a 0.22 μM filter and sonicated for 20 minutes to remove air bubbles.

Sample preparation buffer

A 10 mM solution was prepared by dissolving EDTA in 100 ml nanopure water. To make a 90% methanol/water/1 mM EDTA buffer, 5 ml of the 10 mM EDTA solution was mixed with 45 ml of methanol.

Ascorbic acid and uric acid standards

2 mM stock solutions of ascorbic or uric acid were prepared by dissolving 4 mg of either antioxidant in nanopure water. The stock solutions were diluted 1/200 to give a final concentration of 10 μM.

2.2 Methods

2.2.1 Blood collection and plasma preparation

Blood was collected from volunteers by venipuncture using winged infusion sets and a 50 ml syringe (Terumo, USA). The volunteers fasted overnight and were in a healthy condition. 200 ml was collected from each donor into four 50 ml centrifuge tubes containing 0.5 ml 10% ETDA (pH 7.4) as an anti coagulant.

The tubes were centrifuged at 4100 g for 20 minutes at 4˚C with the brake off to separate the cells from the plasma. The plasma was then transferred to 50 ml round

bottom centrifuge tubes and centrifuged at 11, 000 g for 30 minutes in a fixed angle rotor to remove the cellular components reminiscent in the plasma. The plasma from four-six donors was pooled together and 50% sucrose solution was added to make a final concentration of 0.6%. The plasma was stored in 20 ml aliquots in the -80 freezer for up to three months until required.

2.2.2 Plasma and protein oxidation

Pure bovine serum albumin (BSA) solution and human plasma were incubated in the presence and absence of 10 mM 2,2'-azobis(amidinopropane) dihydrochloride (AAPH) in an incubator (Bioline, Edwards Instrument Company, Australia) at a temperature of 37˚C in an orbital motion at a speed of 80 rpm. The constant motion ensured oxygenation of the solutions. At set times points, samples were removed and analysed for the concentration of PrOOHs, cholesterol dienes or α-tocopherol concentration by the methods described in sections 2.2.3, and 2.2.8 respectively.

2.2.3 Protein hydroperoxide determination by the FOX assay

A) 25 mM Sulfuric acid FOX assay

PrOOHs on BSA were measured by the sulphuric acid FOX assay, as modified by Gay *et al*. (1999a and b). As it lacks a delipidation procedure, this assay is only able to measure PrOOH in lipid-free systems like pure BSA. 1 ml samples were removed from BSA solutions in triplicate into 1.8 ml clear centrifuge tubes (Axygen) at selected time points. 140 µl of 72% trichloroacetic acid (TCA) was added and vortexed to mix before being left on ice for five minutes. This is to ensure that there is complete precipitation of the proteins. The eppendorf tubes were subsequently centrifuged at 4˚C, 10, 000 rpm for seven minutes and the supernatant decanted off. 1 ml of 5% TCA was added, the pellet resuspended using sealed Pasteur pipettes (Chase Scientific Glass, Inc) and centrifuged again. This washing step was repeated. After the second wash the supernatant was removed and the pellet resuspended in 900 µl of 25 mM H_2SO_4 using the sealed glass rod before 50 µl of 5 mM xylenol orange was added and mixed by vortexing. 50 µl of 5 mM ferrous ammonium sulfate was added, vortexed and incubated at room temperature in the dark for 30 minutes. The samples were transferred to cuvettes (Sarstedt) and the absorbance read against a water blank at 560 nm on a UV-visible spectrophotometer (model UV-1601PC, Shimadzu) using

the UV Probe software (Shimadzu). The concentration was calculated using the extinction coefficient of 35, 500 $M^{-1}cm^{-1}$ for BSA hydroperoxides in H_2SO_4.

B) Acetic acid FOX assay

The acetic acid FOX assay was modified by Pearson, (2002) and can detect PrOOHs produced in AAPH-treated lipoproteins and AAPH-treated plasma. 200 µl of sample was removed from plasma solutions in triplicate into 1.8 ml clear centrifuge tubes (Axygen) at selected time points and diluted 1/5 with nanopure water. 140 µl of 72% TCA was added to sample, vortexed to mix and then left on ice for five minutes. The samples were centrifuged at 10, 000 rpm for seven minutes at 4˚C to pellet the protein and then the supernatant was decanted. The pellets were resuspended in 1 ml of 1:1 methanol/chloroform solution using sealed Pasteur pipettes, before being centrifuged again. The supernatant was decanted once more and the pellets dried by placing the 1.8 ml centrifuge tubes upside down for five minutes. 900 µl of 50% aqueous acetic acid was then added to the dried pellets and also to the blank eppendorf tubes. The pellets were resuspended and vortexed briefly to mix. 50 µl of 5 mM xylenol orange was added to all the 1.8 ml centrifuge tubes and mixed to distribute evenly. 50 µl of 5 mM ferrous ammonium sulfate was then added and also vortexed to mix. The samples were incubated for 30 minutes in the dark at room temperature before reading the absorbance at 560 nm against the blank. Using the coefficient 32, 500 $M^{-1}cm^{-1}$ and the dilution factor of 1/5, the concentration of PrOOH in plasma was calculated.

2.2.4 Plasma dialysis for protein oxidation

The dialysis bags were made from 14 kDal dialysis tubing (14.3 mm in diameter, supplied by Medicell International, Ltd) having previously been treated by boiling in 5% (w/v) $NaHCO_3$ and 1 mM EDTA before extensive washing and further boiling in nanopure water. The tubing was stored in 50% ethanol solution and was thoroughly washed with distilled water when required for dialysis.

PBS for dialysis was prepared the previous day and left stirring for 24 hours. The plasma was placed inside the prepared dialysis bag and the top layered with argon gas. Plasma was dialysed against four 1 litre changes of chelex-treated, nitrogen-degassed

PBS over a 24 hour period in a darkened room with the temperature maintained at 4˚C.

2.2.5 HPLC analysis of ascorbic acid and uric acid

Detection of ascorbic acid and uric acid was achieved by ion-pair liquid chromatography on a Shimadzu (New Zealand, Ltd) SIL-10A automated high-performance liquid chromatography (HPLC) machine with electrochemical detection at +60 volts. This protocol was adapted from Frei *et al*. (1988). The mobile phase was filtered through a 0.22 μM filter, sonicated for 20 minutes and pumped though the column overnight for a minimum of 16 hours at 1 ml/min. The column used for the detection was a reverse phase phenosphere 5 μm C_{18}, 250 x 4.60 mm with a guard column. The column was kept in a Shimadzu CTO-10A column oven at a temperature of 30˚C while being used. Uric acid and ascorbic acid standards were freshly prepared at the beginning of the analysis, and 100 μl of the standard and samples were placed into autosampler vials with 10 μl injected into the HPLC. Ascorbic acid and uric acid eluted as single peaks, with uric acid at five minutes and ascorbic acid at seven minutes. The peak areas were calculated using Shimadzu Class VP (Version 6.12 SP4) software.

2.2.6 Determination of uricase concentration

Different concentrations of uricase were prepared and added to ascorbic acid and uric acid standards. The solutions were incubated for 30 minutes to determine the concentration that blocked uric acid. As 1 mg/ml uricase was subsequently found to block uric acid formation, this concentration was incubated with plasma at 37˚C for 30 minutes. 200 μl of sample was then added to 800 μl of 90% methanol/water/EDTA and left on ice for five minutes in the dark to precipitate protein. Samples were centrifuged at 10, 000 rpm for ten minutes at 5˚C before being analysed for uric acid content by HPLC.

2.2.7 The role of amino acids in protein oxidation

Plasma was dialysed, to remove free amino acid and specified amino acids were then added back to the dialysed plasma at 250 or 500 mM. The control was also dialysed but with PBS rather than amino acids to ensure the plasma in all treatments was of a

consistent dilution. The role of amino acids in plasma protein oxidation was examined by exposing each treatment to AAPH before measuring PrOOHs via the acetic acid FOX assay.

2.2.8 Determination of cholesterol diene and α-tocopherol concentration by HPLC

Cholesterol diene formation in LDL was carried out by modifying the originally developed by Kritharides *et al*. (1993). 500 µl of plasma sample was removed from plasma solutions in triplicate into 15 ml black-top glass tubes. 20 µl of 20 mg/ml EDTA, 20 µl of 100 mg/ml BHT and 1 ml of cold methanol was added to the test tubes and vortexed for ten seconds. 5 ml of hexane was added and vortexed for 30 seconds before the tubes were sealed with parafilm and stored in the -80˚C freezer until analysis. On analysis, the samples were removed from the freezer and thawed. They were vortexed for 30 seconds and then centrifuged for three minutes at 5˚C, 1, 000rpm. The top 4 ml of hexane was transferred into a tapered glass test tube and dried down under nitrogen in an oxygen-free hot water bath. The residue was reconstituted in 100 µl of mobile phase and placed in an autosampler vial. A 20 µl injection was passed through an Econosphere C18 5µm column (Alltech Associates Inc., U.S.A), maintained at 35˚C inside a Shimadzu CTO-10A column oven. The mobile phase, consisting of acetonitrile/isopropanol/water in a ration of 44:54:2, was pumped at a flow rate of 1 ml/min. Cholesterol dienes were detected by UV absorbance using a SPD-10A ultraviolet detector at a wavelength of 234 nm. This was joined in series to a fluorescence detector, with an excitation of 292 nm and an emission of 335 nm, to also enable for α-tocopherol detection. A colesteryl linoleate standard was prepared prior to analysis and stored in the -80˚C freezer, while a 3 µM α-tocopherol standard was made up by diluting the 100 µM stock solution (stored in the -20˚C freezer) in cold methanol. The peak areas were calculated using Shimadzu Class VP (Version 6.12 SP4) software and the concentration of dienes and α-tocopherol was determined quantitatively by comparison with the known standard of the dienes or α-tocopherol.

2.2.9 Irradiation of BSA

5 ml of the 1 mg/ml solution of BSA in PBS was transferred to a 20 ml scintillation vial and placed in the X-ray source (Philips model PW2264-20 tungsten x-ray generator, 35kV, 30mA) directly below the aperature. A small tube connected to an oxygen cylinder was placed inside the scintillation vial to allow constant bubbling of oxygen through the BSA over the radiation period. The PB-DOPA content of the irradiated BSA was analysed by the ED-DOPA assay or HPLC fluorescence analysis for PB-DOPA after acid hydrolysis.

2.2.10 Fricke Dosimeter Assay

Under aerobic conditions, irradiation produces a constant flux of hydroxyl radicals ((Halliwell and Gutteridge, 1990; Cudina and Josimovic, 1987). The Fricke dosimeter assay is used to determine the rate of this flux and was performed to calibrate the X-ray source. 10 ml of 1 mM ferrous ammonium sulfate, 0.6 mg of sodium chloride and 0.22 ml of concentrated sulfuric acid were mixed to form the Fricke solution. The Fricke solution was exposed to irradiation for ten minutes and the absorbance was measured at 304 nm against a Fricke solution not exposed to X-rays.

Dose rate (in Gy/min) = 286 x absorbance / time of exposure

G = 5.8 Gy/min.

2.2.11 PB-DOPA production via Fenton reaction

The analysis of PB-DOPA generated on BSA exposed to the Fenton reaction was modified from the method used by Gieseg *et* al. (1993). BSA at a concentration of 1 mg/ml, was made up using 10 mM chelexed sodium phosphate buffer. 15 ml centrifuge tubes consisting of 0, 40, 70, or 100 μM copper chloride were prepared containing BSA and 5 mM hydrogen peroxide. The tubes were covered with tin foil to permit the continual circulation of oxygen while being gently mixed at 100 rpm for the duration of the 30 minute incubation at 37˚C. 25 μl of 5μg/ml catalase and 0.25 g of washed chelex-100 were then added to stop the reaction and remove free metal ions. The tubes were rotated in a 4˚C room for two hours, and then centrifuged at 1, 500 rpm for five minutes to pellet the chelex. 200 μl of supernatant was placed in Durham tubes and freeze dried in a Speed Vac in preparation for acid hydrolysis and

HPLC analysis. 500 µl of sample was placed in labelled eppendorfs for analysis by the ED-DOPA assay.

2.2.12 ED – DOPA assay

This assay was originally developed by Sutherland *et al*. (2003) and modified by Parkes, (2005). 500 µl of irradiated BSA was placed in triplicate in labelled 1.8 ml clear centrifuge tubes and 500 µl PBS, 50µl ethylenediamine dihydrochloride and 70 µl ethylenediamine added. DOPA standards of 0, 5, 10, 25 and 50 µM were made up and also mixed with 500 µl PBS, 50µl ethylenediamine dihydrochloride and 70 µl ethylenediamine. All samples and standards were vortexed and incubated in a 50˚C incubator. After a one hour incubation, the fluorescence was measured against a water blank using a fluorescence spectrophotometer (Cary Eclipse) at an excitation of 430 nm and an emission of 545 nm. The fluorescence (y) was recorded by operating the Advanced Reads software (Cary Eclipse), with the concentration of PB-DOPA (x) calculated using the slope (m) and y-intercept (c) from the standard curve of the standards and the equation x=(y-c)/m.

2.2.13 HPLC analysis of PB-DOPA

A) Acid hydrolysis

Acid hydrolysis and HPLC analysis of the hydrolysates is based on a method developed by Gieseg *et al*. (1993), with further adaptation by Parkes, (2005). 200 µl of sample was placed in triplicate in labelled Durham tubes (7.5 x 50 mm, Biolab) and dried in the speed vacuum. Once dry, the Durham tubes were placed in Pico-Tag reaction vials (Millipore, USA), with 1 ml 6M HCl with 1% w/v phenol and 50 µl mercaptoacetic acid in the bottom of the reaction vials. The vials were flushed with argon then evacuated by a vacuum pump before closing the valve and incubating at 110˚C for 16 hours. Subsequently, the vials were removed and cooled before opening. 200 µl of 0.1% TFA was added to each Durham tube, vortexed to dissolve hydrolysate residue and then centrifuged for 15 minutes at 10 000 rpm to pellet undissolved residues. The supernatant was transferred into labelled 1.8 ml clear centrifuge tubes and centrifuged. 100 µl of the supernatant was placed in an autosampler vial with 10 µl injected into the HPLC for analysis.

B) HPLC analysis of acid hydrolysates

10 µl samples were injected and run through an Aqua C18 125A 250 x 4.6 mm HPLC column (Phenomenex, New Zealand) that was maintained at 35˚C in the column oven. The combination of 0.1% TFA as mobile phase A and acetonitrile as mobile phase B was used and run at 1 ml/min. Mobile phase B was run at 1% for the first ten minutes, and changed to 5% for the next four minutes, 10% for two minutes, 5 % for seven minutes and 1% for the last seven minutes. PB-DOPA was detected by fluorescence on a Shimadzu fluorescence detector with an excitation of 280 nm and emission of 320 nm. The peak area was determined by Shimadzu Class VP (Version 6.12 SP4) software with the PB-DOPA concentration calculated by comparison of the peak area with the 1 µM DOPA standard peak area.

2.2.14 ED-DOPA assay accuracy

10 ml of 1 mg/ml BSA, in 10 mM non-chelexed sodium phosphate buffer was placed in a scintillation vial and exposed to radiation for 60 minutes.

4.42 ml of irradiated and unirradiated BSA was transferred to falcon tubes. Half the samples in each treatment were mixed with 577 µl of 1 mM copper sulfate while the other half were mixed with 577 µl PBS. All the tubes were mixed and incubated at 37˚C, 110 rpm for two hours. 25 µl of catalase and 0.25 g chelex were added to the tubes containing copper sulfate to stop the reaction and remove free metal ions. The tubes were mixed and placed on the rotator in the dark, 4˚C room for two hours before being centrifuged for five minutes at 1, 500 rpm to pellet the chelex. 200 µl of each sample was placed in Durham tubes to be dried down in the Speed Vac for acid hydrolysis and HPLC DOPA analysis. A further 500 µl of each sample was placed in labelled centrifuge tubes for ED assay analysis.

2.3 Statistical analysis

Results shown were obtained from single experiments representing a minimum of three repetitions of the same experiment under identical conditions. Each data point represents a duplicate or triplicate in each treatment and is expressed in the form of mean value plus standard error of the mean. The standard error is represented on the graphs by symmetrical error bars. The statistical analysis was performed using PRISM software (Version 4, Graphpad Software, Inc). Comparison treatments within

experiments were analysed using one-way analysis of variance (ANOVA) followed by Tukey's multiple comparisons test. Statistical significance is shown using the following notation * $p \leq 0.05$ (significant), ** $p \leq 0.01$ (very significant), *** $p \leq 0.001$ (highly significant). These notations represent the significance of the data from its zero time value.

RESULTS

3.1 Protein oxidation induced by AAPH

3.1.1 PrOOH formation on BSA

AAPH is a water-soluble azo radical initiator that decomposes at a temperature dependent rate, generating a constant flux of peroxyl radicals (Niki, 1990). The use of AAPH, therefore, enables both lipid and protein oxidation to be studied in a defined system.

The kinetics of PrOOH formation on pure proteins was investigated by incubating 2 mg/ml of pure BSA with and without 10 mM AAPH at 37˚C over a five hour period (Figure 3.1.1). Under such conditions, a linear increase in PrOOH formation was observed over the five hours, whereas BSA incubated with no AAPH displayed very little PrOOH formation over the same period. The 10 mM AAPH-mediated production of PrOOHs occurred at a rate of 1.7 μM during the first hour of incubation, slowing to a rate of 0.8 μM over the next 4 hours.

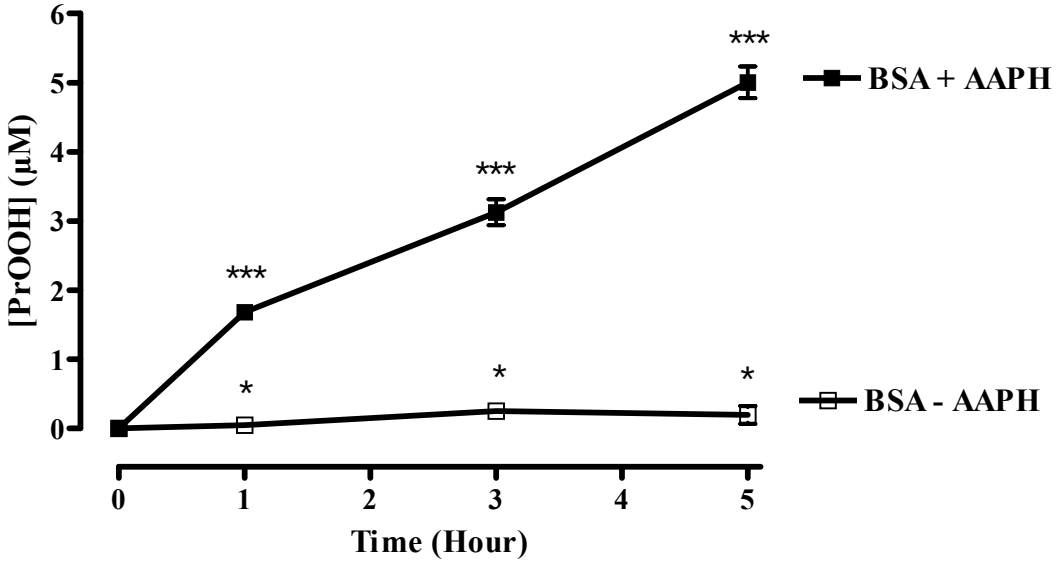


Figure 3.1.1 Peroxyl radical-mediated PrOOH formation on BSA
BSA, at a concentration of 2 mg/ml, was incubated at 37˚C in the presence and absence of 10 mM AAPH. Incubation was over a five hour period, with samples removed for analysis at selected time intervals. Hydroperoxides were measured by the sulfuric acid FOX assay with the PrOOH concentration adjusted against blank controls and time zero PrOOH values. Each data point represents the mean ± SEM of triplicate samples.

3.1.2 PrOOH formation in human plasma

Proteins are thought to be a major target molecule of radical attack in the oxidation process (Gebicki, 1997). The kinetics of PrOOH formation in peroxyl radical oxidized plasma had previously been investigated by Ling, (2004) and Yang, (2005). Both identified a lag phase of six-eight hours when plasma was incubated with 10 mM AAPH. This experiment was reproduced in the current study to confirm the existence of a lag phase, with human blood plasma being incubated in the presence and absence of 10 mM AAPH at 37˚C over a 24 hour period (Figure 3.1.2). Samples were removed every two hours and PrOOH formation measured using the acetic acid FOX assay, which is designed to determine PrOOH formation in lipid containing systems such as plasma. Plasma lacking 10 mM AAPH showed very little PrOOH formation over the 24 hour period, indicating no auto-oxidation of proteins, while plasma incubated with 10mM AAPH produced a six hour lag phase where little or no PrOOH formation occurred. The six hour lag phase was followed by an increase in PrOOH formation, at a rate of 3.7 μM, and ultimately by a decrease/plateau in production between the 12 and 24 hour time points that was attributed to the degradation of PrOOHs. The prominence of this plateau varied between experiments due to the use of different batches of plasma. Likewise, the precise rate of formation also showed some variation. The small increase after two hours observed in Figure 3.1.2, was not significant and may have been an experimental variant as it was not observed in all experiments. The lag phase was, however consistently detected in all experiments. Thus having confirmed its existence the main objective of the current study could begin expanding the work of Ling, (2004) and Yang, (2005), by trying to define the source of the six hour lag phase during AAPH-induced plasma.

The source of the lag phase was initially investigated by dialysing the plasma to remove small molecular weight molecules (Figure 3.1.3). In agreement with Yang, (2005), dialysed plasma was not associated with a lag phase when incubated with 10 mM AAPH. PrOOH formation began immediately in the absence of the plasma's small molecular weight components. Such components include ascorbic acid, uric acid and free amino acids.

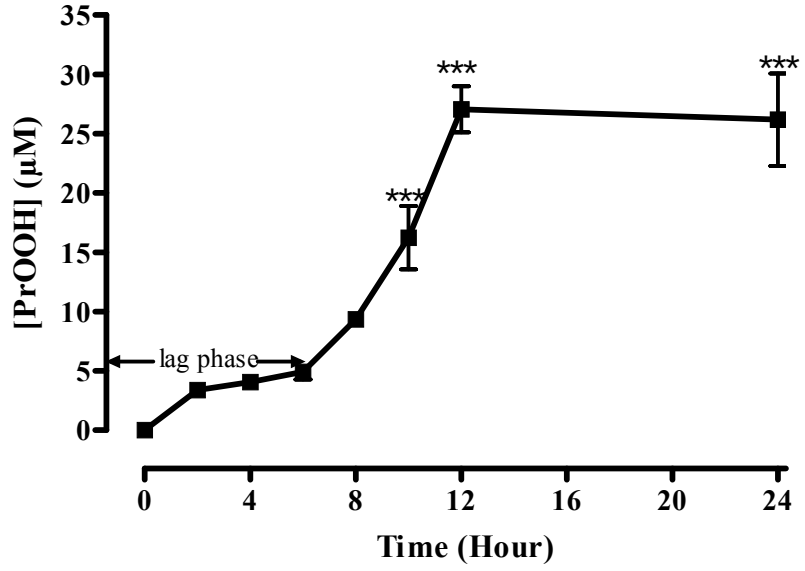


Figure 3.1.2 Peroxyl radical-mediated PrOOH formation in plasma
Plasma was incubated at 37˚C in the presence and absence of 10 mM AAPH. Incubation was over a 24 hour period, with samples removed for analysis at selected time intervals. Protein hydroperoxides were measured by the acetic acid FOX assay and adjusted against blank controls and the corresponding time zero PrOOH values. Each data point represents the mean ± SEM of triplicate samples.

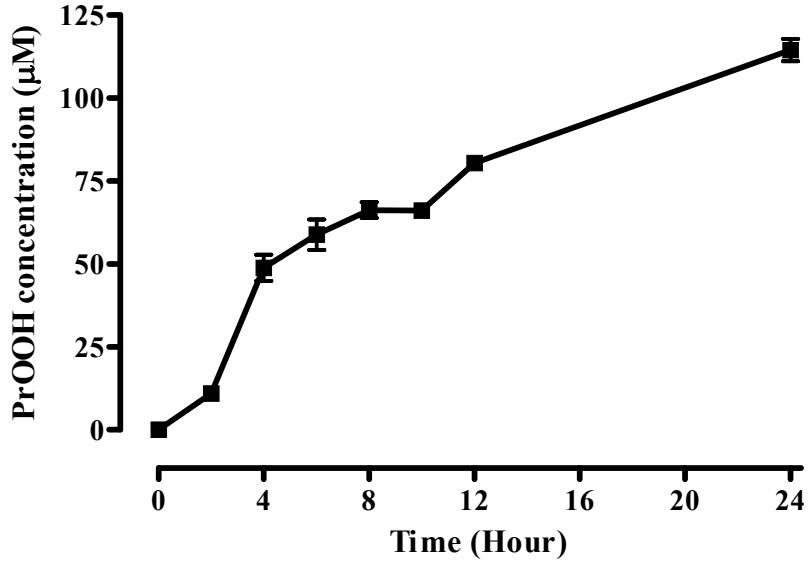


Figure 3.1.3 Peroxyl radical-mediated PrOOH formation in dialysed plasma
Dialysed plasma was incubated at 37˚C with 10 mM AAPH over a 24 hour period. Samples were removed at selected time points for PrOOH analysis by the acetic acid FOX assay and adjusted against blank controls and the corresponding time zero PrOOH values. Each data point represents the mean ± SEM of triplicate samples.

3.2 Concentration of ascorbic acid and uric acid in plasma

To investigate whether ascorbic and/or uric acid were responsible for the lag phase observed when plasma is incubated with 10 mM AAPH (Figure 3.1.2), both antioxidants were first analysed by HPLC in undialysed plasma to monitor their concentration during a 24 hour incubation. It was observed that ascorbic acid became completely depleted in plasma within two hours of exposure to AAPH, suggesting it may play a role in protecting for the first two hours while the ascorbic acid oxidation product, DHA protects during the rest of the lag phase, however cannot be solely responsible for the six hour lag phase (Figure 3.2.1). Uric acid decreased in concentration over time but approximately 15 μM and 7 μM remained even after six hours and 24 hours respectively. This suggests that, although uric acid may provide some protection, it is not responsible for the inhibition of PrOOH formation during the lag phase.

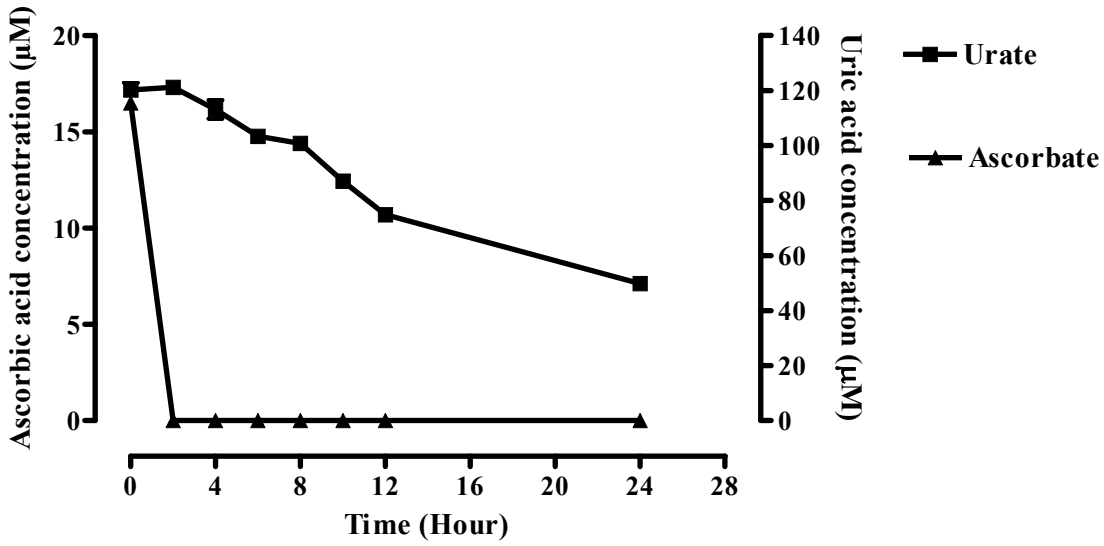


Figure 3.2.1 AAPH decreases the concentration of ascorbic acid and uric acid in plasma

Plasma was incubated at 37˚C in the presence of 10mM AAPH. Incubation was over a 24 hour period, with samples removed for analysis of uric acid and ascorbic acid via HPLC at selected time intervals. Each data point represents the mean ± SE of triplicate samples.

3.3 Effect of plasma antioxidants on the PrOOH lag phase of AAPH-mediated human plasma oxidation

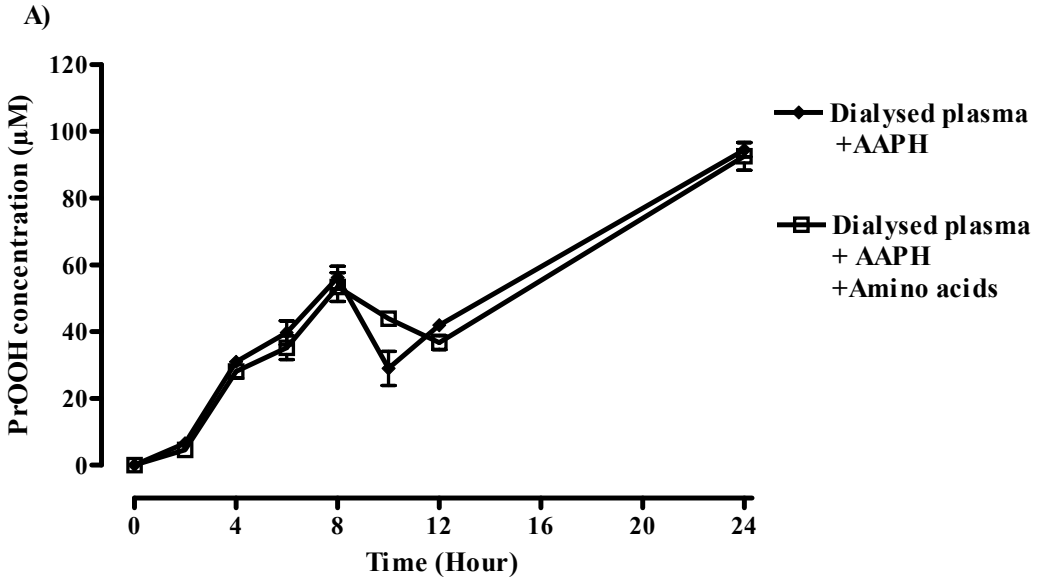
3.3.1 Effect of free amino acids on PrOOH formation in AAPH-mediated oxidation of dialysed plasma

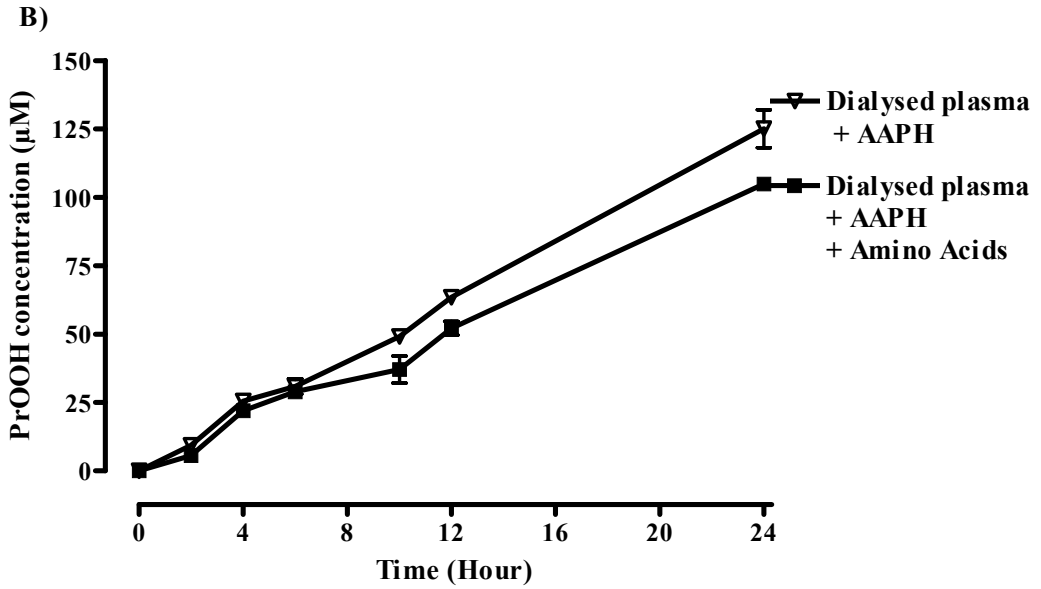
Free amino acids in the plasma are thought to act as sacrificial antioxidants, protecting proteins from free radical attack. Literature searches produced a list of free amino acid concentrations found in human plasma (Wu *et al*., 1998; Chuang *et al*., 2005; Borden *et al*., 1952; Glew *et al*., 2004; Muscaritoli *et al*., 1999; Tcherkas and Denisenka, 2001; Filho *et al*., 1997; Gregory *et al*., 1986; Lepage *et al*., 1997). The five amino acids with the highest concentrations were identified as valine, alanine, glutamine, lysine, and glycine. The effect of these five free amino acids on plasma protein oxidation was then investigated by supplementing dialysed plasma with 250 mM of each of the amino acids. This supplemented dialysed plasma was incubated with 10 mM AAPH at 37˚C and samples were removed for PrOOH analysis using the acetic acid FOX assay, at selected time points over a 24 hour period (Figure 3.3.1A).

Dialysed plasma, supplemented with free amino acids, showed immediate production of AAPH-induced PrOOHs with a rapid increase in formation after two hours. This was identical to the trend observed with dialysed but supplemented plasma, which was also exposed to 10 mM AAPH. At ten and twelve hours there is a decrease in PrOOH formation. There is no significant difference between the dialysed plasma and supplemented dialysed plasma at these time points indicating this is an experimental variant. There is no sign of the six hour lag phase, indicating that under these conditions free amino acids do not play a role in the protection of the plasma proteins by acting as sacrificial antioxidants for the first six hours of peroxyl radical attack.

To determine whether a higher concentration of free amino acids could inhibit AAPH-induced protein oxidation in plasma, the experiment was repeated but this time dialysed plasma was supplemented with 500 mM of each of the five amino acids (Figure 3.3.1B). However, the rate of PrOOH formation in dialysed plasma remained unchanged from the rate in dialysed plasma supplemented with this higher concentration of amino acids.

Some amino acids are more susceptible to peroxide formation and may therefore be more efficient as sacrificial antioxidants than their less susceptible counterparts. To investigate whether the more reactive free amino acids in plasma were contributing to the lag phase, a literature search was undertaken to identify the free amino acids in order of reactivity (Gebicki and Gebicki, 1993). Tyrosine, proline, leucine, isoleucine, arginine, and histidine were the six amino acids with the highest reactivity, and were subsequently added to dialysed plasma at a concentration of 500 mM each. The supplemented plasma was incubated with 10 mM AAPH at 37˚C for a 24 hour period, with samples analysed via the acetic acid FOX assay at selected time points (Figure 3.3.1C). This result again showed no difference in the rate of PrOOH formation between dialysed plasma and supplemented plasma. This indicates that free amino acids do not act as sacrificial antioxidants and do not contribute to the six hour lag phase in peroxyl radical-induced PrOOH formation in plasma.


A)
PrOOH concentration (µM)
120
100
80
60
40
20
0
0
4
8
12
16
20
24
Time (Hour)
Dialysed plasma +AAPH
Dialysed plasma + AAPH +Amino acids


B)
PrOOH concentration (µM)
150
125
100
75
50
25
0
0
4
8
12
16
20
24
Time (Hour)
Dialysed plasma + AAPH
Dialysed plasma + AAPH + Amino Acids

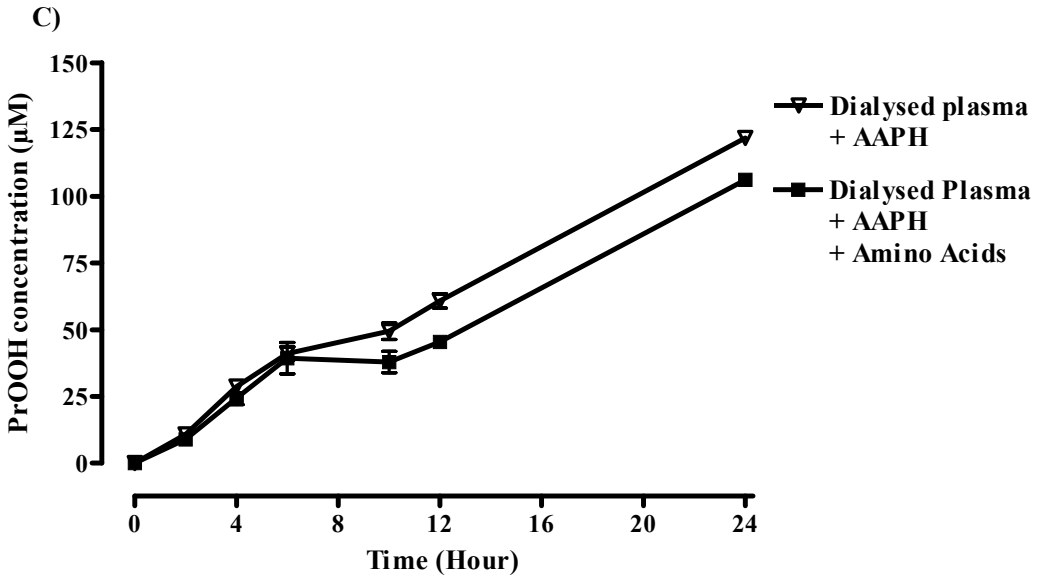


Figure 3.3.1 Peroxyl radical-mediated PrOOH formation in plasma incubated with and without free amino acids

Plasma was incubated at 37˚C in the presence of 10 mM AAPH with and without free amino acids. A) The free amino acids valine, alanine, glutamine, lysine and glycine were added to dialysed plasma at a concentration of 250 mM each. B) The free amino acids valine, alanine, glutamine, lysine and glycine were added to dialysed plasma at a concentration of 500 mM each. C) The free amino acids tyrosine, proline, leucine, isoleucine, arginine and histidine were added to dialysed plasma at a concentration of 500 mM each. Incubation was over a 24 hour period, with samples removed for analysis at selected time intervals. Protein hydroperoxides were measured by the acetic acid FOX assay and adjusted against blank controls and the corresponding time zero PrOOH values. Each data point represents the mean ± SEM of triplicate samples.

3.3.2 Effect of ascorbic acid and uric acid on AAPH-mediated PrOOH formation in dialysed plasma

Although free amino acids could not account for the lag phase during the AAPH-mediated oxidation of plasma proteins, other small molecular weight molecules remained as viable candidates. Yang, (2005) had previously added the antioxidants glutathione, bilirubin, ascorbic acid and uric acid to dialysed plasma, both separately and together, to observe their effect on the lag phase. Glutathione and bilirubin did not restore the lag phase but ascorbic acid supplementation created a two hour lag phase, while uric acid supplementation was associated with a four hour lag phase. HPLC analysis shows ascorbic acid has disappeared from plasma within 30–60 minutes, and so, is unlikely to be the sole instigator of the lag phase (Figure 3.2.1). From these results, it was suggested ascorbic acid and uric acid together might be producing the lag phase.

In the current study, ascorbic acid and uric acid were both added at a physiological concentration of 50 μM and 300 μM respectively, to the same dialysed plasma and then incubated for one hour to allow equilibration. The one hour equilibration period was required because without it, the uric acid and ascorbic acid did not appear to be present in the plasma. This was shown by adding uric acid and ascorbic acid to dialysed plasma and then measuring the antioxidants via HPLC prior to the one hour incubation. Only low levels of uric acid were detectable under such conditions (data not shown). This solution was then incubated with 10 mM AAPH at 37˚C for a 24 hour period, with samples removed at selected time points for PrOOH analysis using the acetic acid FOX assay. Results show that when uric acid and ascorbic acid are added to dialysed plasma, the six hour lag phase is restored (Figure 3.3.2). The result is identical during the first twelve hours to that observed in the undialysed plasma, but the dialysed plasma supplemented with uric acid and ascorbic acid subsequently keeps increasing while the undialysed plasma plateaus.

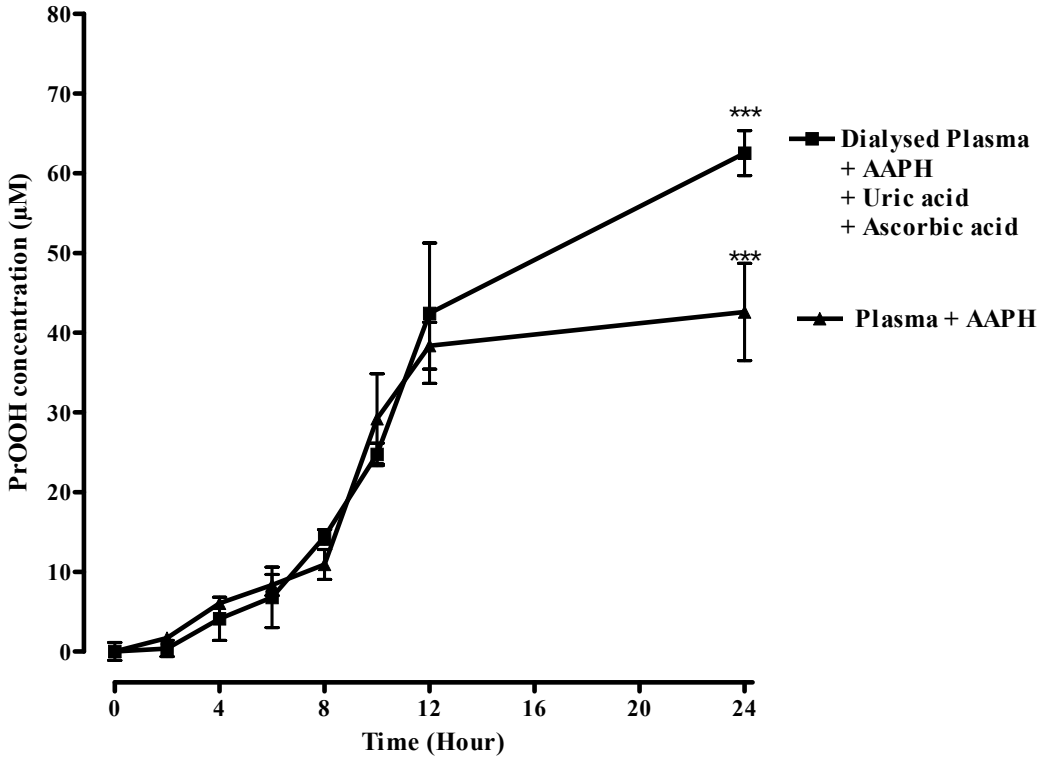


Figure 3.3.2 Peroxyl radical-mediated PrOOH formation in dialysed plasma supplemented with uric acid and ascorbic acid

Dialysed plasma supplemented with ascorbic acid and uric acid was incubated with 10 mM AAPH at 37˚C. Incubation was over a 24 hour period, with samples removed for analysis at two hour time intervals. Protein hydroperoxides were measured by the acetic acid FOX assay and adjusted against blank controls and the corresponding time zero PrOOH values. Each data point represents the mean ± SEM of triplicate samples. Significance values are shown between treatments.

3.3.3 Effect of uricase on AAPH-mediated PrOOH formation in plasma oxidation

Uric acid is a well-known, major water-soluble antioxidant found in human plasma. As Yang, (2005) and Figure 3.3.2 illustrate, uric acid also appears to contribute to the protection of plasma proteins from oxidative attack. To more fully confirm this antioxidants involvement in the PrOOH lag phase, the uric acid in undialysed plasma needed to be enzymatically removed and the loss of the lag phase subsequently noted. Uricase breaks down uric acid to produce allantoin and hydrogen peroxide and as a result, is a useful tool for blocking uric acid activity without removing or disrupting other molecules.

To distinguish whether uric acid was the source of the lag phase by removing it from the plasma, plasma was incubated at 37˚C with 1 mg/ml of uricase for 30 minutes, before adding 10 mM AAPH and incubating at 37˚C for a further 24 hour period. Samples were removed at selected time points and analysed for PrOOHs by the acetic acid FOX assay. Plasma incubated with 1 mg/ml uricase and no AAPH showed very little PrOOH formation, demonstrating that uricase was not causing autoxidation of the plasma (Figure 3.3.3). Unexpectedly, AAPH-induced PrOOH formation in plasma exposed to 1 mg/ml uricase was associated with a six hour lag phase and this was not significantly different from the lag phase for control uricase-free plasma. After this six hour lag phase, the increase of PrOOHs in the uricase treatment was almost double the level of protein peroxidation in uricase-free plasma. The rapid increase continued up to 24 hours and was followed by a decrease between 24 and 48 hours. Uricase-free plasma incubated with 10 mM AAPH showed the characteristic six hour lag phase and then the formation of PrOOHs between six and 24 hours, with a plateau occurring between 24 and 48 hours.

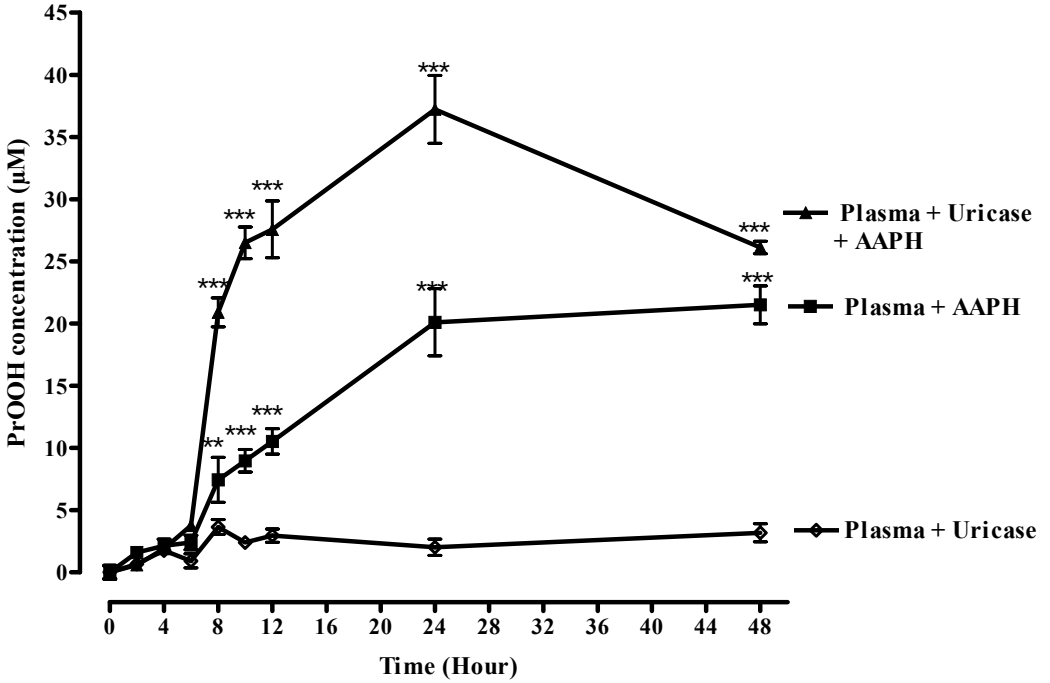


Figure 3.3.3 Peroxyl radical-mediated PrOOH formation in plasma incubated with and without 1 mg/mL uricase

Plasma was incubated at 37˚C in the presence and absence of 10 mM AAPH and 1 mg/mL uricase. Incubation was over a 24 hour period, with samples removed for analysis at selected time intervals. Protein hydroperoxides were measured by the acetic acid FOX assay and adjusted against blank controls containing and the corresponding time zero PrOOH values. Each data point represents the mean ± SEM of triplicate samples.

3.3.4 Effect of excess urate on AAPH-mediated PrOOH formation in undialysed plasma

If uric acid is responsible for the lag in AAPH-induced PrOOH formation, excess uric acid should extend this lag phase beyond six hours. To further explore the effect of uric acid on the six hour lag phase, excess uric acid was therefore added to undialysed. 600 µL of uric acid, which is double the normal physiological concentration found in plasma, was added to undialysed plasma and incubated for 60 minutes to allow the uric acid to equilibrate. The plasma was then incubated with 10 mM AAPH over a 24 hour period, with samples removed at selected time points for analysis of PrOOH formation using the acetic acid FOX assay. Results show that excess uric acid extended the lag phase from six hours to twelve hours, with very little PrOOH formation occurring during that time. The slight increase in PrOOH formation at six hours was not significant and had disappeared by eight hours, suggesting it was an experimental variant. From this result, we are able to conclude that uric acid contributes to the lag phase in PrOOH formation that is observed when plasma is oxidized by AAPH.

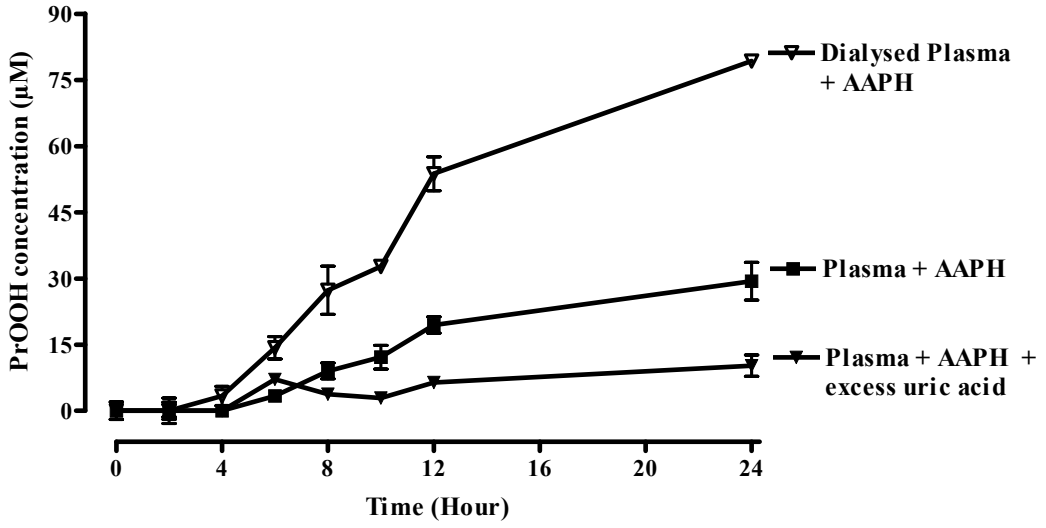


Figure 3.3.4 Peroxyl radical-mediated PrOOH formation in undialysed plasma incubated with excess uric acid

Excess uric acid was added to undialysed plasma and incubated with 10 mM AAPH at 37˚C. Incubation was over a 24 hour period, with samples removed for analysis at selected time intervals. Protein hydroperoxides were measured by the acetic acid FOX assay and adjusted against blank controls and the corresponding time zero PrOOH values. Each data point represents the mean ± SEM of triplicate samples.

3.4 Effect of AAPH-mediated lipid oxidation in human plasma

Through continuous monitoring of lipid oxidation products, Esterbauer *et al*. (1989) was able to determine that diene formation consists of three phases in LDL. The first is a lag phase where very few or no dienes are produced, followed by a propagation phase where dienes rapidly increase, and finally a decomposition phase where dienes decrease. Continuous measurement of diene formation via spectrophotometer is a good marker of lipid oxidation, as is the more specific measurement of cholesterol dienes via HPLC. The HPLC measurement was therefore used to investigate the phases of lipid oxidation, and to observe if there is a link between protein oxidation and lipid oxidation, plasma was incubated with 10 mM AAPH over a 24 hour period. Samples were removed at selected time points for the measurement of cholesterol dienes formation by HPLC analysis at an absorbance of 234 nm. The diene concentration was determined by the comparison with a standard of known concentration.

The results show a two hour lag phase where no cholesterol diene formation occurs, followed by a rapid increase in cholesterol diene formation over the rest of the 24 hours (Figure 3.4.1). Although the concentration continued to increase over the time course, there was a slowing of formation between 12 and 24 hours. Plasma incubated without AAPH showed no cholesterol diene formation confirming that no auto-oxidation was occurring in the plasma. AAPH-induced lipid oxidation commencing in the plasma after a two hour lag phase suggests that a difference exists between lipid and protein oxidation, with the latter commencing only after a six hour lag phase (Figure 3.4.2).

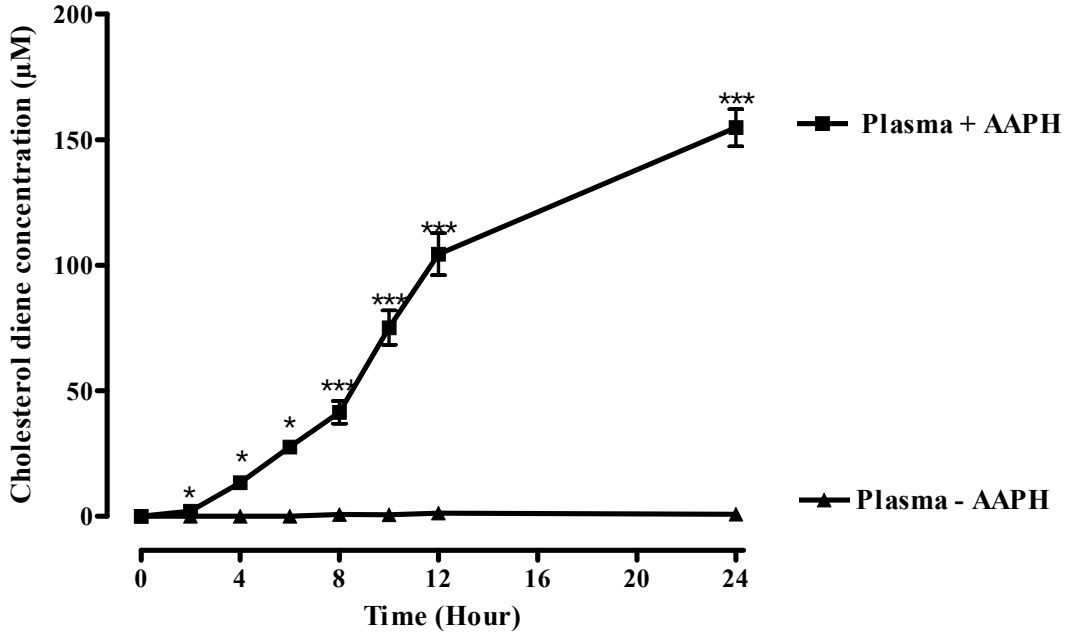
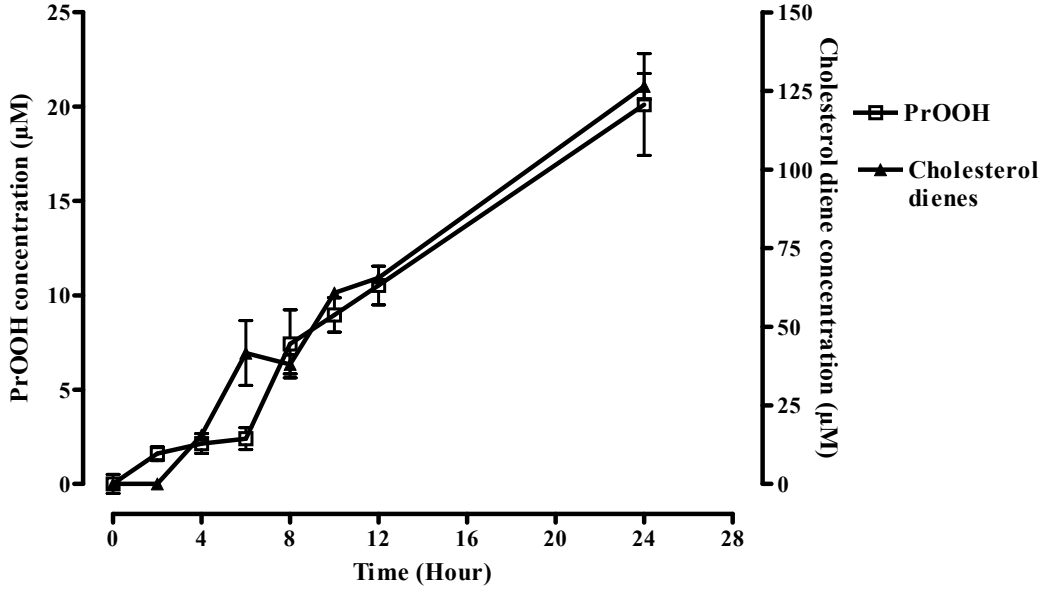


Figure 3.4.1 Peroxyl radical-mediated cholesterol diene formation in plasma
Human plasma was incubated in the presence and absence of 10 mM AAPH at 37˚C. Incubation was over a 24 hour, period with samples removed for analysis at selected time intervals. Cholesterol dienes were measured by HPLC analysis at an absorbance of 234 nm. Each data point represents the mean ± SEM of triplicate samples.



3.4.2 Peroxyl radical-mediated PrOOH formation and cholesterol diene formation in human plasma
Human plasma was incubated at 37˚C in the presence of 10 mM AAPH for a 24 hour period. Samples were removed at selected time intervals for analysis of PrOOH formation by the acetic acid FOX assay and cholesterol diene formation by HPLC analysis at an absorbance of 234 nm. Each data point represents the mean ± SEM of triplicate samples.

3.4.2 Cholesterol diene formation in dialysed plasma exposed to peroxyl radicals

Dialysing the plasma before incubation with 10 mM AAPH caused a loss of the six hour PrOOH lag phase (Figure 3.1.3), but it was not known whether a similar result would be observed for lipid oxidation. Examining the effect of dialysis on cholesterol diene formation may indicate whether the lag phases for the protein and lipid oxidation systems are connected. Human plasma was therefore dialysed in PBS and then incubated with 10 mM AAPH at 37˚C over a 24 hour time period. Samples were removed at selected time points for analysis of cholesterol diene concentration via HPLC analysis at an absorbance of 234 nm.

The two hour lag phase, where no cholesterol diene formation occurred, disappeared when the dialysed plasma was incubated with 10 mM AAPH (Figure 3.4.3). This agrees with the loss of the PrOOH lag phase (Figure 3.1.3). Cholesterol dienes formed immediately, and the concentration continued to increase in a linear fashion over the 24 hour period. The concentration of cholesterol dienes was also double the concentration usually observed in undialysed plasma incubated with 10 mM AAPH (Figure 3.4.1 compared to Figure 3.4.3). This indicates that one of the small molecule antioxidants must be causing the two hour lag phase and also reducing the radical flux, giving a lowered cholesterol diene concentration. Furthermore, dialyzable antioxidants appear to be the source of both the PrOOH and cholesterol diene lag phases. The rate of cholesterol diene formation over the first six hours is 19 μM/hour and, after the depletion of α-tocopherol in dialysed plasma at eight hours, there is a reduction in the rate of formation to 10.24 μM/hour (Figure 3.5.2). Perhaps the α-tocopherol acts as a pro-oxidant while present, and once depleted, the rate of oxidation can then reduce.

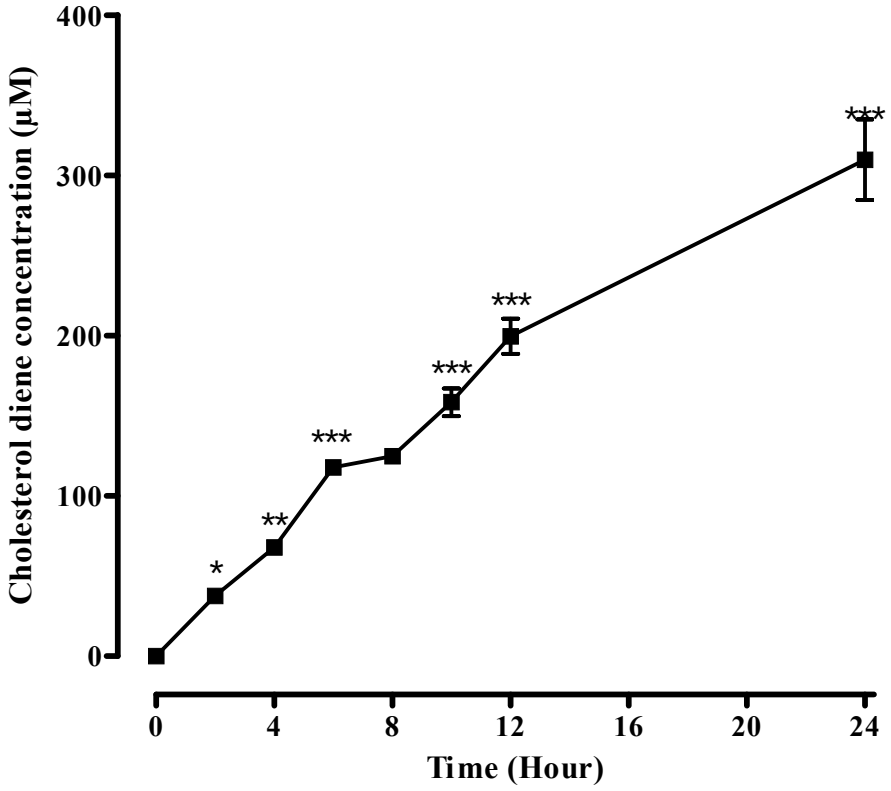


Figure 3.4.3 Peroxyl radical-mediated cholesterol diene formation in dialysed plasma

Human plasma was dialysed and incubated in the presence of 10 mM AAPH at 37˚C. Incubation was over a 24 hour period, with samples removed for analysis at selected time intervals. Cholesterol dienes were measured by HPLC analysis at an absorbance of 234 nm. Each data point represents the mean ± SEM of triplicate samples.

3.4.3 Effect of ascorbic acid on AAPH-mediated cholesterol diene formation in dialysed plasma

HPLC analysis of ascorbic acid established that this antioxidant was completely depleted from plasma after a 30 minute incubation at 37˚C. The removal of the two hour lag in of cholesterol diene formation by dialysis coincides approximately with the depletion of ascorbic acid in plasma. This suggests that ascorbic acid may be causing the two hour lag phase and reducing the radical flux. To investigate this theory, ascorbic acid was added to dialysed plasma and incubated at 37˚C for one hour to allow the ascorbic acid to equilibrate with the dialysed plasma. The dialysed plasma was then incubated with 10 mM AAPH at 37˚C over a 24 hour period. Samples were removed at selected time points, with cholesterol diene concentration measured by HPLC analysis at an absorbance of 234 nm.

Dialysed plasma, supplemented with ascorbic acid, showed very little cholesterol diene formation in the first two hours (Figure 3.4.4). This restoration of the lag phase indicates that ascorbic acid is responsible for protecting the plasma lipids from radical attack during the first two hours. Although the two hour lag phase reproduces the result in undialysed plasma, the AAPH-induced formation of cholesterol dienes after the lag phase is more similar to the undialysed but unsupplemented plasma. Due to there being no change in the rate of the propagation phase, ascorbic acid must not be reducing the radical flux post-lag phase. Ascorbic acid has been found to act as a co-antioxidant regenerating α-tocopherol from the α-tocopherol radical and therefore inhibiting tocopherol-mediated peroxidation (TMP) (Upston *et al*., 1999). Perhaps ascorbic acid is fulfilling a similar function in this study, regenerating α-tocopherol and thereby inhibiting lipid oxidation while the ascorbic acid is present.

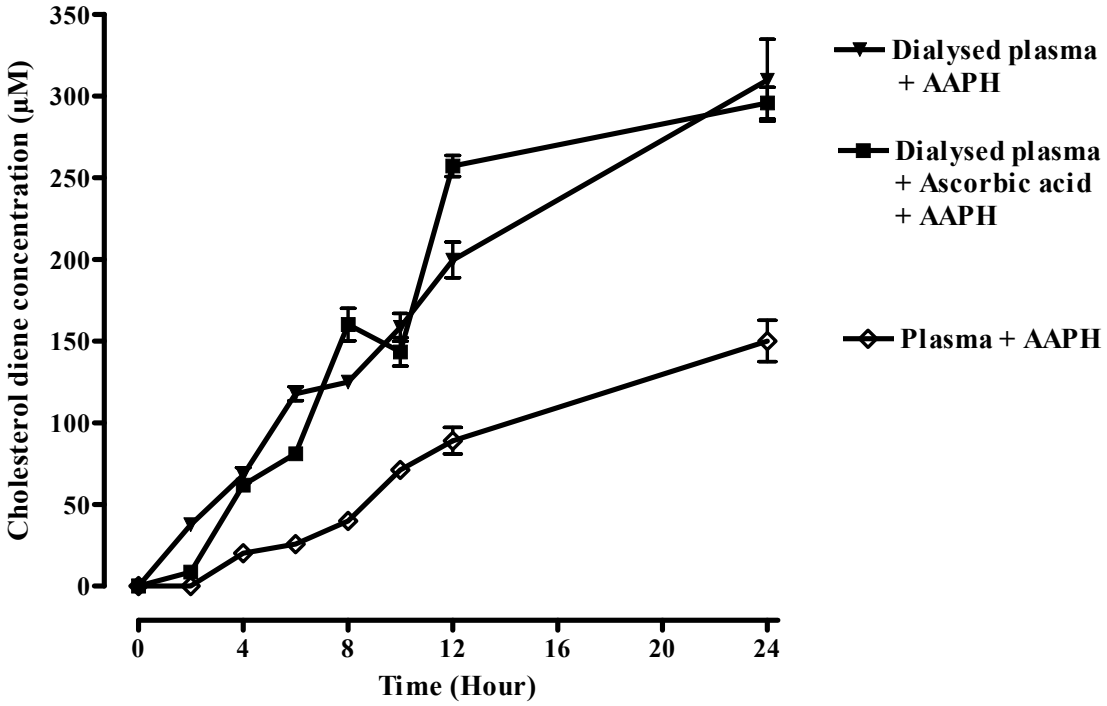


Figure 3.4.4 Peroxyl radical-mediated cholesterol diene formation in dialysed plasma supplemented with ascorbic acid

Dialysed plasma was supplemented with ascorbic acid and incubated with 10 mM AAPH at 37˚C. Incubation was over a 24 hour period, with samples removed for analysis at selected time intervals. Cholesterol dienes were measured by HPLC analysis at an absorbance of 234 nm. Each data point represents the mean ± SEM of triplicate samples.

3.4.4 Effect of uric acid on AAPH-mediated cholesterol diene formation in dialysed plasma

Figure 3.4.4 shows a return of the lag phase when ascorbic acid is present in plasma but does not reduce the rate of cholesterol diene formation post-lag phase. This suggests another small molecular weight antioxidant is responsible for the reduction in the rate of cholesterol diene formation once ascorbic acid is depleted and lipid peroxidation begins. Plasma protein oxidation experiments found that ascorbic acid and uric acid were responsible for the six hour PrOOH lag phase, with uric acid providing the antioxidant activity after the depletion of ascorbic acid (Figure 3.3.2). This suggests uric acid may also be responsible for the reduction in the rate of cholesterol diene formation in plasma post-lag phase. This involvement of uric acid in the reduction of the radical flux was therefore investigated by adding uric acid to dialysed plasma and incubating at 37˚C for one hour to allow equilibration. The supplemented dialysed plasma was then incubated with 10 mM AAPH at 37˚C over a 24 hour period. Samples were removed at selected time points, with cholesterol diene concentration measured by HPLC analysis at an absorbance of 234 nm.

There was a reduction in the rate of cholesterol diene formation during the first two hours compared to dialysed but unsupplemented plasma but, unlike ascorbic acid supplementation, the lag phase was not restored (Figure 3.4.5). The reduced rate of cholesterol diene formation continued over the 24 hour period and was more similar to the trend in undialysed plasma than dialysed but unsupplemented plasma, especially between two and twelve hours. During this time dialysed but unsupplemented plasma produced 12. 2 μM cholesterol dienes per hour, whereas dialysed plasma supplemented with uric acid and undialysed plasma produced 11 μM and 6.64 μM cholesterol dienes per hour respectively. Between 12 to 24 hours the cholesterol diene concentration in dialysed, supplemented plasma remained significantly below the concentration in the dialysed unsupplemented plasma. It did, however, increase significantly above the level of cholesterol dienes in undialysed plasma. This suggests that uric acid is not responsible for the two hour lag phase but is responsible for reducing the radical flux by scavenging the radicals after the depletion of ascorbic acid.

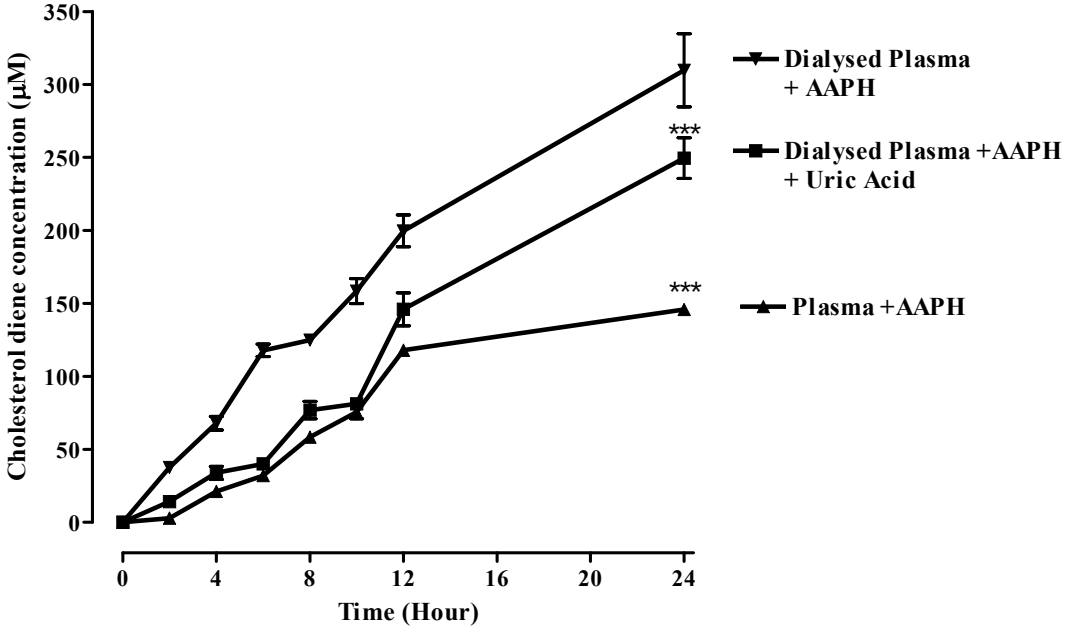


Figure 3.4.5 Peroxyl radical-mediated cholesterol diene formation in dialysed plasma supplemented with uric acid

Dialysed plasma was supplemented with uric acid and incubated with 10 mM AAPH at 37˚C. Incubation was over a 24 hour period, with samples removed for analysis at selected time intervals. Cholesterol dienes were measured by HPLC analysis at an absorbance of 234 nm. Each data point represents the mean ± SEM of triplicate samples. Significance values shown are between treatments.

3.4.5 Effect ascorbic acid and uric acid on AAPH-mediated cholesterol diene formation in dialysed plasma

The previous two experiments (Figures 3.4.4 and 3.4.5) indicate that ascorbic acid is responsible for the two hour lag phase, when no cholesterol dienes are formed. Uric acid was found to be responsible for the lowered rate of cholesterol diene formation post-lag. It was possible that incubating dialysed plasma with both antioxidants simultaneously would restore all kinetics observed with undialysed plasma. To investigate this theory, dialysed plasma was supplemented with both ascorbic acid and uric acid and equilibrated for one hour at 37˚C. The supplemented plasma was then incubated with 10 mM AAPH over a 24 hour time period, with samples removed at selected time intervals for cholesterol diene formation by HPLC analysis at an absorbance of 234 nm.

The two hour lag phase was largely restored, and a reduction in cholesterol diene formation was also observed, when dialysed plasma was supplemented with both ascorbic acid and uric acid (Figure 3.4.6). The trend was very similar to that observed when undialysed plasma is incubated with AAPH. However, as observed in Figure 3.4.5, the cholesterol diene concentration in the dialysed supplemented plasma did become significantly elevated compared to the undialysed plasma treatment between 12 and 24 hours.

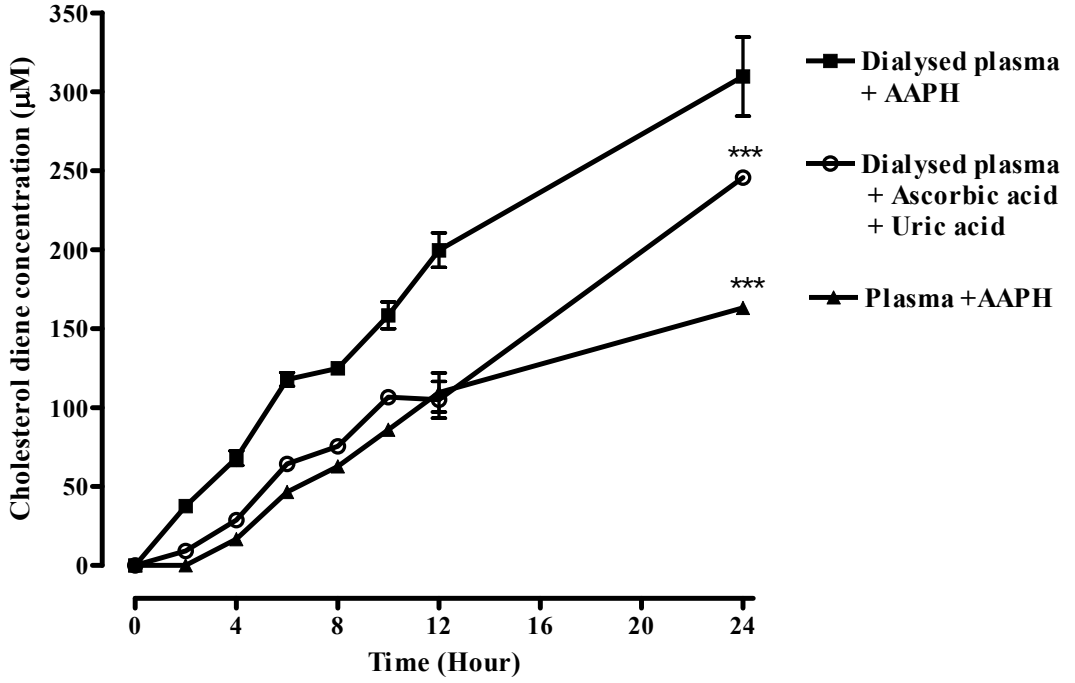


Figure 3.4.6 Peroxyl radical-mediated cholesterol diene formation in dialysed plasma supplemented with uric acid and ascorbic acid

Dialysed plasma was supplemented with uric and ascorbic acid and incubated with 10 mM AAPH at 37˚C. Incubation was over a 24 hour period, with samples removed for analysis at selected time intervals. Cholesterol dienes were measured by HPLC analysis at an absorbance of 234 nm. Each data point represents the mean ± SEM of triplicate samples. The significance values shown are for between treatments.

3.5 α-Tocopherol (Vitamin E) concentration in plasma exposed to peroxyl radicals

3.5.1 α-Tocopherol concentration in plasma

α-Tocopherol is the main antioxidant in LDL, with an average of six molecules per lipoprotein particle (Esterbauer *et al*., 1989). As such, it is also expected to be a significant antioxidant in plasma. To investigate the activity of α-tocopherol in plasma undergoing oxidation, plasma was incubated with and without 10 mM AAPH at 37˚C over a 24 hour time period. Samples were removed at selected time points and solvent-extracted α-tocopherol measured by HPLC analysis using the fluorescence detector with an excitation at 292 nm and an emission at 335 nm.

Plasma incubated in the absence of 10 mM AAPH shows very little change in α-tocopherol concentration with the concentration staying relatively constant over the 24 hour incubation period (Figure 3.5.1). The slight increase in α-tocopherol concentration within the first two hours of incubation is too small to be significant. In the presence of AAPH, the concentration of α-tocopherol declines but this only begins after two hours. By eight hours the AAPH-induced loss of α-tocopherol reaches a plateau, with a very small and insignificant decrease observed between eight and 24 hours.

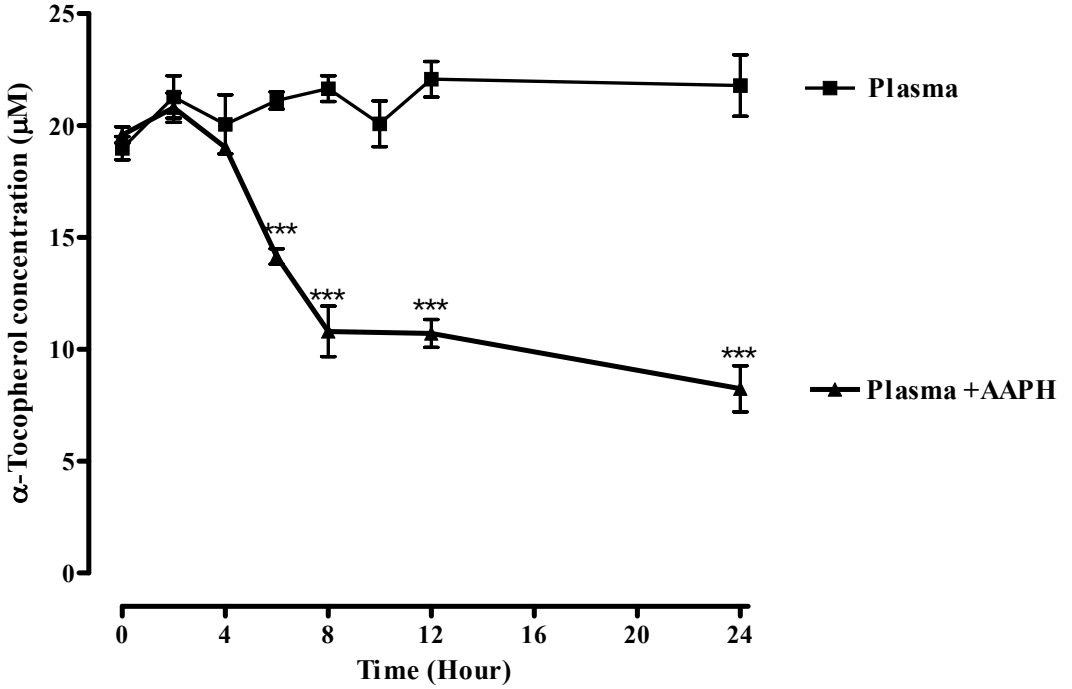


Figure 3.5.1 α-Tocopherol loss in plasma oxidized by the peroxyl radical initiator AAPH

Plasma was incubated in the presence and absence of 10 mM AAPH at 37˚C. The incubation was over a 24 hour period, with samples removed for analysis at selected time intervals. α-Tocopherol was measured by HPLC analysis, using the fluorescence detector with an excitation of 292 nm and an emission of 335 nm. Each data point represents the mean ± SEM of triplicate samples.

3.5.2 α-Tocopherol concentration in dialysed plasma oxidized by AAPH

To investigate the effect of plasma dialysis on α-tocopherol levels, dialysed plasma was incubated with 10 mM AAPH over a 24 hour period with α-tocopherol monitored by HPLC analysis. Surprisingly, the α-tocopherol concentration started declining immediately and was completely depleted by eight hours (Figure 3.5.2). This suggested that dialysis removed a small molecular weight molecule and that this molecule was responsible for protecting α-tocopherol from peroxyl radical attack. An interaction between α-tocopherol and a soluble co-antioxidant has previously been described during studies using purified lipoprotein (Esterbauer *et al*., 1992). This type of interaction could explain the source of the cholesterol diene lag phase.

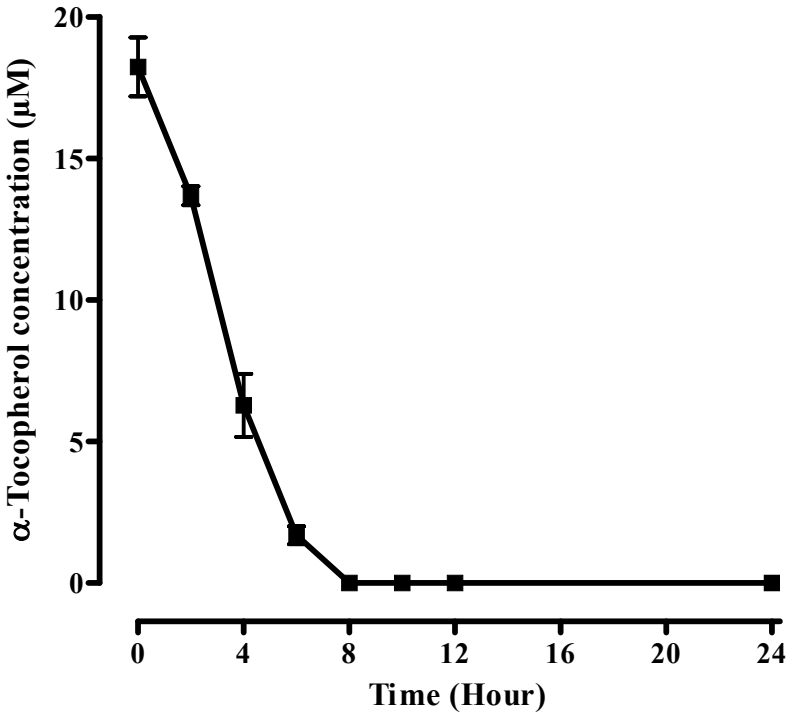


Figure 3.5.2 α-Tocopherol concentration in dialysed plasma oxidized by the peroxyl radical initiator AAPH
Dialysed plasma was incubated with 10 mM AAPH at 37˚C. Incubation was over a 24 hour period, with samples removed for analysis at selected time intervals. α-Tocopherol was measured by HPLC analysis, using the fluorescence detector with an excitation of 292 nm and an emission of 335 nm. Each data point represents the mean ± SEM of triplicate samples.

3.5.3 Effect of ascorbic acid on α-tocopherol concentration in dialysed plasma oxidised by AAPH

Due to the known ability of ascorbate to interact with the α-tocopherol radical and recycle the radical back to α-tocopherol (Mendiratta *et al*., 1997; Schafer *et al*., 2003; Retsky *et al*., 1993; Suh *et al*., 2003; Nieto *et al*., 2000; Gebicki *et al*., 2000b) the effect of supplemented ascorbate on plasma α-tocopherol levels was examined. Dialysed plasma was therefore, supplemented with ascorbic acid and incubated for one hour at 37˚C to allow time for equilibration. The supplemented plasma was then incubated with 10 mM AAPH over a 24 hour time period and samples removed at selected time points for the measurement of α-tocopherol by HPLC.

When the dialysed plasma was supplemented with ascorbic acid, the slight but significant increase in α-tocopherol concentration within the first two hours of incubation was restored (Figure 3.5.3). However, the concentration dropped dramatically after the two hours with complete depletion of α-tocopherol levels by ten hours. This was two hours longer than in the dialysed plasma incubated with 10 mM AAPH, indicating that the ascorbic acid contributes to the protection of α-tocopherol in the first two hours of incubation, but does not reduce the radical flux post-lag phase. This is further evidence of α-tocopherol and ascorbic acid interacting, thereby restoring the α-tocopherol levels for the period ascorbic acid is present in the plasma.

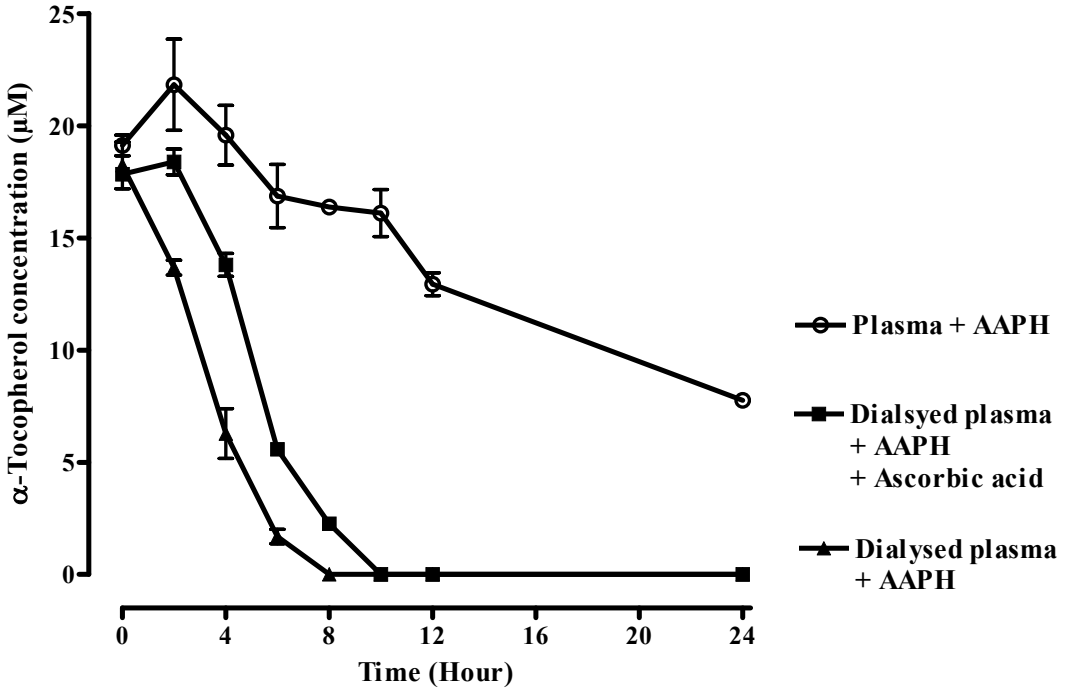


Figure 3.5.3 α-Tocopherol concentration in dialysed plasma, supplemented with ascorbic acid, and oxidized by the peroxyl radical initiator AAPH

Dialysed plasma was supplemented with ascorbic acid and incubated with 10 mM AAPH at 37˚C. Incubation was over a 24 hour period, with samples removed for analysis at selected time intervals. α-Tocopherol was measured by HPLC analysis, using the fluorescence detector with an excitation of 292 nm and an emission of 335 nm. Each data point represents the mean ± SEM of triplicate samples.

3.5.4 Effect of uric acid on the α-tocopherol concentration in dialysed plasma oxidised by AAPH

Uric acid, in combination with ascorbic acid has previously been shown to protect plasma proteins from peroxyl radical attack during the first six hours of incubation, producing a lag phase (Figure 3.3.2) Uric acid also appears to reduce the radical flux post-lag phase, lowering the rate of both PrOOH and cholesterol diene formation in plasma exposed to AAPH (Figures 3.3.2 and 3.4.5). The preceding experiment (Figure 3.5.3) showed that ascorbic acid is only responsible for an initial two hour protection of α-tocopherol but not the rest of the 24 hours. To investigate whether uric acid plays a role in sparing α-tocopherol directly or indirectly, dialysed plasma was supplemented with uric acid and incubated for one hour at 37˚C to allow equilibration. The supplemented dialysed plasma was then incubated with 10 mM AAPH over a 24 hour period and samples were removed at selected time points to be measured for α-tocopherol by HPLC.

AAPH-mediated α-tocopherol loss was significantly slower in dialysed plasma, supplemented with uric acid, than in dialysed but unsupplemented plasma (Figure 3.5.4). By eight hours, all α-tocopherol in the latter treatment. Even after 24 hours, α-tocopherol in the dialysed supplemented plasma had not been completely depleted. The loss of α-tocopherol did, however occur at a slightly faster rate in this treatment than in the undialysed plasma. Despite this, the above observations suggest that uric acid is able to protect α-tocopherol from attack by free radicals.

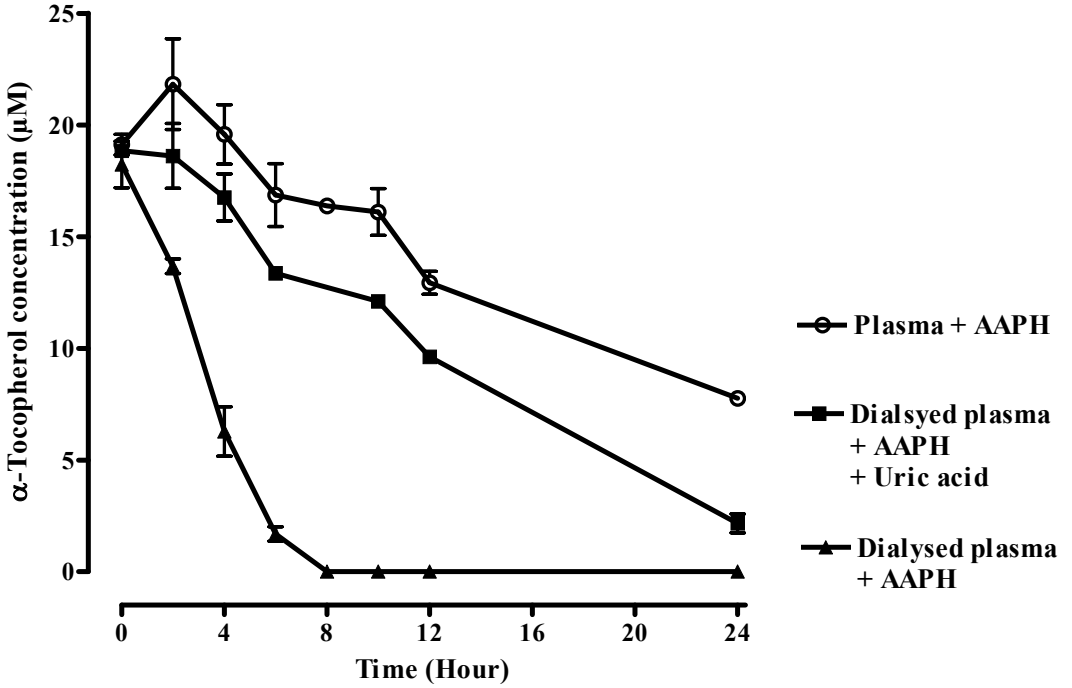


Figure 3.5.4 α-Tocopherol concentration in dialysed plasma supplemented with uric acid, and oxidized by the peroxyl radical initiator AAPH

Dialysed plasma was supplemented with uric acid and incubated with 10 mM AAPH at 37˚C. Incubation was over a 24 hour period, with samples removed for analysis at selected time intervals. α-Tocopherol was measured by HPLC analysis, using the fluorescence detector with an excitation of 292 nm and an emission of 335 nm. Each data point represents the mean ± SEM of triplicate samples.

3.5.5 Effect of uric acid and ascorbic acid together on the α-tocopherol concentration in dialysed plasma oxidized by AAPH

The finding that ascorbic acid protected α-tocopherol for the first two hours (Figure 3.5.3) and uric acid slowed α-tocopherol loss over the 24 hours (Figure 3.5.4), suggested that both ascorbic acid and uric acid together may completely account for the protection of α-tocopherol. To investigate this, dialysed plasma was supplemented with both ascorbic acid and uric acid and incubated for one hour at 37˚C to allow the uric acid and ascorbic acid to equilibrate with the dialysed plasma. This dialysed and supplemented plasma was then incubated with 10 mM AAPH over a 24 hour period, with samples removed at selected time points to be measured for α-tocopherol by HPLC.

Dialysed plasma, supplemented with both ascorbic acid and uric acid, showed a protection of α-tocopherol so strong that these antioxidants almost completely restored α-tocopherol to the levels observed when undialysed plasma was incubated with 10 mM AAPH (Figure 3.5.5). In fact, there was no statistical difference until the 24 hour time point. The lack of statistical significance until 24 hours was previously noted when comparing PrOOH formation between undialysed plasma and dialysed plasma supplemented with both ascorbic and uric acid (Figure 3.3.2).

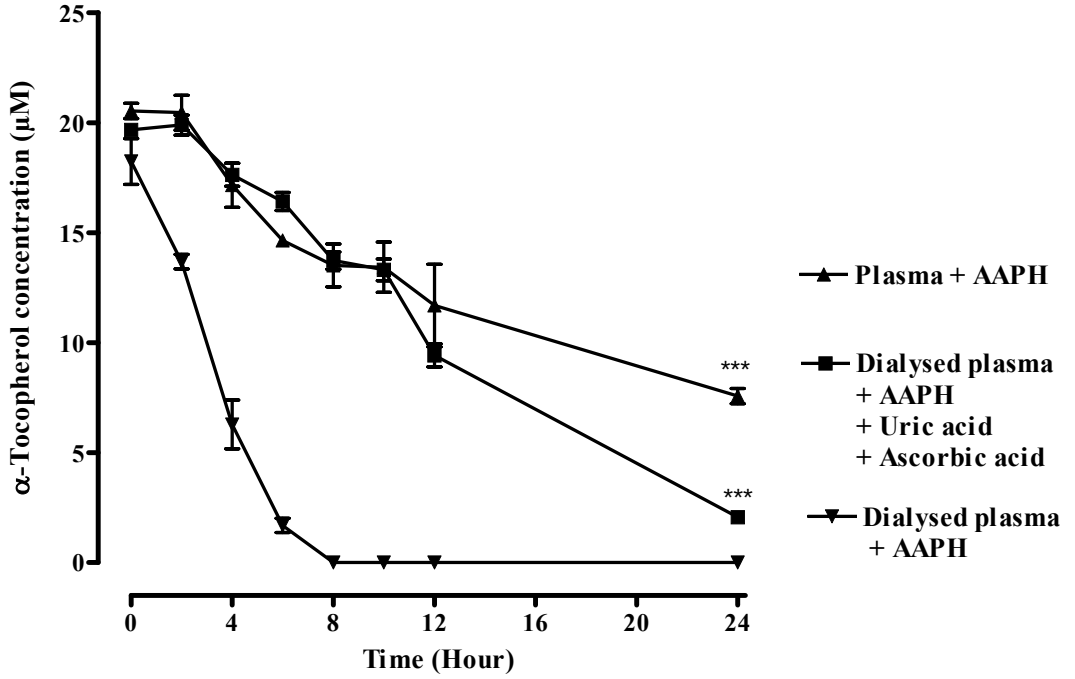


Figure 3.5.5 α-Tocopherol concentration in dialysed plasma supplemented with uric acid and ascorbic acid, and oxidized by the peroxyl radical initiator AAPH
Dialysed plasma was supplemented with uric acid and ascorbic acid and incubated with 10 mM AAPH at 37˚C. Incubation was over a 24 hour period, with samples removed for analysis at selected time intervals. α-Tocopherol was measured by HPLC analysis, using the fluorescence detector with an excitation of 292 nm and an emission of 335 nm. Each data point represents the mean ± SEM of triplicate samples. Significance shown for between treatments.

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3.6 Protein-bound 3,4-dihydroroxyphenylalanine (PB-DOPA) production on BSA

3.6.1 PB-DOPA production on BSA exposed to X-ray radiolysis

Protein-bound 3,4-dihydroroxyphenylalanine (PB-DOPA) is a major product of radical attack on tyrosine residues in peptides and proteins (Simpson *et al*., 1992; Gieseg *et al*., 1993). X-rays are one way of producing radicals that oxidise the tyrosine residues on proteins to PB-DOPA. A previous investigation by Parkes, (2005), using both HPLC analysis and the ED-DOPA assay, showed that the formation of PB-DOPA in BSA exposed to X-rays is linear and lacks a lag phase. In order to reproduce the results observed by Parkes, (2005), BSA solutions in PBS were irradiated and exposed to a constant source of oxygen. BSA solutions of 1 mg/ml and 8 mg/ml of BSA solution were used by Parkes, (2005) to investigate the formation of PB-DOPA and both will be used in this study to determine which concentration permits the best detection of PB-DOPA. BSA was placed in an X-ray machine (Philips model PW2264-20 tungsten x-ray generator, 35kV, 30mA) and exposed to a dose rate of 5.8 Gy/min, under constant oxygen bubbling, for 30 and 60 minutes. Samples were then analysed for PB-DOPA formation by HPLC analysis and the ED-DOPA assay.

Both 1 mg/ml and 8 mg/ml produced PB-DOPA in sufficient concentrations to be detected by both types of analysis (Figure 3.6.1). In agreement with Parkes, (2005), the concentration of PB-DOPA increased as the length of irradiation increased and it did so in a linear fashion with no detectable lag phase. Detection of PB-DOPA by the ED-DOPA assay gave an r^2 value = 0.989 for 8 mg/ml BSA solution, while the 1 mg/ml BSA solution had an r^2 value = 0.993. HPLC analysis gave an r^2 value of 0.882 for the 8 mg/ml BSA solution, while the 1 mg/ml BSA solution had an r^2 value of 0.894. ED-DOPA analysis detected a much higher concentration of DOPA than the HPLC analysis, 30 times higher for 1 mg/ml and 12.5 times higher for 8 mg/ml.

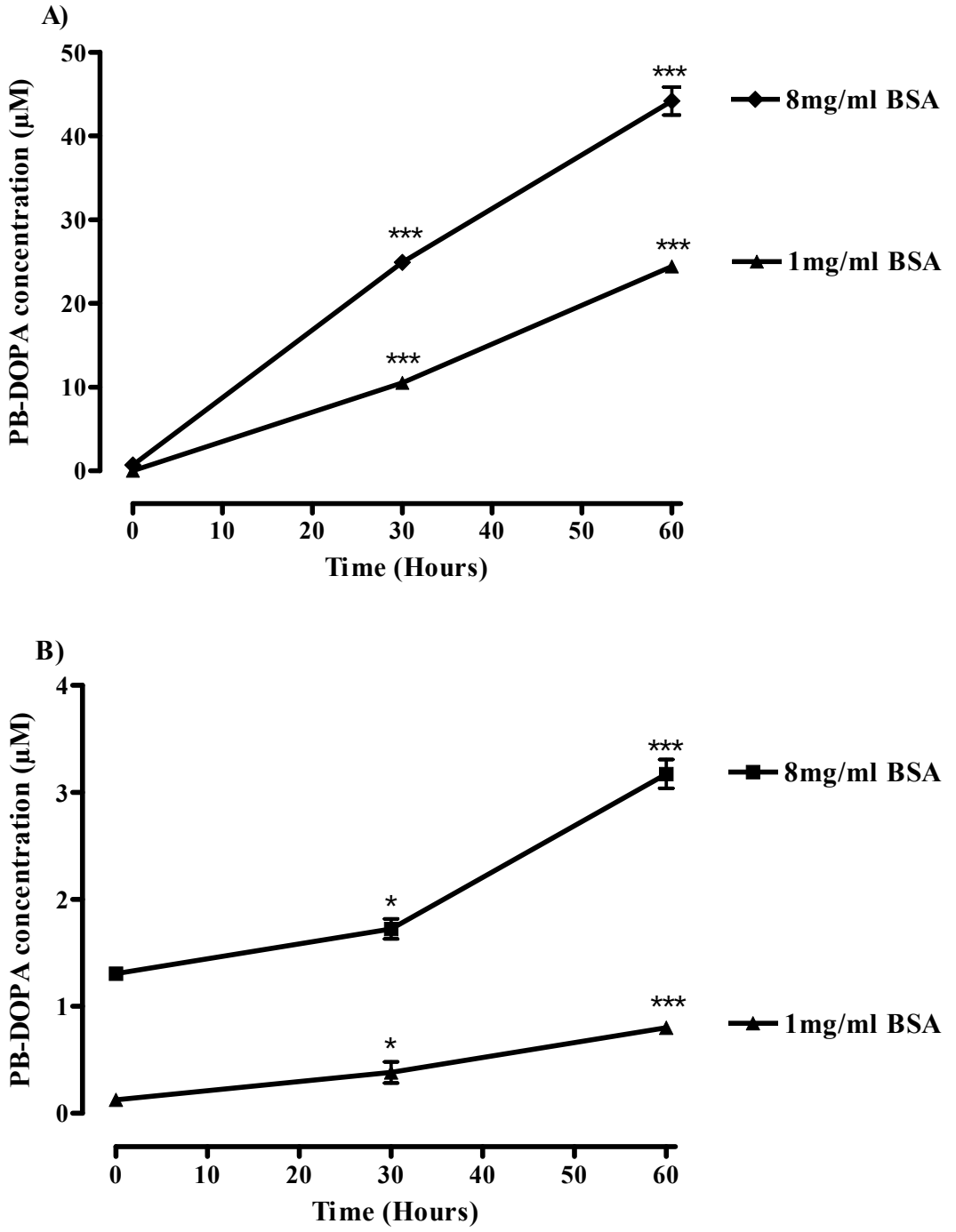

Figure 3.6.1 PB-DOPA production in irradiated BSA
1 mg/ml and 8 mg/ml BSA were exposed to X-ray radiation. Samples from 0, 30 and 60 minutes of exposure were analysed by A) ED-DOPA and B) HPLC. Time can be converted to dose rate using the dose rate of 5.8 Gy/min. Each data point represents the mean ± SEM of triplicate samples.

3.6.2 PB-DOPA production on BSA exposed to X-rays over a 60 minute time period

Although 1 mg/ml and 8 mg/ml BSA were both shown to be suitable concentrations for detecting PB-DOPA (Figure 3.6.1), additional experiments were conducted using only 1 mg/ml BSA. Figure 3.6.1 did support the existence of a linear trend in irradiation-induced PB-DOPA formation on BSA, with a lack of detectable lag phase. However, very few sample time points were collected in that preliminary study. PB-DOPA formation during exposure to X-rays was therefore more thoroughly investigated in the additional experiments by sampling eight time points between zero and 60 minutes. Furthermore, measuring PB-DOPA concurrently by both ED-DOPA and HPLC during these experiments enabled a correlation between the two assays to be drawn. This is of interest because Parkes, (2005) and Figure 3.6.1 noted that PB-DOPA values are higher when evaluated by ED-DOPA compared to HPLC. BSA was therefore placed in eight labelled vials at a concentration of 1 mg/ml, and exposed to various radiation times between zero and 60 minutes before being analysed PB-DOPA by both the ED-DOPA assay and HPLC analysis.

The correlation between the ED-DOPA assay and HPLC analysis was high, with an r^2 value = 0.989 (Figure 3.6.2). The line of best fit for these two assays yielded an equation of $y=7.841x + 0.108$. Irradiation at selected time points over 60 minutes confirms more fully that the PB-DOPA concentration measured by both the ED-DOPA assay and HPLC analysis increases in a linear fashion over time (Figure 3.6.3). However, the PB-DOPA measured by the ED-DOPA assay is almost eight times higher than the concentration measured by HPLC analysis at each corresponding irradiation time point.

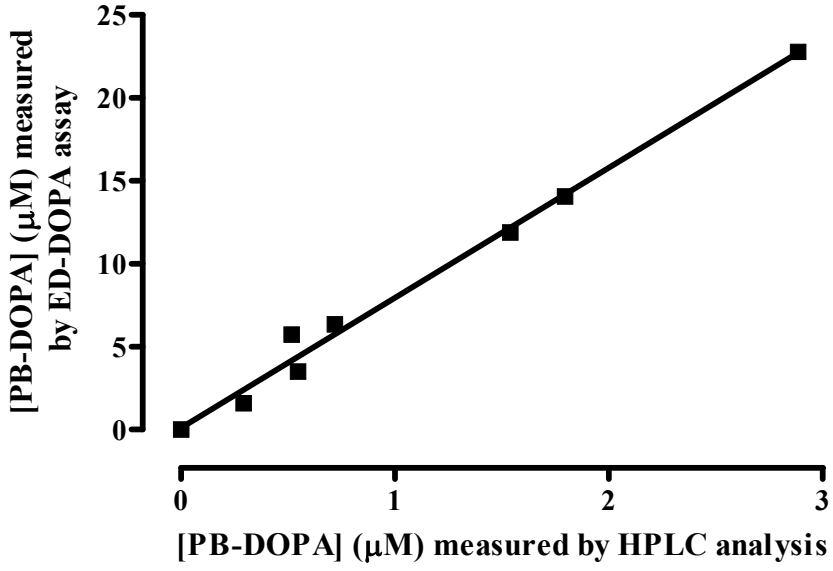

Figure 3.6.2 Correlation between ED-DOPA assay and HPLC analysis
The equation of this best fit line is y=7.84x + 0.108 with an r^2 value = 0.989.

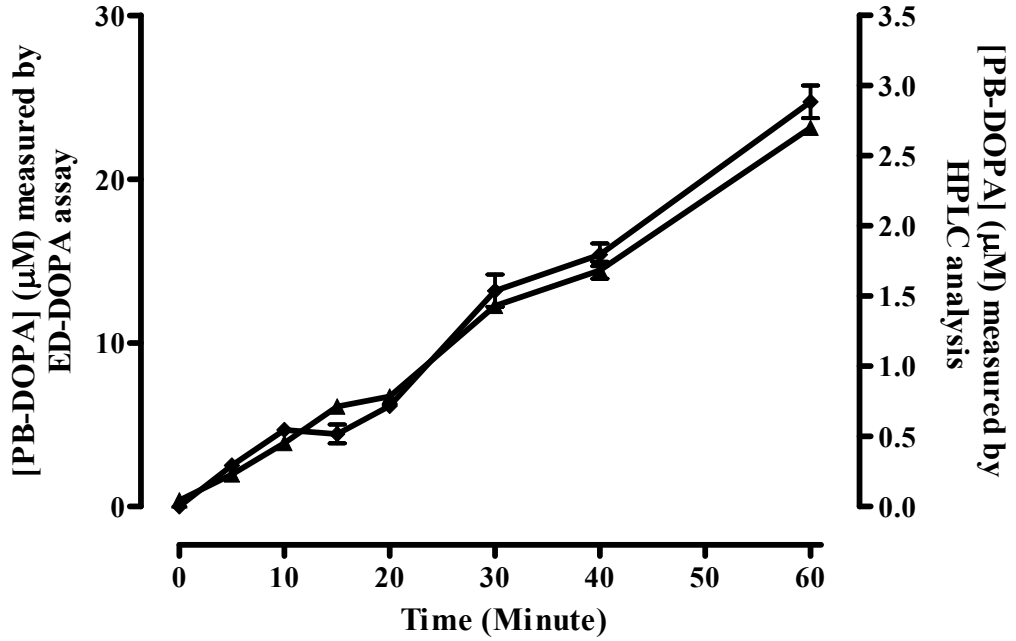

Figure 3.6.3 PB-DOPA production in irradiated BSA
1 mg/ml BSA was exposed to radiation. Samples were collected at selected exposure times, up to 60 minutes, and analysed for PB-DOPA formation by both the ED-DOPA assay and HPLC analysis. Time can be converted to dose rate using a dose rate of 5.8 Gy/min. Each data point represents the mean ± SEM of triplicate samples.

3.6.3 PB-DOPA formation on BSA exposed to radicals produced by the Fenton reaction

Oxidative damage is often related to the Fenton reaction, which is the one electron reduction of hydrogen peroxide (H_2O_2) by transition metal ions to produce the most reactive and damaging ROS, the hydroxyl radical ($OH^{\cdot}$) (Reaction 21). The resulting Fenton-derived radicals are thought to cause additional injury to the cell components especially proteins.

(21) $Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^- + OH^{\cdot}$

Since oxidative damage can be initiated by the Fenton reaction, it has been proposed that exposing BSA to Fenton radicals may produce PB-DOPA. This was investigated by Simpson and Dean (1990) and Gieseg *et al*. (1993), who found that PB-DOPA was indeed formed on BSA exposed to radicals derived from the Fenton reaction. In order to reproduce the results of these previous investigations, 1 mg/ml of BSA was incubated with different concentrations of copper chloride and hydrogen peroxide for 30 minutes at 37˚C. Catalase was the added to stop the reaction and remove the hydrogen peroxide, followed by the addition of chelex to remove the free metal ions. Samples were analysed for PB-DOPA, using the ED-DOPA assay and HPLC analysis.

In agreement with previous findings, significant concentrations of PB-DOPA were produced when BSA was exposed to radicals derived from the Fenton reaction (Figure 3.6.4). Both the ED-DOPA assay and HPLC analysis show PB-DOPA formation increasing with increasing concentrations of copper in a linear fashion between 40 μM and 100 μM of copper. The concentration of PB-DOPA was almost 25 times higher, when measured by the ED-DOPA assay. This, along with Figures 3.6.1 and 3.6.3 indicate that the ED-DOPA assay is measuring PB-DOPA and its oxidation products while HPLC analysis detects only PB-DOPA.

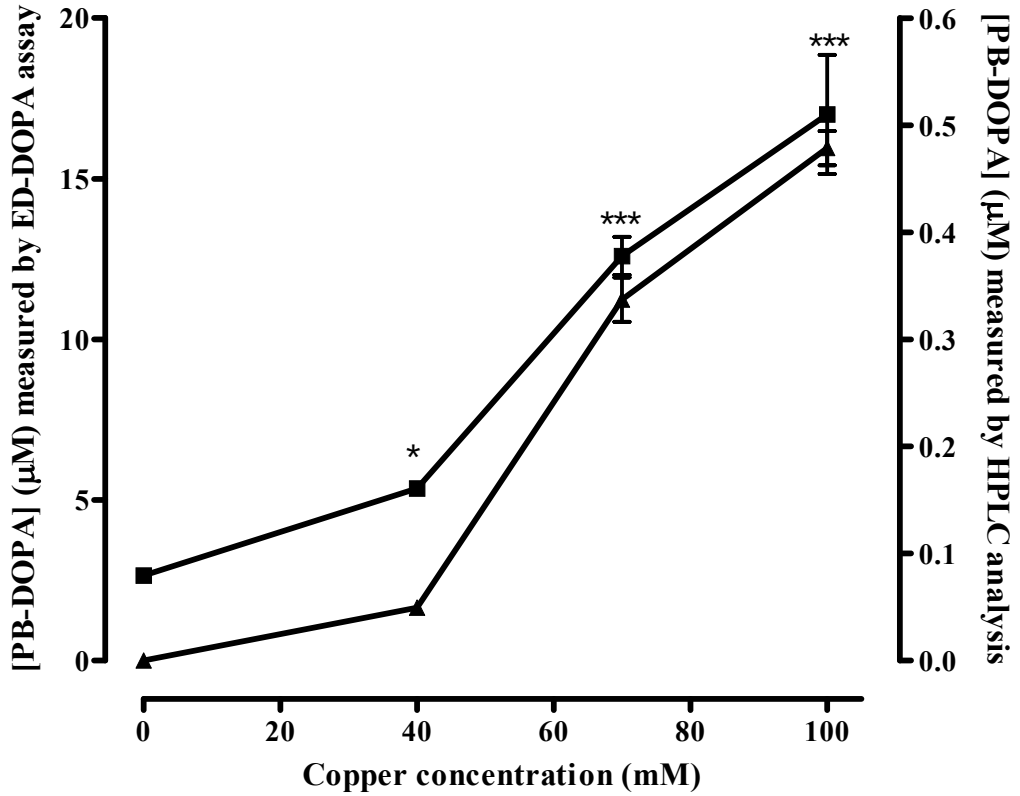

Figure 3.6.4 PB-DOPA production on BSA exposed to copper chloride
1mg/ml BSA was exposed to 0, 40, 70, and 100 μM of copper chloride and analysed for PB-DOPA production by the A) ED-DOPA assay and B) HPLC analysis. Time can be converted to dose rate using the dose rate of 5.8 Gy/min. Each data point represents the mean ± SEM of triplicate samples.

3.6.4 Comparison of the techniques for detecting PB-DOPA: ED-DOPA assay and HPLC analysis.

Irrespective of the oxidation source, the ED-DOPA assay consistently detected higher concentrations of PB-DOPA than HPLC analysis. This result indicates that the ED-DOPA assay does not specifically measure PB-DOPA, but is measuring PB-DOPA oxidation products as well. This can make results inaccurate and misleading, leading to false high readings of "PB-DOPA". Therefore, it was investigated to see if this higher concentration of PB-DOPA was correct or whether this ED-DOPA assay was indeed picking up other products and giving deceptive results of the concentration of PB-DOPA present.

To investigate the inaccuracy of the ED-DOPA assay, BSA was dissolved at 1 mg/ml in unchelexed sodium phosphate buffer and exposed to X-rays under constant oxygen bubbling for 60 minutes. 110 μM copper sulphate was subsequently added to half the samples that had been irradiated for 60 minutes and half the samples that had not been exposed to radiation. The remaining half of each time point (0 and 60 minutes) served as controls, with no copper sulphate added. Catalase was added to all treatments to remove hydrogen peroxide and chelex was added to remove transition metals before analysing samples using the ED-DOPA assay and HPLC analysis.

Incubating the irradiated BSA with copper sulfate promotes the oxidation of any PB-DOPA that has formed. The HPLC method is specific for PB-DOPA, not its oxidation products and as expected, this assay detected less PB-DOPA after treating the BSA with copper (Figure 3.6.5). By contrast, the ED-DOPA assay is thought to measure both PB-DOPA and its oxidation products. Indeed, after incubating irradiated BSA with copper an increase in "PB-DOPA" concentration was observed by the ED-DOPA assay. From these results it can be concluded that the ED-DOPA assay does not present a representative concentration of PB-DOPA in samples. The trend is accurate but the concentration is clearly not. However, the correlation between the ED-DOPA assay and the HPLC PB-DOPA analysis is linear, producing and equation of $y=7.841x + 0.108$ (Figure 3.6.2). The PB-DOPA detected by ED-DOPA can therefore be easily converted to a more representative concentration.

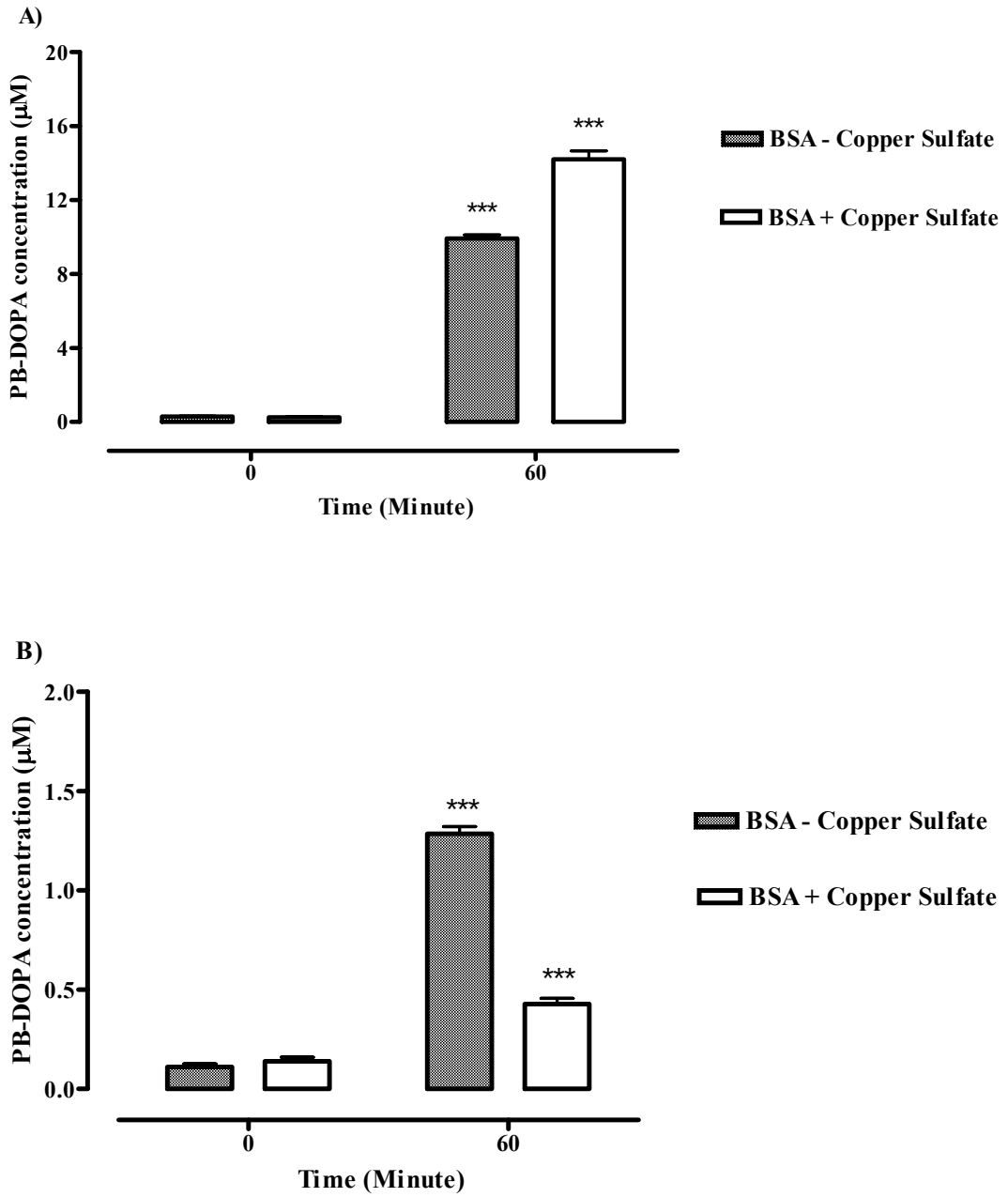

Figure 3.6.5 PB-DOPA production on BSA exposed to copper sulfate
1mg/ml BSA was exposed to radiation for 60 minutes and then incubated in the presence or absence of 110 µM copper sulphate. Samples were analysed for PB-DOPA production by the A) ED-DOPA assay and B) HPLC analysis. Time can be converted to dose rate using the dose rate of 5.8 Gy/min. Each data point represents the mean ± SEM of triplicate samples. Significance is between treatments.

DISCUSSION

4.1 AAPH-mediated protein oxidation

4.1.1 PrOOH formation on BSA

When incubated at 37˚C under aerobic conditions, AAPH will decompose to release radicals at a relatively constant rate of 1.36 x 10^{-7} Ms^{-1} (Niki, 1995). In a solution of BSA exposed to 10 mM AAPH, PrOOHs were found to form in a linear fashion with r^2=0.9796 (Figure 3.1.1). This shows that PrOOH are formed on pure proteins exposed to peroxyl radicals with no observable lag phase. Using the rate of radical generation and the equation rate =2 x K_d x [AAPH], formation can be calculated at 3.19 x 10^{-9} Ms^{-1}. This equates to be 13 PrOOHs generated for every 100 peroxyl radicals formed, and is comparable to previous work in the laboratory (Ling, 2004; Yang, 2005).

4.1.2 Protein oxidation in human plasma exposed to peroxyl radicals

AAPH-mediated PrOOH formation in plasma was associated with a six hour lag phase during which, very few PrOOHs were detected, followed by a rapid increase in PrOOH production and finally a plateau in PrOOH levels after 12 hours (Figure 3.1.2). This result was comparable to previous work in the laboratory (Ling, 2004; Yang, 2005). PrOOH formation has been found to form on pure proteins and in plasma but, contradictory to the results observed in this study, PrOOH formation occurred immediately with no observable lag phase (Simpson *et al*., 1992; Gebicki *et al*., 2000a and b; Gieseg *et al*., 2000). This may be due to these investigations using radiation-induced hydroxyl radicals to initiate PrOOH formation, whereas the current investigation uses less reactive peroxyl radicals. Hydroxyl radicals are produced at a rate of 3.4 x 10^{-8} Ms^{-1} (Simpson *et al*., 1992), whereas peroxyl radicals are produced at a rate of 1.36 x 10^{-7} Ms^{-1} (Niki, 1995) suggesting hydroxyl radicals may have a faster reaction rate than peroxyl radicals in this study. This may explain the immediate production of PrOOH in the presence of hydroxyl radicals compared to the peroxyl radicals used in this investigtion. The lag phase observed when plasma is exposed to peroxyl radicals suggests that the protection of the plasma proteins is provided by the antioxidants present in human plasma.

4.1.3 Loss of PrOOH lag phase in dialysed plasma exposed to peroxyl radicals

Plasma dialysis removes low molecular weight antioxidants while retaining larger molecules. Dialysis of human plasma and then incubation with 10 mM AAPH produces PrOOHs immediately, with no six hour lag phase (Figure 3.1.3). This result is comparable to that observed by Yang, (2005) and indicates that small molecular weight molecules protect the plasma proteins during the lag phase. Although this eliminates protein thiols as the source of the lag phase, it was initially suggested that small molecular weight thiols like GSH could play a role in protecting the plasma proteins. However, Yang, (2005) disproved this theory when dialysed plasma, supplemented with GSH, failed to restore the PrOOH lag phase during incubation with AAPH.

4.2 Involvement of low molecular weight molecules involvement in the protection of plasma proteins

4.2.1 The role of uric acid and ascorbic acid in preventing protein oxidation

Yang, (2005) also supplemented dialysed plasma with uric acid or ascorbic acid. Under each of these conditions, the lag phase was partially restored, leading to the suggestion that uric acid and ascorbic acid might play a role in the six hour lag phase. To further investigate this, uric acid and ascorbic acid levels were monitored in undialysed plasma incubated with 10 mM AAPH (Figure 3.2.1). As ascorbic acid is depleted within two hours of incubation this antioxidant cannot be solely responsible for the lag phase. Nevertheless, the dramatic loss within two hours indicates it plays a role in protection. Uric acid is lost slowly over time but 50% still remains after 24 hours suggesting that, while uric acid may play a role during the lag phase, its antioxidant role during the rest of the oxidation period may be just as critical. This may be important for the long term prevention of protein oxidation with PrOOH levels in dialysed plasma being twice as high as in undialysed plasma (Figure 3.1.1). It is possible uric acid scavenges free radicals limiting the oxidation of plasma proteins.

Further support for the contribution of uric acid and ascorbic acid to the lag phase arises from the observation that supplementation of dialysed plasma with both antioxidants concurrently reproduces the lag phase to what was detected in undialysed plasma incubated with 10 mM AAPH (Figure 3.3.2). The supplemented dialysed plasma result matches the plasma result exactly until between the 12 and 24 hour time points. After 12 hours, the plasma sample reaches a plateau whereas the dialysed plasma supplemented with the antioxidants continues to increase. This may have been due to the supplemented uric acid being lost quicker and eventually being depleted by 12 hours compared to uric acid in undialysed plasma, which is not depleted during the incubation period. Yang, (2005) finding a two hour lag phase was restored when dialysed plasma was supplemented with ascorbic acid, suggests the relative reactivity of ascorbic acid is greater than that of uric acid and as a result is responsible for protection during the first two hours of incubation. Ascorbic acid is known to be the most effective antioxidant in plasma (Retsky *et al*., 1993) and can consume the peroxyl radicals produced by AAPH. This transforms them to less potent forms, preventing protein attack and significantly reducing the production of PrOOHs (May *et al*., 1998). Yang, (2005) also found a partial restoration of the lag phase when dialysed plasma was incubated with uric acid. Although not a complete restoration of the observed lag phase, it did account for four of the six hours of protection. This further supports the theory that uric acid is protecting the plasma proteins once ascorbic acid is depleted. The reduction in PrOOH concentration when uric acid is present in the plasma compared to the concentration found in dialysed plasma indeed indicates uric acid is reducing the radical flux not only for the six hour lag phase but also for the incubation period.

To further investigate the role of uric acid in the six hour lag phase, undialysed plasma was supplemented with excess uric acid. This supplementation doubled the uric acid concentration in the plasma and was hypothesised to extend the lag phase. As expected, the lag phase was indeed extended from six hours to 12 hours (Figure 3.3.4) indicating uric acid plays a vital role in protecting the plasma proteins during the lag phase and reducing the overall radical flux during the incubation period once ascorbic acid had been consumed.

Furthermore, incubating undialysed plasma with uricase was expected to remove uric acid from the plasma and therefore remove all but two hours of the lag phase. Uricase is an enzyme that breaks uric acid down to allantoin, carbon dioxide and hydrogen peroxide. Unexpectedly the lag phase during AAPH-mediated oxidation was not eliminated (Figure 3.3.3) with six hours of lag observed. This in contrast to what has been observed in prior experiments supplementing dialysed plasma with uric acid (Figure 3.3.2), and adding excess uric acid to plasma (Figure 3.3.4). The result was so similar to control plasma incubated with AAPH, that at first it appeared as though the uricase had no effect. However, upon closer inspection, it was noted that PrOOH formation after the six hour lag phase occurred at a faster rate in the uricase-treated plasma than in the control. This increased rate resulted in almost twice the concentration of PrOOHs by eight hours compared to the control, confirming that uricase was removing uric acid from the undialysed plasma. It is contradictory to the previous results which showed a return of the lag phase (Figure 3.3.2) when dialysed plasma was supplemented with ascorbic acid and uric acid and an extended lag phase when excess uric acid was added to undialysed plasma (Figure 3.3.4). It could be that the uric acid break down product, allantoin, exhibits antioxidant activity by scavenging radicals and providing the same effect as uric acid. In addition, maybe this scavenging activity ultimately converts allantoin back to uric acid. Patterson *et al*. (2003) also used uricase to removed uric acid from plasma finding the degradation of uric acid by uricase, removed the protection effect suggesting uric acid was the major antioxidant in the ultra filtrate of plasma. This is contradictory to the results observed here as no effect on protection of plasma proteins was observed when uric acid was broken down.

4.2.2 Free amino acids as antioxidants in human plasma

Free amino acids in the plasma are also thought to be capable of protecting plasma proteins as they are small enough to be removed by dialysis and are present in sufficient concentrations. To investigate this further, dialysed plasma was supplemented with 250 μM and 500 μM of the free amino acids valine, alanine, glutamine, lysine and glysine (Figures 3.3.1A and B). These are the amino acids of highest concentration found in plasma but yet did not show any significant difference from control plasma incubated with AAPH. This suggests that the five most

commonly identified amino acids in plasma are not acting as sacrificial antioxidants protecting plasma proteins under the conditions of this study. To further investigate the result observed in Figures 3.3.1A and B, the six amino acids found to be the most vulnerable to free radical attack, tyrosine, proline, leucine, isoleucine, arginine and histidine, were supplemented to dialysed plasma but also failed to restore the six hour lag phase (Figure 3.3.1C). In light of these results it can be concluded that free amino acids in the plasma, both the highest concentration and the most reactive with free radicals, do not contribute to the protection of plasma proteins during the first six hours of incubation with AAPH. Uric acid and ascorbic acid are clearly more reactive towards peroxyl radicals than free amino acids when supplemented at the concentrations described in this study.

4.3 Effect of peroxyl radical attack on lipids in human blood plasma

The kinetics of AAPH-induced lipid oxidation are described by a two hour lag phase in cholesterol diene formation followed by a rapid linear increase for the rest of the 24 hour period (Figure 4.4.1). This is in contrast with the lack of cholesterol diene formation in plasma incubated in the absence 10 mM AAPH and confirms that the increase due to peroxyl radical attack is not auto-oxidation of the plasma lipids. The result further indicates that an unidentified antioxidant is inhibiting lipid oxidation during the first two hours of incubation with peroxyl radicals but, upon depletion of this substance lipid oxidation proceeds. Lipid peroxidation in human blood plasma exposed to peroxyl radicals has been previously investigated by other groups and is in agreement with the results observed here. In these studies a lag phase during which no or very few lipid hydroperoxides formed was observed over the first 50-60 minutes (Thomas *et al*., 1997; Neuzil *et al*., 1994; Frei *et al* 1988). The kinetics of lipid oxidation in a pure lipoprotein solution are also described by a lag, propagation and decomposition phase (Esterbauer *et al*., 1989). However, the end of the lag phase was observed to coincide with the complete consumption of α-tocopherol in this system. The results in this investigation show the depletion of ascorbic acid coincides with the end of the two hour lipid peroxidation lag phase (Figure 3.2.1). Also coinciding with the end of the lag phase in LDL is the end of the PrOOH lag phase (Gieseg *et al*., 2003). The continued close linkage between lipid peroxidation and protein

peroxidation throughout the propagation and decomposition phases of LDL oxidation has led to the suggestion that PrOOHs are formed from a lipid-derived radical. Comparing the kinetics of lipid and protein oxidation in Figure 3.3.1 at first showed no connection between the two processes.

4.3.1 Role of uric acid and ascorbic acid in reducing lipid oxidation in human plasma

The results found in this investigation indicate ascorbic acid and uric acid protect plasma lipids in plasma exposed to peroxyl radicals (Figure 3.4.6). Supplementation of dialysed plasma with both antioxidants concurrently showed a return of the two hour lag phase and an overall reduction in cholesterol diene concentration to that observed in the undialysed and unsupplemented plasma. The results indicate that ascorbic acid is responsible for the two hour lag phase by inhibiting lipid oxidation while present in the plasma, as the two hour lag phase returned when dialysed plasma was supplemented with ascorbic acid (Figure 3.4.4). There was no reduction in the cholesterol diene concentration after the two hours. Alternatively, ascorbic acid could be acting as a co-antioxidant for α-tocopherol. This type of role has been described for ascorbic acid (Mendiratta *et al*., 1997; Schafer *et al*., 2003; Retsky *et al*., 1993; Suh *et al*., 2003; Nieto *et al*., 2000; Gebicki *et al*., 2000b). By reacting with the α-tocopheroxyl radical ascorbic acid can regenerate α-tocopherol and export the radical from within the oxidising lipid particles to the aqueous phase. The regenerated α-tocopherol molecule can meanwhile continue inhibiting lipid oxidation. Once the ascorbic acid is depleted the α-tocopherol can no longer be regenerated. Under these conditions of low radical flux, this reaction results in the induction of α-tocopherol's pro-oxidant activity and therefore lipid oxidation.

After the depletion of ascorbic acid, uric acid then becomes the main antioxidant and appears to do so by reducing the overall radical flux. The general ability of uric acid to reduce the radical flux explains why it effectively reduces the rate of both PrOOH and cholesterol diene formation. Uric acid's role in reducing the overall radical flux for the incubation period is confirmed in Figure 3.4.5 where there is an observable reduction in cholesterol diene concentration over the incubation period in dialysed plasma supplemented with uric acid compared to control unsupplemented dialysed

plasma. The concentration was reduced almost to the level observed in oxidised plasma. Uric acid is described as an excellent scavenger of peroxyl radicals but is unable to prevent lipid oxidation agreeing with the results observed in this study (Frei *et al*., 1989; Stocker *et al*., 1991; May, 1998; Suara *et al*., 1995; Nieto *et al*., 2000; Frei *et al*., 1988; Ames *et al*., 1981). Gebicki *et al*. 2000a removed ascorbic acid and uric acid from the plasma by dialysis and found that lipid peroxides in the resulting plasma formed immediately upon exposure to radiation. However, it is not in agreement with Esterbauer *et al*, (1989), finding uric acid added to isolated LDL increased the lag phase of lipid oxidation where no oxidation occurred. This investigation was carried out on isolated LDL indicating the reaction may be faster enabling uric acid to inhibit lipid oxidation.

4.4 α-Tocopherol (Vitamin E) loss in AAPH-induced plasma oxidation

α-Tocopherol (α-TOH) is the major lipid soluble antioxidant in LDL (Terentis *et al*., 2002). It is thought to protect LDL against lipid peroxidation by directly scavenging free radicals and reacting rapidly with chain-carrying lipid peroxyl radicals (LOO˙) to break propagation (Kontush *et al*., 1996; Niki *et al*., 2005; Thomas and Stocker, 2000; Bowry *et al*., 1992). In the absence of AAPH, the α-tocopherol concentration remains relatively constant during the 24 hour incubation period (Figure 3.5.1). In the presence of AAPH, the α-tocopherol level is unchanged for the first two hours, but this is followed by a decrease between two and eight hours and plateaus from ten hours onwards (Figure 3.5.1). This result agrees with a study by Ling, (2004) and suggests that the α-tocopherol was being consumed while protecting the plasma lipids from oxidation by its chain-breaking antioxidant activity.

4.4.1 The ability of uric acid and ascorbic acid to protect α-tocopherol from free radical attack in human plasma

The results found in this investigation indicate that ascorbic acid and uric acid protect the α-tocopherol levels in plasma exposed to peroxyl radicals (Figure 3.5.5). It can also be concluded that in the absence of ascorbic acid, α-tocopherol acts via TMP. It switches from an antioxidant to pro-oxidant state, with the α-tocopheroxyl radical replacing the lipid peroxyl radical as the species initiating lipid oxidation. It contrasts,

however with Yeum *et al*, (2003), who indicated α-tocopherol was the first line of defence against oxidative damage and uric acid the second. The concentration of AAPH was double that used in this investigation suggesting the faster reaction was the cause for this. Other laboratories investigating α-tocopherol's role in the protection of lipid oxidation have drawn similar conclusions (Stocker, 1999; Upston *et al*., 1999; Thomas *et al*., 1997). The TMP theory is further supported by the loss of α-tocopherol after eight hours of incubation when dialysed plasma is exposed to peroxyl radicals (Figure 3.5.2). This depletion coincides with a 50% reduction in the rate of cholesterol diene formation and supports the theory that, in the absence of a co-antioxidant, α-tocopherol drives lipid peroxidation. This pro-oxidant activity is in agreement with Bowry *et al*, (1992), and Neuzil and Stocker, (1994) also finding that α-tocopherol gave a pro-oxidant effect when both isolated LDL and plasma are oxidised by peroxyl radicals. Peroxidation was faster in the presence of α-tocopherol so, as α-tocopherol was consumed, peroxidation declined. Additional evidence for TMP arises from the fact that supplementation of dialysed plasma with ascorbic acid delays the loss of α-tocopherol for two hours (Figure 3.5.3). This result highlights the ability of ascorbic acid to serve as a co-antioxidant by recycling the α-tocopheroxyl radical back to α-tocopherol. While ascorbic acid is present in the plasma, the depletion of α-tocopherol inhibited. After ascorbic acid is depleted from the plasma, the protection of α-tocopherol is by uric acid (Figure 3.5.4). Uric acid is reducing the overall radical flux to α-tocopherol and therefore delaying the depletion of the antioxidant. It is not a co-antioxidant for α-tocopherol, as it is unable to recycle the α-tocopheroxyl radical back to α-tocopherol. As discussed earlier, uric acid is unable to trap and remove radicals like ascorbic acid. Instead, it scavenges the peroxyl radicals, and by doing so, intercepts the oxidation chain reaction (Frei *et al*., 1989; Thomas *et al*., 1997).

4.5 Protein-bound 3,4-dihydroroxyphenylalanine (PB-DOPA) production on BSA irradiated by x-rays

4.5.1 PB-DOPA production on irradiated BSA

Protein-bound 3,4-dihydroroxyphenylalanine (PB-DOPA) is a major product of radical attack on tyrosine residues in peptides and proteins. Its formation has been studied by several other laboratories, who noted its production in a range of oxidising

systems and on a variety of substrates (Fu *et al*., 1998; Gieseg *et al*., 1993; Sutherland *et al*., 2003). These radicals are generated by metal-catalysed Fenton systems, gamma radiation and UV light.

The current study has confirmed once again the formation of PB-DOPA on BSA exposed to both irradiation and the Fenton system. In the case of the former, PB-DOPA was produced in a linear fashion during a 60 minute exposure to X-ray radiolysis (Figures 3.6.1 and 3.6.3). The lack of a lag phase indicates that a certain concentration of radicals does not have to be generated before PB-DOPA is produced, it is formed as soon as the radicals are created. It also suggests that BSA is very susceptible to PB-DOPA formation. These results are in agreement with Parkes, (2005) who also observed a linear formation of PB-DOPA on BSA during radiolysis. Sutherland *et al*. (2003) also found that with increasing amounts of irradiated BSA, PB-DOPA formation increased linearly.

The Fenton system also generates radicals that are capable of reacting with proteins to form PB-DOPA. This confirms the results originally noted by Simpson and Dean (1990), and Gieseg *et al*. (1993) and has now been confirmed in the current study (Figure 3.6.4). This indicates that during the Fenton reaction the radicals produced are capable of reacting with proteins to form reaction products, in this case PB-DOPA. The results also show that copper is capable of being the transition metal ion producing free radicals via the Fenton reaction. Ranges of copper concentrations were investigated and all were shown to promote PB-DOPA formation on BSA by both assays. Both the ED-DOPA assay and HPLC analysis detected PB-DOPA, with the ED-DOPA assay detecting a much higher concentration of DOPA than HPLC analysis. This is also suggesting what was observed in Figures 3.6.1, 3.6.3, and 3.6.4 that the ED-DOPA assay is measuring the oxidation products of PB-DOPA as well as PB-DOPA produced in the investigation. Although the PB-DOPA concentration determined by each assay is significantly different, the two assays remain highly correlated with and r^2=0.989 (Figure 3.6.2).

4.6 Specificity of the ED-DOPA assay

Throughout the fluorometric research of PB-DOPA formation on BSA, two assays were always used. One measures PB-DOPA by HPLC analysis of acid hydrolysates while the other measures PB-DOPA by fluorometric analysis. PB-DOPA was found to be present at consistently higher concentrations when measured by the ED-DOPA assay compared to HPLC analysis. This trend was observed when oxidation was from exposure to X-ray radiolysis (Figures 3.6.1 and 3.6.3) or the Fenton reaction (Figure 3.6.4). An experiment was therefore undertaken to determine whether the ED-DOPA assay was actually specific for PB-DOPA and the results were compared to HPLC analysis (Figure 3.6.5). If both systems for measuring PB-DOPA were specific, a decrease in PB-DOPA concentration should have been observed when irradiated BSA was subsequently incubated with copper sulfate compared to the concentration observed in the control, irradiated BSA incubated in the absence of copper sulfate. Incubating with copper sulfate promotes the oxidation of any PB-DOPA that has formed, so if oxidation products are being detected in the assay, a higher concentration will be observed. As expected, HLPC analysis of PB-DOPA showed a decrease in PB-DOPA concentration under these conditions. By contrast, the ED-DOPA assay showed a higher concentration than the control despite being treated identically to the HPLC samples. Ethylenediamine reacts with *o*-diphenols to form a fluorescent derivative, which is measured at an excitation of 430 nm and an emission of 545 nm. This result suggests that PB-DOPA is not the only *o*-diphenol able to react with ehtylenediamine, illustrating that the ED assay is detecting oxidation products of PB-DOPA in addition to the PB-DOPA itself. The oxidation products are probably what the ED-DOPA assay is detecting as well as the PB-DOPA in the sample. The assay may also be detecting other oxidized amino acids, particularly tryptophan and phenylalanine.

It can be suggested from these results that the ED-DOPA assay cannot be solely used for measuring PB-DOPA as other products may be measured along with PB-DOPA. The HPLC analysis of PB-DOPA is a more accurate measurement of PB-DOPA as it measures only PB-DOPA and not its oxidation products or other oxidised amino acids. Despite this, the ED-DOPA assay may still be useful. It is cheaper and less time consuming than the HPLC method. Accurate PB-DOPA measurements can be

derived from ED-DOPA reading because the concentrations of PB-DOPA detected by HPLC and ED-DOPA are highly correlated. When a standard curve was created, using concentrations obtained during the irradiated BSA (Figure 3.6.2), a linear trend was observed (r^2=0.989). With HPLC and ED-DOPA measurements recorded on the x and y axes, respectively, an equation could be derived from this linear trend: y=7.84x + 0.11. Any concentration recorded by the ED-DOPA assay can be converted to a more accurate concentration that is more representative of the PB-DOPA concentration that would be detected by the HPLC analysis.

4.7 Summary

In this study, the kinetics of plasma protein and lipid oxidation were investigated in the presence of the peroxyl radical generator, AAPH. PrOOH formation on plasma proteins and cholesterol diene formation on plasma lipids were associated with lag phases of six and two hours respectively. During these lag phases, very little oxidation was detected but was followed by a rapid increase in PrOOHs and cholesterol dienes formation. The different lengths of the lag phases meant that, initially the two processes did not appear to be related. However, further investigation illustrated that the antioxidants, ascorbic acid and uric acid, were responsible for both lag phases and also for the reduction in overall radical flux throughout the incubation period. Uric acid in particular, accounts for this latter activity. It can be concluded that ascorbic acid is protecting the target molecules in the plasma during the two hours of oxidation as during the first two hours it is depleted from the plasma. With lipid oxidation there is no cholesterol diene formation during the two hour lag phase indicating ascorbic acid is recycling the α-tocopheroxyl radical back to α-tocopherol to inhibit lipid oxidation during this period. Protein oxidation however, is not inhibited by ascorbic acid during the lag phase, the PrOOH formation is significantly reduced during this period. Ascorbic acid and uric acid not only protect plasma proteins and lipids from oxidative attack, but also protect α-tocopherol from being consumed. It can be concluded that in the absence of the co-antioxidant ascorbic acid, α-tocopherol exhibits TMP activity. In this pro-oxidant state, the α-tocopheroxyl radical is not recycled back to α-tocopherol, but instead replaces the lipid peroxyl radical as the species that initiates lipid oxidation.

The overall radical flux over the incubation period is being reduced by uric acid to delay the consumption of α-tocopherol. Overall, it can therefore be concluded that the antioxidants, ascorbic acid and uric acid, play a pivotal role in the protection of plasma proteins, lipids and α-tocopherol from peroxyl radical attack.

In a separate study, it was confirmed that PB-DOPA is formed on BSA during exposure to X-rays radiolysis and Fenton reagents. Although both HPLC analysis and the ED-DOPA assay detected PB-DOPA in these systems, it can be concluded that the ED-DOPA assay is not sufficiently specific to enable the concentration of PB-DOPA to be accurately determined. Compared to the HPLC analysis, ED-DOPA measurements are always significantly larger. Additional experiments indicated that the ED-DOPA assay measures both PB-DOPA and its oxidation products, therefore accounting for the larger readings when using this assay. By contrast, the HPLC analysis of acid hydrolysates only detects PB-DOPA and so, is a much more reliable assay as it only measures PB-DOPA.

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