ANALYSIS OF PLASMA HYDROPEROXIDES: METHODOLOGICAL ASPECTS AND LEVELS IN HEALTH AND DISEASES

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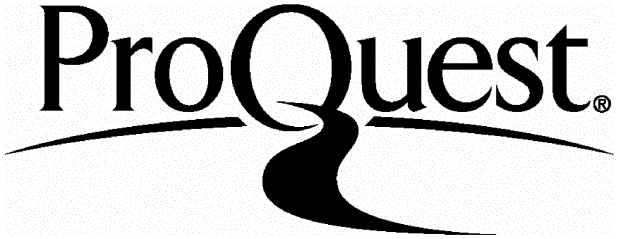

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ABBREVIATION

13-HPODE	[9Z, 11E, 13(S)]- 13-Hydroperoxyoctadecadien-1-oic acid
$^1O_2$	Singlet oxygen
2-ME	2-mercaptoethanol
5-HPETE	[5(S),6E,8Z,11Z,14Z]-5-Hydroperoxyeicosatetraenoic acid
9-HPODE	[9(S), 10E, 12Z]-9-Hydroperoxyoctadeca dien-1-oic acid
AA	Arachidonic acid
AA-PH	2,2-Azobis(2-amidinopropane) hydrochloride
AH	Ascorbate
Apo-T	Apo-transferrin
BHT	Butylated hydroxytoluene
BSA	Bovine serum albumin
CD	Conjugated diene
Ch-OOH	Cholesterol hydroperoxide
CU-OOH	Cumene hydroperoxide
DETPAC	Diethylenetriaminepenta-acetic acid
EDTA	Ethylenediaminetetra-acetic acid
FAME	Fatty acid methyl ester analysis
FOX	Ferrous Oxidation in Xylenol Orange
G/PX	Glutathione peroxidase
GC	Gas-chromatography
GSH	Glutathione (reduced form)
GSSG	Glutathione (oxidised form)
H_2O_2	Hydrogen peroxide
HDL	High density lipoprotein
Holo-T	Holo-transferrin
HPLC	High-performance liquid chromatography
$L^{\bullet}$	Allyl radical
LA	Linoleic acid

LA-OOH	Linoleic acid hydroperoxide
LN	Linolenic acid
LDL	Low density lipoprotein
LH	Unsaturated fatty acid
$LO^{\bullet}$	Alkoxyl radical
$LOO^{\bullet}$	Peroxyl radical
LOOH	Hydroperoxide
NIDDM	Non insulin-dependent diabetes mellitus
$OH^{\bullet}$	Hydroxyl radicals
PA_2	Phospholipase A_2
PBS	Phosphate-buffer saline
Ph-OOH	Phosphatidyl choline hydroperoxide
PUFAs	Polyunsaturated fatty acids
SOD	Superoxide dismutase
TB-OOH	Tert-butyl hydroperoxide
TBA	Thiobarbituric acid
TCA	Trichloro acetic acid
THF	Tetrahydrofuran
TPP	Triphenylphosphine
VLDL	Very low density lipoprotein
XO	Xylenol orange

Abstract

Increased lipid peroxidation is thought to play an important role in the pathogenesis of atherosclerosis. However, such elevation in lipid peroxidation has been difficult to assess because specific and reproducible methods for the measurement of products of lipid peroxidation have been unavailable.

The aim of this thesis is to show the development of a simple and reliable method to measure of plasma hydroperoxides, initial products formed during peroxidation of unsaturated lipids (e.g. fatty acids and cholesterol). Using this as an index of oxidative injury in vivo, I have shown that it feasible to measure plasma hydroperoxides using Ferrous Ion Oxidation in Xylenol Orange (FOX) version 2. The assay is based upon the oxidation of ferrous to ferric ions by hydroperoxides under acidic conditions. The generated ferric ion is complexed by the ferric ion indicator xylenol orange yielding a blue-purple complex with an absorbance maximum at 550-600 nm. Authentic plasma hydroperoxides are determined by a strategy in which the specific hydroperoxide reductant, triphenylphosphine, is used to discriminate between the background signal generated by ferric ions present in plasma and that generated by hydroperoxide in plasma. The method is specific and sensitive; detection limit <1.0 μM. Inter- and intra assay coefficients of variation are (<5%). Intra laboratory coefficient of variation is <10%. Using this assay, I have shown that plasma hydroperoxides were elevated in individuals with non-insulin-dependent diabetes mellitus (NIDDM) (9.4 ± 3.5 μM;(n=110) compared to those in healthy controls (3.9 ± 2.2 μM; n=60). Relationships between measures of plasma oxidation, metabolic control and selected complication in NIDDM and location of plasma hydroperoxides are discussed with relevance to atherosclerosis.

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Chapter I INTRODUCTION

1. Free radicals and human diseases

The concept that free radicals play an important role in the pathogenesis of certain human diseases such as atherosclerosis, diabetes, ischaemic heart disease and rheumatoid arthritis is becoming increasingly recognised (Halliwell and Gutteridge, 1991; Simic et al. 1988). This has led to increased need for techniques to measure free radicals and their reaction specifically in clinical situation. Free radicals have very short life-time and direct determination of them is difficult. The only analytical technique that can directly measure free radicals is electron spin resonance (ESR) spectrometry (Janzen, 1990). However, biologically important reactive oxygen radicals (ROS) do not accumulate in sufficiently high concentrations to be directly seen by ESR (Pou and Rosen, 1990). Thus, free radical activity is usually assessed by indirect methods such as measurement of various end products as a result of interaction of free radicals with lipids, proteins and DNA (Slater, 1984).

Lipid peroxidation has been studied intensively as a marker of free radical activity in tissue injury. Halliwell and Gutteridge (1990) have reported that "*The detection and measurement of lipid peroxidation is the evidence most frequently cited in supporting the involvement of free radical reactions in toxicology and human disease*". There are several reasons why attention has been focused on this issue. First, if free radicals are formed in biological system, then there is a strong possibility that peroxidation of lipids will be initiated where polyunsaturated fatty acids are abundant. Second, lipid peroxidation is a important process in free radical pathology as it is damaging to cells. Furthermore, vast array of techniques has been developed to measure lipid peroxidation (Holley and Cheeseman, 1993; Coghlan et al. 1991; Grootveld and Halliwell, 1986). However, applying these techniques for measurement of lipid peroxidation in a biological system such as plasma must be carried out very carefully, since the presence of interfering factors specially in clinical situations may lead to misunderstanding of the relation of free radicals to disease.

1.1. Biochemistry of free radicals

Definition

Free radicals can be defined as a chemical species which contain an unpaired electron and are generally reactive. Examples are superoxide ($O_2^{\bullet -}$) an oxygen centred radical, thiyl ($RS^{\bullet}$) a sulphur centred radical, trichloromethyl ($CCl_3^{\bullet}$) a carbon centred radical and nitric oxide ($NO^{\bullet}$), in which the unpaired electron is delocalised between both atoms (Halliwell et al. 1992). The hydrogen atom is also considered as a radical, since it has a single electron. Hence abstracting a hydrogen atom from a biological molecule leaves an unpaired electron on the atom from which hydrogen has been removed. Free radicals can be formed in different ways:

(1) by homolytic or heterolytic cleavage of a molecular bond:

Homolytic cleavage	$A{:}B \longrightarrow A^{\bullet} + B^{\bullet}$	radical species
Heterolytic cleavage	$A{:}B \longrightarrow A^{+} + {:}B^{-}$	anion species

In homolytic cleavage both $A^{\bullet}$ and $B^{\bullet}$ have unpaired electrons and are called free radicals. In heterolytic cleavage the cation A^{+} lacks an electron, while the cation $:B^{-}$ contains an added electron.

(2) by reduction oxidation reaction:

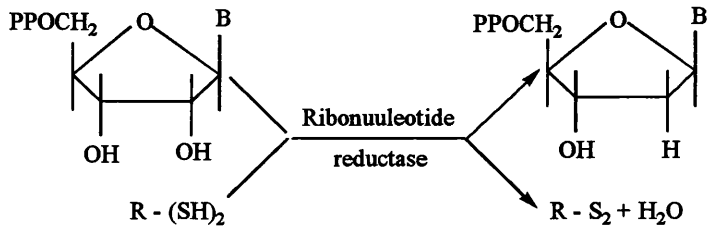
Reduction	$A + e^{-} \longrightarrow A^{-\bullet}$	anion radical
Oxidation	$A - e^{-} \longrightarrow A^{+\bullet}$	cation radical

These species are called anion and cation radicals. They are chemically unstable and less reactive than uncharged free radicals, but they can react with other radicals or can abstract atoms from other molecules. Free radicals can be positively charged, negatively

charged or electrically neutral and conventionally indicated with superscript dot(˙) (Simic et al. 1988; Sies, 1991).

1.1.1. Production of free radicals

Free radicals can be generated in the environment, foods, chemical reactions and living systems. Production of free radicals in animal cells is mediated via several biochemical pathways, both by enzymes and non-enzymatically, often by the redox chemistry of transition metal ions and can either be accidental or part of a cell programme. Some enzymes utilise a free radical at their active site in the process of catalysis; for example ribonucletoide reductases. These enzymes have been purified to homogeneity from a number of sources and they differ substantially in structure (subunit size and organisation) and in cofactor requirement (Stubbe, 1990). In Eschericia coli and in higher cells ribonucleotide reductase consists two nonidentical subunit in a 1:1 complex, named proteins B1 and B2. The two catalytic sites of the enzyme are made up from parts of each subunit. This enzyme catalyses the enzymatic formation of deoxyribonucleotides, a necessary step in the synthesis of DNA. The active form of enzyme from Eschericia coli or from mammalian sources contains as part of its polypeptide structure a free tyrosyl radical, stabilised by an iron centre. This radical participates in the catalytic process during the replacement of the hydroxyl group at C_2 of ribose sugar ring by hydrogen atom (Reichard et al. 1983). The reaction simply can be shown as following:



In most cases $R(SH)_2$ is thioredoxin, a small protein with two cysteine - SH groups in close proximity. The oxidised thioredoxin (RS_2) is re-reduced by a reductase enzyme at the expense of NADPH (Halliwell and Gutteridge, 1991). Treatment of ribonculeotide

reductase with hydroxyurea (an inhibitor of the enzyme) inactivates the enzyme by reacting with the tyrosyl radical (Barlow et al. 1983). The regeneration of the tyrosyl radical occurs by an enzyme system consisting of superoxide dismutase, flavin reductase and a third unknown protein (Fontecave et al. 1987).

Phagocytic cells play an important role in the body defence against bacterial attack via generation of reactive oxygen species. The killing mechanism of phagocytes is mostly oxygen-dependent. The killing mechanism of phagocytes in the absence of oxygen is less well understood (Gabig and Babior, 1981). In the presence of oxygen, exposure of phagocytes to variety of natural and synthetic stimuli, leads to a marked increase over basal oxygen metabolism (the respiratory burst) (Babior, 1978). Activation of the respiratory burst lead to generation of superoxide and H_2O_2 via following reactions:

$$2O_2 + NADPH \longrightarrow 2O^-_2 + NADP^+$$

$$2O^-_2 + 2H^+ \longrightarrow H_2O_2 + O_2$$

Superoxide radical and H_2O_2 are thought to be the intermediate products of respiratory burst and not particularly powerful oxidants. Hence, superoxide-dependent killing mechanism are postulated occur via production of hypochlorous acid and hydroxyl radical (Babior, 1984). The enzyme myeloperoxidase (MPO), which is secreted by activated neutrophils into the surrounding medium (Heinecke et al. 1993), uses H_2O_2 to oxidase Cl^- ions to hypochlorous acid (HOCl), which has been shown play an important role in bacterial killing (Mckenna and Davies, 1988). The formation of the hydroxyl radical from O^-_2 and H_2O_2 in the presence of metal ions is the more important factor in the killing mechanism of phagocytes (Halliwell et al. 1985). Although deliberate production of free radicals is limited for special circumstances and because of their short life-time and tight control the damage is very limited, some leakage of superoxide, hydrogen peroxide and other reactive oxygen species is inevitable.

Under normal circumstances, the electron transport system in the mitochondria and endoplasmic reticulum are the major source of the free radicals in cells. Another source of superoxide in animal cells is the autoxidation of certain compounds including ascorbic acid (Puget and Michelson, 1974; Hirata and Hayaishi, 1975) thiols (e.g. glutathione,

cysteine), adrenaline and flavin co-enzymes (Fridovich, 1975). These autoxidation reactions can be greatly enhanced by the involvement of transition metal ions. Nevertheless, production of superoxide radical from ascorbic acid is doubtful. Halliwell and Foyer (1976) have shown there is no evidence for production of superoxide radical during autoxidation of ascorbic acid at alkaline pH.

1.1.2. Fate of free radicals

In biologic systems the accidental production of free radicals is normally kept to a minimum by the function of various antioxidant systems. Transition metal ions are kept tightly bound by proteins. In addition, animals have evolved not only enzymatic and non-enzymatic defence against this metabolic pathway, but also repair systems that prevent accumulation of oxidatively damaged molecules (Sies, 1991; Fridovich, 1989). However, under pathophysiological conditions, deficiencies of antioxidants, or following killing or damaging of living tissues, the normal control mechanisms may be disturbed. Under these circumstances, all of the major classes of biomolecules, such as lipids, proteins, and nucleic acids may be attacked by free radicals.

1.1.2.1. Interaction with proteins

Proteins are major structural and functional constituents of cells. Hence, they are also significant targets of free radical attack (Davies, 1987). Free radical damage to proteins becomes significant if it is somehow focused on specific sites of particular proteins. It has been shown that copper or iron ions can bind to a metal binding site of some proteins. This metal-protein complex can then react with H_2O_2 to generate a more reactive radical such as hydroxyl radical, which attack the side chains of amino acid residues at the metal binding site (Stadtman, 1990). These modifications cause significant changes on functional properties of proteins. In case of enzymes, they become inactive or less active (Davies, 1987; Aruoma and Halliwell, 1989; Marx and Chevion, 1986; Stadtman and Oliver, 1991; Stadtman, 1990). It has been proposed that accumulation of inactive or less active enzymes in cells during aging are a result of free radical-mediated damage to proteins (Stadtmann, 1992; Fucci et al. 1983). Some amino acid residue are converted to

carbonyl derivatives (Levine, 1983; Amici et al. 1989). Oxidative damage to lens crystalline and site-specific modification of albumin by free radicals have been proposed to be involved in formation of cataract (Spector, 1985; Seccia et al. 1991).

Measurement of carbonyls is a useful tool for assessing oxidative damage to proteins. These measurement are: reaction with tritiated borohydride, reaction with 2,4-dinitrophenylhydrazine (DNPH), and reaction with fluorescein thiosemicarbazide for gel electrophoresis (Levine et al. 1990). The spectrophotometric method is probably the most convenient procedure, to be used for the detection of reaction of DNPH with protein carbonyls to form protein hydrazones (Reznick and Packer, 1994).

1.1.2.2. Interaction with DNA

Free radicals can cause damage to DNA by a variety of mechanisms, such as base lesions, sugar lesions, single strand breaks and double strand breaks (Breen and Murphy, 1995). It has been shown that these types of damage to DNA can cause mutagenic effects and may be involved in cancer (Meneghini and Martins, 1993; Imlay and Linn, 1988). It has been shown that neither $O_2^{\bullet -}$ nor H_2O_2 directly cause damage to DNA, and much of the damage to DNA results from the hydroxyl radical ($OH^{\bullet}$) formed as a consequence of interaction of these species with metal ions such as copper and iron (Halliwell and Aruoma, 1991; Aruoma et al. 1991). Furthermore, carbon-centred radicals, although less reactive than the hydroxyl radicals, can cleave the DNA and alkylate DNA bases (Augustu, 1993). Because of the short life-time and rapid removal of free radicals by antioxidant defence systems, DNA is readily attacked only if the oxidising radicals are formed in its vicinity, and then DNA becomes a vulnerable and important target. Significant damage to DNA occurs, when it is 'site-specific' with high intensity, as was shown for copper-protein (see 1.1.2.1.). This can lead to strand breaks and mutation. The amount of damage is limited, because of the large capacity of the repair system for DNA. Lack of the repair system in mitochonderial DNA makes it more susceptible than nuclear DNA to free radical attack (Fukanaga and Yielding, 1979; Richter et al. 1988). Nevertheless, even with a high level of repair efficiency, sufficient damage may accumulate over a lifetime to lead to mutation and cancer (Guyton and Kensler, 1993). Oxidised nucleobases have been detected in human urine,

which indicates continual oxidative attack on DNA (Fraga et al. 1990; Kasai and Nishimura, 1991). 8-hydroxyguanine (8-OHG) is one of the major products of base damage as a result of exposure of DNA to hydroxyl radicals. High performance liquid chromatography (HPLC) or gas chromatography-mass spectrometry (GC-MS)-based measurement of 8-OHG are frequently used as a method of assessment of radical mediated damage to DNA in cells subjected to oxidative stress (Halliwell, 1993).

1.1.2.3. Effect on lipids

Lipids containing PUFAs and their esters are among the most susceptible molecules to oxidative degradation present in living tissues (Ursini et al. 1991). The oxidative destruction of PUFAs known as lipid peroxidation, is particularly damaging because it proceeds as a self-continuing chain-reaction (Porter, 1990). lipid peroxidation leads to loss of normal structure and function of biomembranes (Recknagel et al. 1991). The mechanism and biomedical significance of lipid peroxidation will be discussed later (see 1.3.).

1.2. Antioxidant defence against free radicals

A number of systems have evolved that allow oxidation of substrates and reduction of oxygen in a controlled system with only minimal radical production. Some radicals, however, still escape and can be very damaging. As a result, defences against the harmful actions of free radicals have evolved, especially in organisms that live in an oxygen rich environment. These are known as antioxidant defences and can be divided into two main categories, the first whose role is to prevent the generation of free radicals and second, those that intercept any that have been generated (Cotgreave et al. 1988). They exist in both the aqueous and membrane compartments of cell and can be enzymes or non-enzymes.

1.2.1. Preventive antioxidants

Preventive, or primary antioxidants, reduce the rate of initiation of free radical chains usually by converting the free radical producing products, such as ROOH to non-radical products, such as alcohols. They can be classified as an enzymes, metal binding proteins and small molecules.

1.2.1.1. Enzymes

The main protective mechanisms used by cells in vivo against the toxic effects of free radicals are superoxide dismutase, catalase, and glutathione peroxidase enzymes (Flohe, 1982; Michiels et al. 1994).

Superoxide dismutases (SODs)

The superoxide dismutases are a family of metalloenzymes that catalyse the dismutation of $O^{\bullet -}_2$. The most prevalent enzyme is the Cu-Zn SOD which is found in the cytosol of cells. The copper atom is essential to the catalytic activity, whilst the zinc atom gives stability to the enzyme. A second enzyme containing manganese (Mn SOD) is found in mitochondria (Steinman, 1982). A microbial form of SOD contains iron. Another form of enzyme has been found in blood plasma (Marklund, 1984). This enzyme is also called extracellular superoxide dismutase (EC-SOD). It contains four copper atoms and possibly four zinc atom (Marklund, 1982). Although its activity like Cu-Zn SOD is inhibited by cyanide (Marklund, 1982), yet no relation to Cu-Zn SOD has been found in amino acid composition, antigenic properties, or probable chromosomal localisation (Marklund, 1984).

The Cu-Zn SOD enzymes prevent the direct initiation of lipid peroxidation by the perhydroxyl radical (HOO$^{\bullet}$). In addition, they accelerate the dismutation of $O^{\bullet -}_2$ to H_2O_2, thereby preventing the iron redox cycling reaction and the iron-dependent Haber-Weiss reaction, which leads to the formation of highly reactive hydroxyl radicals. The dismutation of $O^{\bullet -}_2$ by Cu-Zn SOD occurs in two steps via the following reactions:

$$SOD\ Cu^{++} + O^{\bullet -}_2 \longrightarrow SOD\ Cu^{+} + O_2$$

$$SOD\ Cu^{+} + O^{\bullet -}_2 \xrightarrow{2H^{+}} SOD\ Cu^{++} + H_2O_2$$

Cu-Zn SOD activity can be inhibited by H_2O_2 (Bray et al. 1974; Hogson and Fridovich, 1975). This inactivation is thought to be due to site-specific formation of $OH^{\bullet}$, which reacts at the copper ion in the active site (Mavelli et al. 1983).

Catalase

Catalase is an enzyme which catalyses the decomposition of H_2O_2 to water and oxygen by following reaction:

$$2H_2O_2 \longrightarrow 2H_2O + O_2$$

Mammalian catalase is a complex of four identical subunits. Each subunit contains a single hematin (FeIII-protoporphyrin IX) group. Decomposition of H_2O_2 by catalase occurs in two subsequent steps:

$$Catalase\text{-}Fe^{3+} + H_2O_2 \longrightarrow Compound\ I + H_2O$$

$$Compound\ I + H_2O_2 \longrightarrow Catalase\text{-}Fe^{3+} + H_2O + O_2$$

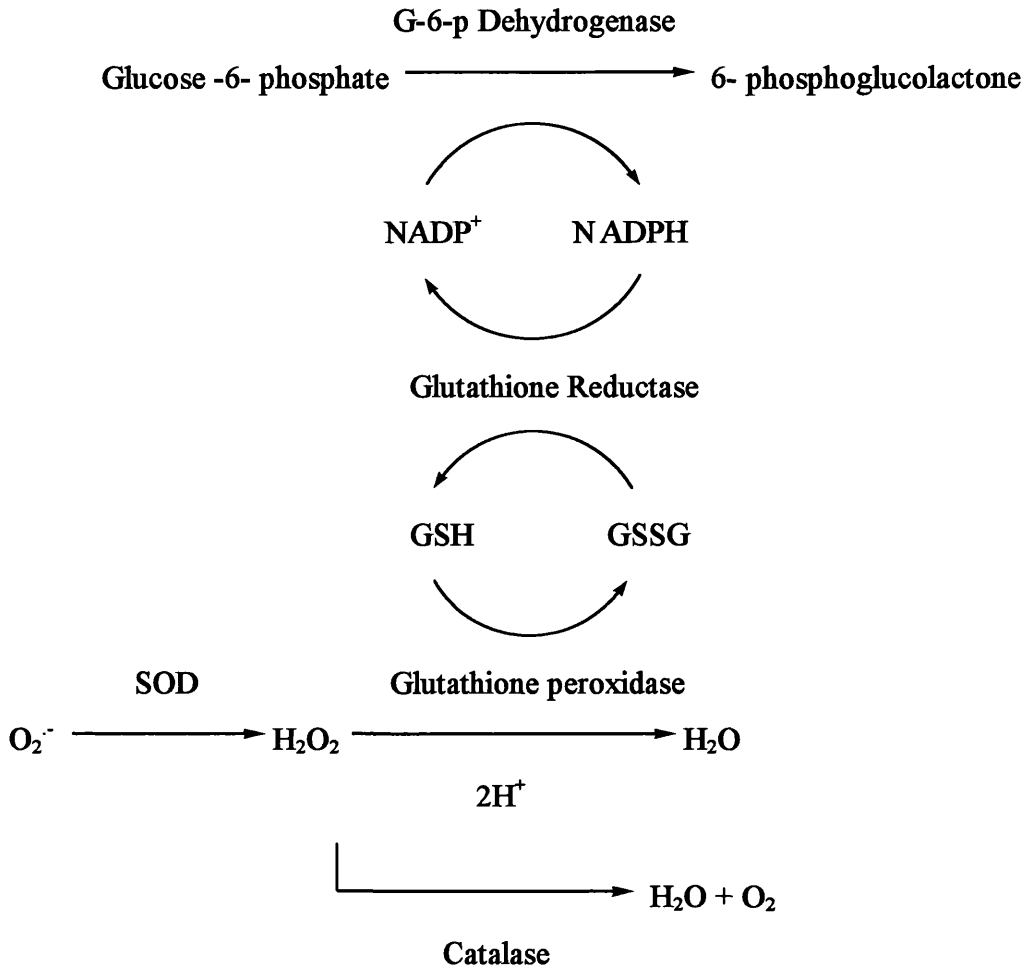
Compound I can be defined as an intermediate reactive molecule. The exact structure of compound I is uncertain. It is probably is intermediate in structure between a ferric peroxide (FeIII-HOOH) and Fe(V)=O (Halliwell and Gutteridge, 1991).

Glutathione peroxidase

Glutathione peroxidase is a tetrameric protein containing four atoms of selenium bound as selenocysteine moieties, which is involved in the catalytic activity of GSH/PX and traces of this element are essential to the human diet (Flohe, 1982). This enzyme is found in the cytosol and mitochondria of animal cells. Glutathione, the substrate for the enzyme, is present at millimolar concentration in vivo in the cytosol of the cells and its function is to maintain the cell in a proper oxidation/reduction state.

Glutathione peroxidase is activated by H_2O_2 to an active compound which is then reduced to the native enzyme by glutathione. The hexose monophosphate shunt is the source of generation of reducing power in the form of NADPH, which is used in the glutathione peroxidase system for the removal of hydrogen peroxide (Loven et al. 1983). Glutathione peroxidase also catalyses the reduction of hydroperoxides including those derived from unsaturated lipids (Little and O'Brien, 1968; Biaglow et al. 1988).

Figure 1.2.1.1: Mechanism of superoxide anion and H_2O_2 removal



1.2.1.2. Metal binding proteins

Because of the risk of metal-catalysed Fenton-type reactions, which lead to the formation of very reactive species such as the hydroxyl radical, biological systems possess a variety of mechanisms which minimise the levels of free metal ions such as iron and copper. These include a number of binding proteins that act as intracellular storage sites and intercellular transport carriers. Transferrin, which functions as a carrier molecule, is a glycoprotein and each molecule has two separate binding sites to which Fe^{3+} attaches extremely tightly at physiological pH. Under normal conditions only 20 to 30 percent of transferrin is loaded with iron on average, which means that the amount of free iron in plasma would be expected to be essentially zero. Lactoferrin, is a similar protein to transferrin, and is found in some body fluids, and in milk, and is produced by phagocytic

cells. Lactoferrin also binds two molecules of Fe^{3+} per mol of protein. Excessive iron in the circulation , which is not required is stored in intracellular protein ferritin, which possesses 24 subunits and a capacity of 4500 metal-binding sites (Aisen and Liskowsky, 1980). Similarly, copper is bound in blood plasma both to albumin and to the specific copper-binding protein, caeruloplasmin, a glycoprotein with 6 to 7 metal-binding sites (Gutteridge and Stocks, 1981).

1.2.1.3. Small molecules

Glutathione

Glutathione is a water soluble antioxidant with the tripeptide structure gamma-glutamyl-cysteinylglycine, at concentrations that are usually in the millimolar range (1-10 mM) in the cytosol of cells (Meister and Anderson, 1983). Body fluids such as bile, glomerular filtrate, blood plasma and epithelial cell lining also contain GSH. It exists in reduced form, GSH, and oxidised (disulphide) GSSG form. Most of the free glutathione in vivo is present as GSH rather than GSSG. In the oxidised form, GSSG, two GSH molecules join together as the -SH groups of cysteine are oxidised to form a disulphide bridge, -S-S. Glutathione can react chemically with free radicals in a number of ways. It may act as a reductant, reducing species such as H_2O_2 directly to water with the formation of GSSG (Ross et al. 1985) or it may react directly with free radicals such as $O^{\cdot-}_2$, $OH^{\cdot}$ and $RO^{\cdot}$ by a radical transfer process, yielding the thiyl radical of GSH, $GS^{\cdot}$ and eventually, GSSG. Finally, GSH may react with electrophiles to form covalent adducts. These reactions are catalysed by a group of glutathione transferase enzymes (Orrenius and Moldeus, 1984).

Vitamin C (Ascorbic acid)

Vitamin C (AH_2, at physiological pH mostly present at AH^-) is an important antioxidant both within cells and in the plasma (Stocker et al. 1991). Like GSH, ascorbate may directly reduce free radicals with the concurrent formation of dehydroascorbate via the

semidehydroascorbate free radical. Vitamin C can also react with superoxide (Nishikimi, 1975) and hydroxyl radicals (Ferrendsen and Verma, 1978) which results in formation of dehydroascorbic acid (A) and monohydroascorbate radical ($AH^{\cdot}$) via following reactions:

$$AH^{-} + O_2^{\cdot -} + 2H^{+} \longrightarrow AH^{\cdot} + H_2O_2$$

$$AH^{\cdot} + O_2^{\cdot -} + H^{+} \longrightarrow A + H_2O_2$$

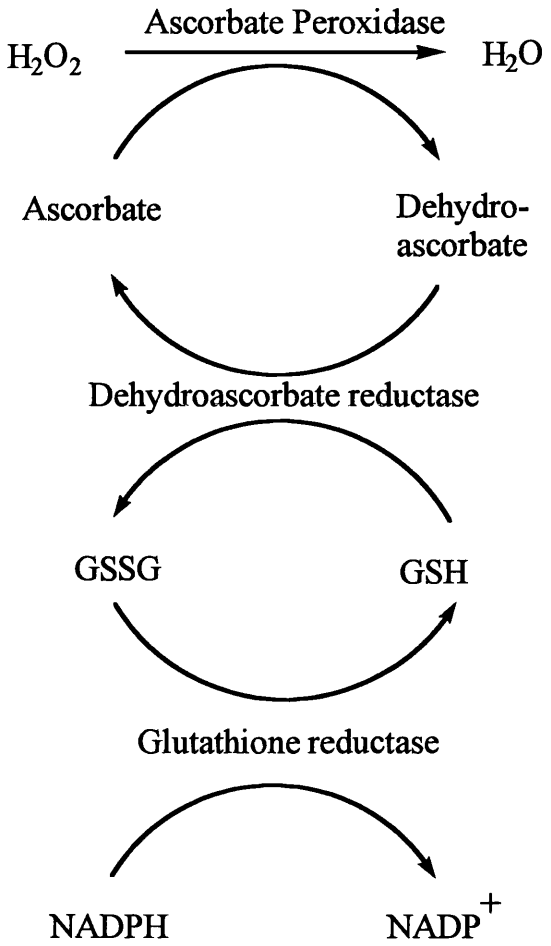
$$AH^{-} + OH^{\cdot} \longrightarrow AH^{\cdot} + OH^{-}$$

$$AH^{\cdot} + OH^{\cdot} \longrightarrow A + H_2O$$

The repair of oxidatively damaged proteins (Hoey and Butler, 1984) and quenching of singlet oxygen (Chou and Khan, 1983) by ascorbic acid has been reported. It has been shown that ascorbic acid can protect α_1-antiprotease "the enzyme, which protects tissue against proteases such as elastases" by reacting with hypochlorous acid (Halliwell et al. 1987). Hypochlorous acid is a powerful oxidant that inactivates α_1-antiprotease. Furthermore, ascorbic acid may participate in the restoration of α-tocopherol radical to reduced form (see Fig 1.2.2. b).

Because of lack of the ascorbate peroxidase enzyme in animal cells, removal of H_2O_2 and other reactive oxygen species by ascorbic acid may be occurring non-enzymatically. In plants, where ascorbate is present at high concentration (up to 50 mM), the removal of H_2O_2 by ascorbate is catalysed via ascorbate peroxidase in chloroplasts (Halliwell, 1984; Gillham and Dodge, 1986; Gillham and Dodge, 1987). The 'ascorbate and GSH cycle' in chloroplasts has been depicted in figure 1.2.1.3. Glutathione may reduce the semidehydroascorbate free radical with the formation of $GS^{\cdot}$ (McCay, 1985).

Figure 1.2.1.3: Glutathione-ascorbate cycle in chloroplasts



Uric aid

Uric aid is a poorly soluble substance of the major end product of purine metabolism. Purine bases themselves arise from break down of nucleic acids which are released during cell destruction and also ingested in the diet. It has been shown that uric acid is a powerful scavenger of singlet oxygen ($^1O_2$), peroxyl radicals ($ROO^\bullet$), and hydroxyl radicals ($OH^\bullet$), and it has been suggested that uric acid functions as an antioxidant in vivo (Ames et al. 1981). Uric acid is excreted unchanged from urine in man, but in most mammals it is further oxidised to allantoin. Hence, it has been proposed that measurement of oxidation product of uric acid, especially allantoin, might be a 'marker'

for oxidant generation in vivo (Grootveld and Halliwell, 1987). The rapid reaction of uric acid with the guanyl radical also suggests that uric acid may act as a repair agent of oxidative damage to DNA bases (Simic and Jovanovic, 1989). In addition uric acid at physiological pH stabilises ascorbic acid in plasma (Sevanian et al. 1985). Urate peroxyl radicals are the main product of reaction of uric acid with $OH^{\bullet}$ which are less reactive than $OH^{\bullet}$ (Halliwell et al. 1985). However, it has been shown that urate radicals accelerate inactivation of alcohol dehydrogenase (Kittridge and Willson, 1984). The urate radicals maybe repaired by ascorbate and hence the suggested damage to alcohol dehydrogenase enzyme by the urate radicals may be prevented in vivo by ascorbate (Simic and Jovanovic, 1989; Maples and Mason, 1988).

1.2.2. Chain-breaking antioxidants

Chain-breaking or secondary antioxidants trap the chain-propagating peroxyl radicals, $ROO^{\bullet}$, and alkoxyl radicals, $RO^{\bullet}$, and thereby reduce the length of the oxidation chains. The majority of chain-breaking antioxidant are phenols (A-OH) or aromatic amines (A-NH). The mechanism of most of them could be described by the following reactions:

$$ROO^{\bullet} + A\text{-}OH \longrightarrow ROOH + A\text{-}O^{\bullet}$$

$$ROO^{\bullet} + A\text{-}NH \longrightarrow ROOH + A\text{-}N^{\bullet}$$

The aromatic oxygen centred and nitrogen centred radicals are less reactive and incapable of abstracting hydrogen atoms because of the delocalisation of the unpaired electron in the aromatic ring structure and they are eventually destroyed, e.g. by reaction with a second peroxy radical or antioxidant radical:

$$\left.\begin{array}{l} ROO^{\bullet} + A\text{-}O^{\bullet} \\ A\text{-}O^{\bullet} + A\text{-}O^{\bullet} \end{array}\right\} \text{non-radical products}$$

Vitamin E

Vitamin E describes a group of compounds which consists of four tocopherols and four tocotrienols, which are chain -breaking antioxidants. Among the vitamin E isomers the most abundant and biologically active isomer is α-tocopherol, which has been found in the hydrophobic interior of biological membranes of most cells (Van Acker et al. 1993). The term α-tocopherol and vitamin E are used in the literature almost interchangeably. However, because of biological activity of other isomers of vitamin E use of this term is incorrect (Halliwell and Gutteridge, 1991). In addition to the α-isomer the other tocopherols with biological activity are β, γ and δ tocopherol. They differ from α- tocopherol only in regard to methyl substitution on the benzene ring (Fig 1.2.2.a. & Table 1.2.2.). The tocotrienols differ from tocopherols in that they have an unsaturated sidechain (Niki, 1987). The unsaturated isomers have not been well studied and only α-tocotrienol appears to have antioxidant activity.

Figure 1.2.2. a: Chemical structure of natural tocopherols

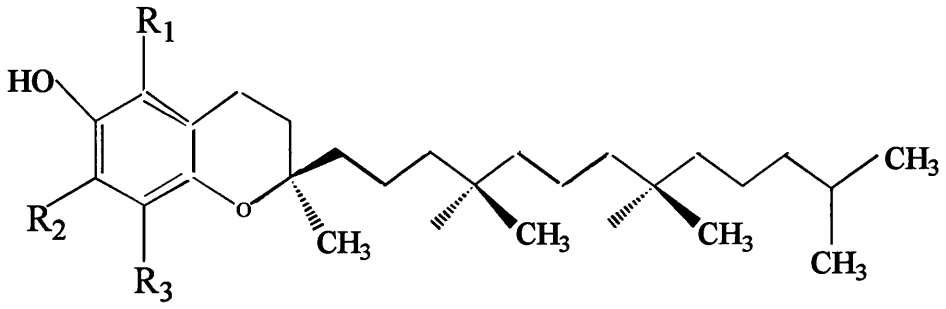


Table 1.2.2 : Natural tocopherols

	R1	R2	R3
α-tocopherol	CH_3	CH_3	CH_3
β-tocopherol	CH_3	H	CH_3
γ-tocopherol	H	CH_3	CH_3
δ-tocopherol	H	H	CH_3

The chroman head group is thought to be responsible for the antioxidant activity (Burton et al. 1980), whereas probably the major function of the phytyl sidechain is the incorporation and retention of the molecule in the membrane (Niki et al. 1985).

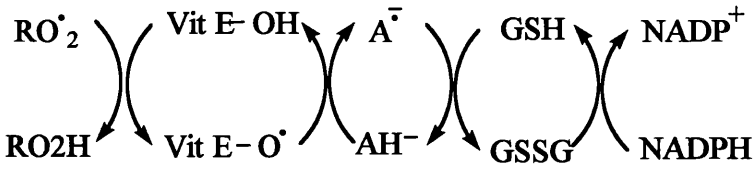
Burton et al (1983) have shown that α-tocopherol is the major peroxyl radical-trapping, chain-breaking antioxidant in human blood, even in the individuals that are suffering from deficiency of vitamin E (Ingold et al. 1987). Although dietary intake of γ-tocopherol is higher than α-tocopherol (Parker, 1989), plasma concentration of α-tocopherol is 5-10 fold higher than γ-tocopherol (Behrens and Madere, 1985; Clevidence and Lehmann, 1989; Traber and Kayden, 1989; Behrens and Madere, 1987). It has been proposed that vitamin E metabolism is regulated by hepatic tocopherol binding protein (Kayden and Traber, 1993). This regulation is possibly specific for α-isomer rather than

other isomers of vitamin E. This protein preferentially transfers the α-isomer of vitamin E to nascent VLDL in the liver for secretion into the plasma, where the normal metabolism of lipoproteins causes α-tocopherol to return to the liver via LDL and HDL (Yoshida et al. 1992). Excess α-tocopherol and other forms of vitamin E are excreted in the bile. Hence, the higher concentration and biologic activity of α-tocopherol in comparison with other isomers of vitamin E is a phenomenon not so much related to absorption but results from differences in the affinity of hepatic tocopherol binding protein for these compounds. The absorption, retention, biologic and antioxidant activity of vitamin E isomers can be summarised as follows:

Absorption	$\alpha \geq \gamma > \beta > \delta$	Biological activity	$\alpha > \beta \approx \gamma > \delta$
Retention	$\alpha > \gamma > \beta > \delta$	Antioxidant activity	$\alpha > \beta \geq \gamma > \delta$

A factor that adds to the efficacy of their antioxidant activity, is the repair of the α-tocopherol radical by ascorbic acid and glutathione (Niki et al. 1984; Packer et al. 1979). This increases the overall in vivo efficiency of the vitamin E system (Fig 1.2.2.b).

Figure 1.2.2. b: Free radicals scavenging by a redox cycle



Carotenoids

Carotenoids are widely distributed in nature, and have an important role in protecting living systems. Among the carotenoids, β-carotene has the advantage of lack of toxicity (Mathews-Roth, 1988). β-carotene along with α-tocopherol has been known as a lipid

soluble chain-breaking antioxidant in human plasma (Burton and Ingold, 1984). This carotenoid accumulates to high concentrations in the membranes of certain tissues such as the retina. β-carotene activity as a peroxyl radical trapping antioxidant varies with the pressure of oxygen. It is more efficient at low oxygen tension against singlet oxygen and peroxyl radicals (Burton, 1989). The proposed mechanism is that β-carotene directly reacts with $ROO^{\bullet}$ to yield a carotenoid radical. The combination of carotenoid radical with oxygen leads to formation of carotenoid-peroxyl radical, which is a reversible reaction and depends on the tension of oxygen in the environment (reaction 2). At low oxygen pressure the production of chain-carrying peroxyl radicals is reduced, while a high tension of oxygen cause the equilibrium of reaction 2 to shift toward the right. Under this condition, because of autoxidation, β-carotene forms a peroxyl radical and can act as a prooxidant (Burton, 1989). Since the oxygen pressure in animal cells is low, β-carotene may be of great importance as an antioxidant in vivo. The reaction is simply shown below:

$$\text{ß-Carotene} + ROO^{\bullet} \longrightarrow \text{ß-Carotene}^{\bullet} + ROOH \qquad (1)$$

$$\text{ß-Carotene}^{\bullet} + O_2 \underset{\text{Low } O_2 \text{ tension}}{\overset{\text{High } O_2 \text{ tension}}{\rightleftharpoons}} \text{ß-Carotene-}OO^{\bullet} \qquad (2)$$

A large number of epidemiological studies support the finding that natural foods containing carotenoids, flavonoids, and other antioxidants have a chemoprotectant effect against cancer (Toma et al. 1995; Van-Poppel, 1993; Durgan et al. 1993). However, epidemiological evidence is not very consistent. On the other hand, a large scale trial (29000 middle-aged male smokers) has shown a higher incidence of lung cancer in the group supplemented with ß-carotene in comparison with those treated with placebo (Albanes and Heinonen, 1994). Hence, these substances may have harmful as well as beneficial effects.

Bilirubin

Bilirubin is the lipid soluble end product of catabolism of the haem of haemoprotein. It is toxic for many tissues in high concentrations. It has chain-breaking antioxidant property at physiological conditions (Stocker et al. 1987). Under physiological oxygen tension, bilirubin reacts directly with $ROO^{\cdot}$ and thus may supplement the activity of β-carotene in many tissues. It has been shown that albumin-bound bilirubin participates in a non-enzymatic antioxidant defence system in plasma (Frei et al. 1988; Lindeman et al. 1989). Bilirubin is also an efficient scavenger of singlet oxygen (Di-Mascico et al. 1989). On the other hand, bilirubin can produce singlet oxygen during photo-oxidation (McDoonagh, 1971). This reaction is of a particular importance during phototherapy of neonatal hyperbilirubinemia. Photo-oxidation causes bilirubin to undergo a wide variety of oxidative and additive reactions which lead to self-destruction of bilirubin (Cohen and Ostrow, 1980). Bilirubin is a rather inefficient sensitiser for singlet oxygen and also the most reactive known acceptor for singlet oxygen, hence, bilirubin possibly controls its own photodestruction in vivo by reacting rapidly with any singlet oxygen it produces (Lightner and Norris, 1974). It has also been proposed that photoisomerisation, where bilirubin undergoes structural rearrangement, is more important than production of singlet oxygen in clearance of bilirubin during phototherapy (Bensasson et al. 1993).

1.3. Lipid peroxidation in biologic systems

Cell membranes contain large amounts of unsaturated lipids, cholesterol and proteins. The stability and balance between lipids and protein is important for proper function of membrane-related enzymes and channels. Abundance of polyunsaturated fatty acids, however, makes the cell membranes susceptible to peroxidation. On the other hand, membranes also contain lipophilic antioxidants (e.g., vitamin E) which stabilise membrane structure and, along with the other antioxidant systems, maintain the biological environment in equilibrium. Enzymatically controlled oxidation of PUFAs is a necessary step in the biosynthesis of essential eicosanoids (e.g., prostacyclin, thromboxane, and leukotriene), which are sufficiently controlled in vivo. Nevertheless, uncontrolled oxidation of PUFAs in vivo causes undesirable physiological effects. This

become important, especially if there is a lack of proper antioxidant mechanism (Ames, 1983).

The peroxidation process

Lipid peroxidation can be described as a free radical chain reaction in terms of initiation, propagation, branching, and termination processes. The initiation step is neither simple nor well understood. However, several reactions have been cited as important in biologic systems. It has been suggested that lipid peroxidation is initiated by the hydroxyl radical ($OH^{\bullet}$), that can be formed from oxygen (Czapski, 1984). Formation of $OH^{\bullet}$ occurs via $O_2^{-\bullet}$ dependent reduction of Fe^{3+} to Fe^{2+} and subsequently cleavage of H_2O_2 by Fe^{2+} to $OH^{\bullet}$:

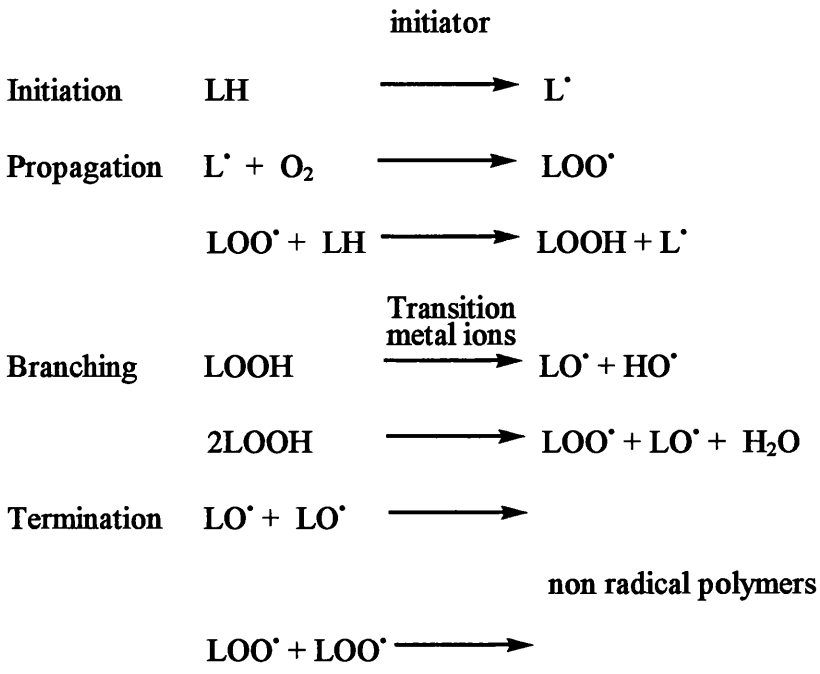
$$O_2^{-\bullet} + Fe^{3+} \longrightarrow O_2 + Fe^{2+} \qquad (1)$$

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

$$Fe^{2+} + H_2O_2 \longrightarrow Fe^{3+} + OH^{-} + OH^{\bullet} \qquad (3)$$

The reaction (3) is known as a Fenton reaction. Various iron-oxygen complexes may also be capable of abstracting a hydrogen atom and initiating lipid peroxidation (Samokyszyn et al. 1990). Abstraction of H atom from methylen group leaves behind a lipid radical group ($L^{\bullet}$). Once a lipid radical is formed, it may enter into a propagation step. The carbon centre radical reacts with molecular oxygen to give a lipid peroxy radical ($LOO^{\bullet}$). The $LOO^{\bullet}$ attacks another lipid molecule and abstracts a hydrogen atom to give a lipid hydroperoxide (LOOH) and other lipid radical ($L^{\bullet}$). The latter reaction carries the lipid peroxidation chain through the propagation sequence. The branching of lipid hydroperoxides often involves transition metal catalysis, yielding lipid peroxyl

(LOO$^{\bullet}$) and lipid alkoxyl radicals (LO$^{\bullet}$). Termination is a bimolecular interaction of lipid alkoxyl and lipid peroxyl radicals which gives non radical products. The whole procedure can be simplified as follows:



Where LH = unsaturated fatty acid, HO$^{\bullet}$ = hydroxyl radical, L$^{\bullet}$ = Lipid radical, LO$^{\bullet}$ = alkoxyl radical, LOO$^{\bullet}$ = peroxyl radical and LOOH = Lipid hydroperoxide.

1.4. Mechanism

The various mechanisms by which the first lipid-free radicals are formed in tissue are described.

1.4.1. Enzymatic pathway

Peroxidation of PUFAs occurs via different enzymes. Among these, cyclooxygenase, cytochrome P450 and lipooxygenase enzymes are well recognised (Oliw, 1994; Anggard and Flower, 1993). These enzymes in the presence of molecular oxygen catalyse the oxidation of free arachidonic acid. Cytochrome P450 and lipooxygenases catalyse biotransformation of PUFAs to a variety of oxygenated products including epoxides and hydroxy fatty acids. Cyclooxygenase catalyses oxidation of arachidonic

acid to form prostaglandin H_2 as a precursor of prostaglandins, prostacyclins and thromboxanes.

1.4.1.1. Cyclooxygenase

This enzyme catalyses controlled enzymatic lipid peroxidation of fatty acid substrates such as arachidonic acid (AA) in vivo. In response to an appropriate stimulus, phospholipase A_2 alone or in combination with other lipases, catalyses the cleavage of esterified arachidonic acid. The free fatty acid can interact with different types of enzymes which are available in biological systems. In the case of cyclooxygenase, this enzyme catalyses the reaction converting arachidonic acid (20:4 n-6 eicosatetraenoic acid) to the unstable cyclic endoperoxides (e.g., PGG_2 and PGH_2). This enzymatic conversion involved activated complexes which, if perturbed, e.g. pathophysiological states, could cause uncontrolled lipid peroxidation. These intermediate products are further metabolised to prostaglandins or thromboxanes by a series of complex reactions (Hamberg and Samuelsson, 1967).

1.4.1.2. Lipooxygenases

Another group of enzymes collectively called lipooxygenases, convert arachidonic acid to a series of hydroperoxyeicosatetraenoic acids (HPETEs), which are then further metabolised to either a series of corresponding HETEs or to the leukotrienes (Kanner et al. 1987). ATP, iron and calcium ions are important cofactors in these metabolic pathways (Yamamoto, 1991). The basic mechanism of catalysis by lipooxygenase appears to be the same as that of cyclooxygenase activation.
The majority of the lipooxygenase products are converted to the corresponding alcohols enzymatically via, e.g. glutathione peroxidase (Siegel et al. 1979).

1.4.1.3. Cytochrome P450

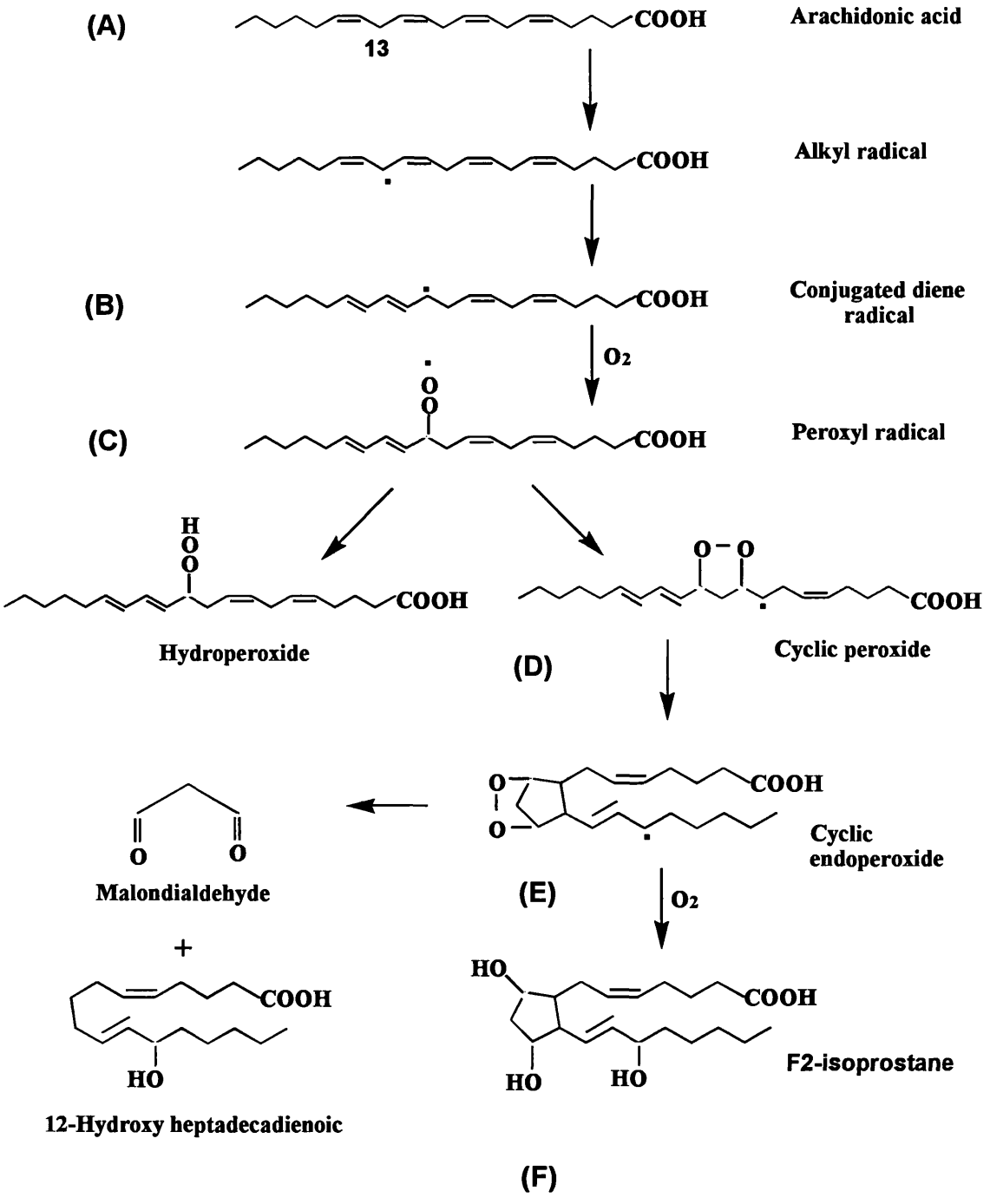
Cytochrome P450 is a heme-containing mixed function oxidase which is responsible for many drug oxidation reactions. The enzyme consists of a family of closely related

isoenzymes located in the membrane of the endoplasmic reticulum, and it exists in multiple forms of monomeric molecular weight of approximately 45000-55000 (Gibson and Skett 1986). It can function as a monooxygenase, requiring NADPH and molecular oxygen to oxidise a substrate. Cytochrome P450 in conjunction with NADPH and cytochrome P450 reductase can catalyse ADP-Fe^{3+} dependent peroxidation of liposomes from different sources (Sevanian et al. 1990). Cytochrome P450 also catalyses the hydroperoxide-dependent oxidation of a variety of endogenous substrates and xenobiotics (White and Coon, 1980).

1.4.2. Non-enzymatic pathway

The non-enzymatic peroxidation of PUFAs such as arachidonic acid occurs via incorporation of oxygen, which is accelerated in the presence of traces of transition metal ions. Fatty acid hydroperoxides can also stimulate the peroxidation of PUFAs via hydroperoxide dependent peroxidation (Girotti, 1985). Hydroperoxides are the initial products of this peroxidation process. These products further decompose to form a variety of secondary products including hydroxylated fatty acids, aldehydes, prostaglandin-like compounds and short chain alkanes which could be measured as a marker of oxidative stress in vivo. As illustrated in figure 1.4.2., the initial step of the reaction, starting with AA, is an abstraction of hydrogen at C-13 (Fig 1.4.2.a). This allylic abstraction of hydrogen can occur at different positions on the carbon chain (e.g., 10 and 7). The resulting allylic radical then undergoes molecular rearrangement to yield a conjugated diene with UV absorbance at 234 nm (Fig 1.4.2.b). Reaction of a conjugated diene radical with an oxygen molecule produces an 11 peroxy radical (Fig 1.4.2.c). This electrophilic oxygen-based radical attacks the electron-rich carbon 9, simultaneously forming a cyclic endoperoxide and an unpaired electron at carbon 8 (Fig 1.4.2.d). This radical attacks carbon 12, closing a 5-membered ring and generating a further allylic radical stabilised at carbon 15 (Fig 1.4.2.e). The cyclic endoperoxide can interact with another molecule of oxygen to yield a prostaglandin-like compound (F_2-isoprostane) or it can be decomposed to malondialdehyde and other polymerisation products (Fig 1.4.2.f).

Figure 1.4.2: Non-enzymatic peroxidation of arachidonic acid



From Nourooz-Zadeh, J.(unpublished data)

1.5. Biomedical significance of lipid peroxidation

Lipid peroxidation has been implicated in a number of pathological reactions in vivo. The occurrence of high levels of lipoperoxides has been shown play an important role in the aetiology of degenerative diseases such as atherosclerosis (Witztum, 1994; Bruckdorfer, 1990) diabetes (Baynes, 1991; Wolff, 1993) and retinal degeneration (Yagi, 1976). There is also a close relationship between lipid peroxidation and aging as indicated by the accumulation of lipofuscin pigments (Pal Yu, 1993). Lipofuscin is defined as a yellowish-brown lipid-soluble cytoplasmic granular pigment having an intense yellow autofluorescence when exposed to ultraviolet light (Tsuchida et al. 1987). This pigment accumulates in various tissues of animals and increases as they age. A variety of secondary products other than MDA such as 4-hydroxynonenal produced during lipid peroxidation are implicated in lipofuscin formation (Esterbauer et al. 1986; Fukuzawa et al. 1985; Shimasaki et al. 1984).

The in vivo peroxidation of lipids is more complex than in pure in vitro systems, because of the variety of PUFAs, presence or absence of transition metal ions and reducing agents. Hence, in biological systems it is not apparent which of the lipid peroxidation products can be formed, in what quantities and which of them have a significant relationship with the pathogenesis of related diseases.

From a molecular point of view, damage to living systems by lipid peroxidation can be direct or indirect. Direct damage due to lipid peroxidation may alter biophysical properties of membranes (Richter, 1987). Disturbances in membrane integrity can cause loss of barrier properties (Mak and Weglicki, 1985), which lead to changes in concentration of intra- and extracellular ions such as calcium (Orrenius et al. 1989). Lipid peroxidation also decreases the activity of endoplasmic reticulum enzymes such as glucose 6-phosphatase and cytochrome P450, which are associated with cell membranes (Glende et al. 1976; Poli et al. 1981). Indirect effects of lipid peroxidation are due to the formation of reactive intermediates and end products. Aldehydes are among the formed end products of lipid peroxidation (Frankel, 1987; Janero and Burghardt, 1989). Among the aldehydes, attention has been focused on malonaldehyde (MDA) and 4-hydroxynonenal (4-HNE), because of their toxicity to cells (Esterbauer et al. 1991). Both MDA and 4-HNE are very reactive and react with amino acids, proteins, nucleic

acids and various enzymes. This leads to inhibition of synthesis of proteins, DNA, RNA and inactivation of enzymes (Esterbauer et al 1991; Benedetti and Comporti, 1987). It has been shown that 4-HNE, which is produced during oxidation of LDL, binds the lysine amino group of the LDL apoprotein (Lang and Esterbauer, 1991). This cause modification of LDL, which can no longer be recognised by the LDL receptor and is instead recognised by the scavenger receptor of macrophages. This finally leads to development of atherosclerosis.

1.6. Atherosclerosis

Atherosclerosis is a progressive and degenerative disease, characterised by a series of cellular changes in the arterial system, which is accompanied by dysregulation of cholesterol metabolism (Ross, 1992; Hajjar and Pomerantz, 1992). These changes involve formation of various different lesions, the composition of which depends on anatomic location, the age and sex of the individual, and the risk factors to which the individual has been exposed. The composition of these lesions vary from fatty streaks to gelatinous lesions to various forms of fibrous plaques and complicated lesions. Clinically fibrous plaque and the complicated lesion are important, since they may partially or fully obstruct the lumen of an artery and lead to the common clinical sequence of disease such as infarction, gangrene, or reduced function of an organ or limb (Ross, 1992).

1.6.1. Risk factors

Hypercholesterolemia, diabetes, hypertension and cigarette smoking have been considered as the major risk factors in the incidence of atherosclerosis (Ross, 1992).

1.6.1.1. Hypertension

Hypertension has been recognised as a risk factor associated with increased incidence of coronary heart disease, cerebrovascular disease, and accelerated atherogenesis.

Functional disturbances in cell membranes and abnormality in lipoprotein phospholipid composition have been shown in patients with essential hypertension (Swales, 1990; Bagdade et al. 1995). The LDL of these patients is more susceptible to oxidative modification than normal LDL (Keidar, 1994). Hypertension is not a direct risk factor for atherosclerosis. However, when accompanied with other risk factors such as diabetes and hypercholesterolaemia, it becomes one the common risk factors for incidence of atherosclerosis (Ross, 1992).

1.6.1.2. Smoking

Cigarette smoking is one of the epidemiologic risk factors for the incidence of atherosclerosis (Ball and Turner, 1974). Cigarette smoke contains carbon and oxygen centred free radicals, which can initiate lipid peroxidation (Church and Pryor, 1985). Modification of LDL and consequent tendency to oxidation has been shown in smokers as a group, compared with non smokers (Sanderson et al. 1995; Harats et al. 1990). Higher lipid peroxide levels and lower antioxidant capacity in arterial tissue of smokers in comparison with non smokers has also been reported (Mezzetti et al. 1995). Cigarette smoke also contains nitric oxide (NO), which can react with oxygen to form nitrogen dioxide (NO_2). Nitrogen dioxide is itself a radical and is capable of attacking compounds $>C=C<$ with double bonds (Halliwell and Gutteridge, 1991).

1.6.1.3. Diabetes

Diabetes mellitus has been known as a complex with insulin dysfunction in conjunction with abnormalities in glucose homeostasis and lipid metabolism. Diabetes has been proposed as an independent factor in the incidence of atherosclerosis, even in the absence of other risk factors such as hyperlipidemia and hypertension (Kannel and McGee, 1979; Wolff et al. 1991). There are many potentially atherogenic factors in diabetes that may participate in the sequence of events which lead to the increased incidence of atherosclerosis (Steiner, 1985). It has been shown that diabetes associated with oxidative stress (an imbalance between reactive oxygen species and the antioxidant system in

favour of former) can be implicated in the complications of diabetes (Baynes, 1991). There is substantial evidence which indicates disturbances of antioxidants and micronutrient status in the diabetic state (Strain, 1991; Asayama et al. 1993; Walter et al. 1991; Collier et al. 1990). Significant disturbance in the metabolic handling of ascorbic acid as indicated by the increased dihydroascorbic acid/ascorbic acid (DHA/AA) ratio has been reported (Sinclair et al. 1991). Furthermore, impaired glutathione and thiol transport has been shown in diabetic patients (Yoshida et al. 1995). Many diabetic individuals are also hypercholesterolaemic. It has been shown that plasma of diabetic patients has increased levels of modified LDL, which causes accumulation of cholesterol in intimal aortic cells in vitro (Sobenin et al. 1993). Some diabetics demonstrate decreased levels of HDL and they are often hypertensive. It has been shown that diabetic patients have higher levels of plasma lipid peroxidation products than normal individuals (Collier et al. 1992; Kaji et al. 1985). Glycosylation of LDL in the presence of high levels of glucose may cause the LDL to become more susceptible to oxidation (Hunt et al. 1990; Sakurai et al. 1991; Doucet et al. 1995; Bowie et al. 1993; Smith et al 1985). Impaired function of vascular endothelial cells, which have a key role in vascular physiology and pathophysiology has been reported in diabetes by several investigators (Ruderman et al. 1992; Cohen and Tesfamariam, 1992). Dysfunction of vascular endothelium in diabetes has been associated with its complications and accelerated atherosclerosis. Different mechanisms such as non-enzymatic glycosylation, autoxidation of monosaccharides, the polyol pathway, increased production of reactive oxygen radicals from damaged tissue and reduction of antioxidant reserve have all been proposed as pathways that may participate in endothelial cell dysfunction as a result of cumulative oxidative stress in diabetes (Tesfamariam, 1994).

Polyol pathway

Under hyperglycaemic conditions production of sorbitol and fructose from glucose is increased due to aldose reductase (AR) and sorbitol dehydrogenase activity (Gonzalez et al. 1986). Studies have shown that AR enzyme is present in endothelial cells (Kern and Engerman, 1982). Aldose reductase utilises reduced nicotinamide-adenine

dinucleotide phosphate (NADPH) to reduce the aldehyde form of glucose to sorbitol. Since AR activity requires cellular NADPH (Fig 1.6.1.3.), the polyol pathway may deplete NADPH. NADPH is essential for many enzyme functions such as cytochrome P450, nitric oxide synthase and glutathione reductase (Cohen, 1993; Gonzalez et al. 1984). Glutathione reductase is required to maintain glutathione in a reduced form (GSH), which is one of the most important antioxidant factors against oxidative damage. Hence depletion of GSH as a result of the polyol pathway may be involved in the tissue injury in diabetes (Wolff, 1985).

Furthermore, depletion of NADPH may lead to lower production or inactivation of nitric oxide (Fig 1.6.1.3.) via increased free radical activity. Nitric oxide ($NO^{\bullet}$) is an endothelium-derived relaxing factor that induces smooth muscle relaxation and is an important mediator in the regulation of vascular tone (Ignarro et al. 1987). Nitric oxide contains an unpaired electron, it can rapidly react with super oxide anion ($O_2^{\bullet -}$) to form inactive nitrite and ,therefore, protects endothelial cells against cytotoxic effect of $O_2^{\bullet -}$. $NO^{\bullet}$ exerts its protective effect against $O_2^{\bullet -}$ in normal physiological conditions (where $NO^{\bullet}$ is present in amounts higher than $O_2^{\bullet -}$). Nevertheless, it has been proposed that under hyperglycaemic conditions the production of ROS such as $O_2^{\bullet -}$, is increased and production of $NO^{\bullet}$ is decreased as a result of the polyol pathway. In this condition, $NO^{\bullet}$ may interact with $O_2^{\bullet -}$ to form peroxynitrite, which then forms more reactive radicals such as nitric oxide (NO_2) and hydroxyl radical ($OH^{\bullet}$).

Autoxidation of glucose

Exposure of plasma and cell membrane proteins to high levels of glucose for a long time causes non-enzymatic glycosylation, cross-linking and formation of advanced glycosylation end products (Brownlee et al. 1988). These products represent the terminal adducts of the non-enzymatic glycation reaction between glucose and the amino groups of protein (Brownlee et al. 1984). It has been proposed that advanced glycosylation end

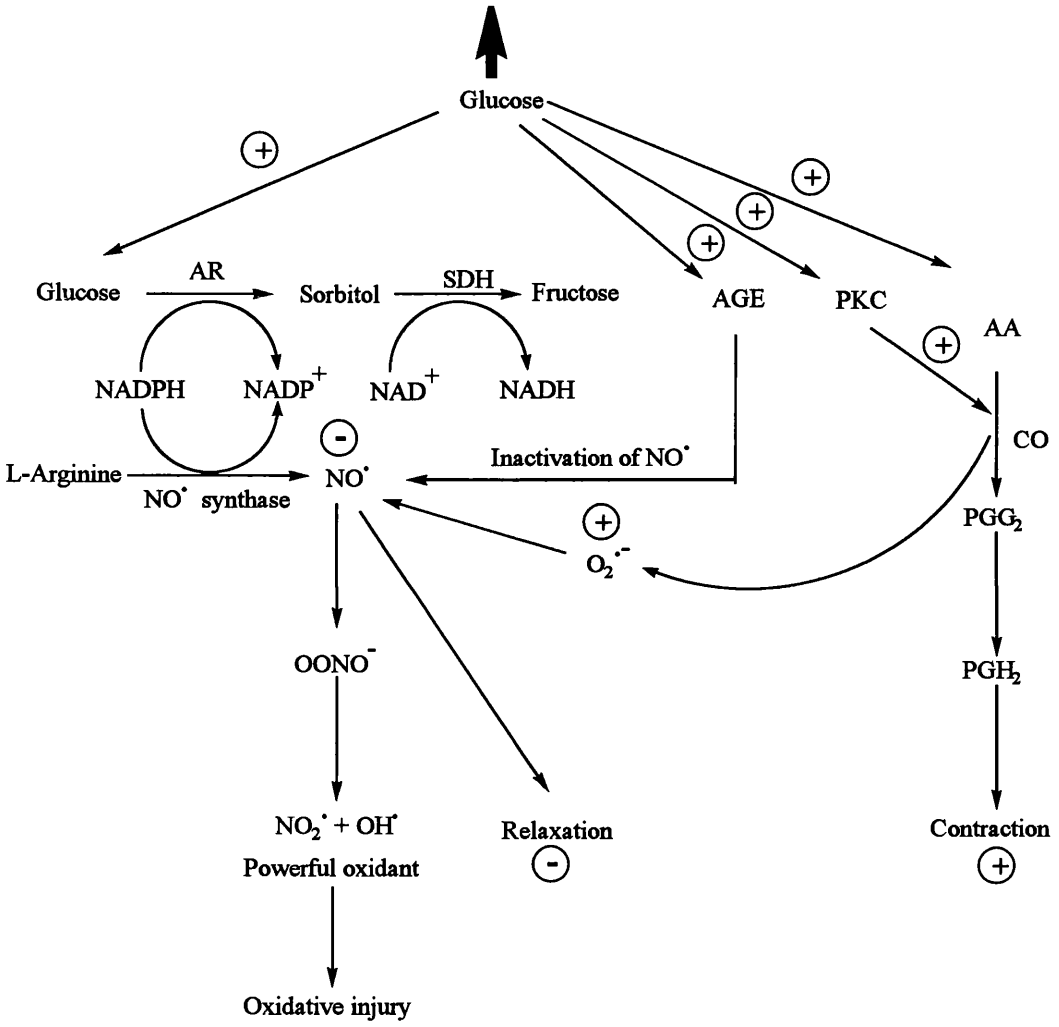
products inactivate nitric oxide via a chemical reaction (Bucala et al. 1991) and may be implicated in further damage to endothelial cells.

Protein kinase C activation

It has been shown that elevated glucose may cause activation of protein kinase C which in turn can play a role in the formation of $O_2^{\bullet -}$ and vasoconstrictor prostaglandins (PGH_2) (Lee et al. 1989). Increased formation of $O_2^{\bullet -}$ and PGH_2 may cause inactivation of $NO^{\bullet}$ and smooth muscle contraction and eventually lead to impaired function of vascular endothelium.

These varied observations indicate that diabetes has been associated with oxidative stress which may be involved in dysfunction of vascular endothelium via various mechanisms. These pathways may cause the development of complications of diabetes and accelerated incidence of vascular disease such as atherosclerosis. The events which may led to endothelial dysfunction and tissue injury in diabetes are depicted in figure 1.6.1.3.

Figure 1.6.1.3: The proposed mechanism for vascular endothelium dysfunction via different pathways in hyperglycaemia



Where AR = aldose reductase, SDH = sorbitol dehydrogenase, NADPH = nicotinamide-adenine dinucleotide phosphate (reduced form), NADH = nicotinamide-adenine dinucleotide (reduced form), AA = arachidonic acid, CO = cyclooxygenase, $NO^{\bullet}$ = nitric oxide, AGE = advanced glycosylation products, PKC = protein kinase C, $O_2^{\bullet -}$ = superoxide, $OONO^-$ = peroxynitrite, $NO_2^{\bullet}$ = nitrogen dioxide.

1.6.1.4. Hypercholesterolemia

All lipids of human plasma are transported as complexes with proteins. Except for fatty acids, which bind chiefly to albumin, the lipids are carried in special macromolecular complexes termed lipoproteins (Malloy and Kane, 1995). Plasma lipoproteins consist of five major classes and several subclasses. Each class, as separated by ultracentrifugation, is heterogeneous in size and composition (Gotto et al. 1986). They are diverse species of particles containing cholesterol, triglycerides, and other lipids, together with a series of specific apoproteins. The composition and properties of plasma lipoproteins are shown in Table 1.6.1.

Plasma lipoproteins are not only responsible for transport of lipids, but also play an important role in metabolism of lipids such as cholesterol and triglyceride (Stein, 1986). Abnormal elevation of lipoproteins in the plasma can result from disturbances in their metabolism such as overproduction, deficient removal, or from combination of both abnormalities (Ross, 1992).

Chylomicrons

Chylomicrons are the largest particles, which are synthesised in the epithelial cells of gut and responsible for the transporting dietary fat. The major lipid fraction is triglyceride, which constitutes over 80% of the total particle by weight. Chylomicrons contain a variety of apolipoproteins (see table 1.6.1.), but only 1-2% of the total particle is made up of apolipoproteins.

Very low density lipoproteins (VLDL)

VLDL is the other triglyceride-rich lipoprotein, which is synthesised and released from liver. VLDL transports hepatic-synthesised triglyceride and cholesterol, that are probably derived from dietary precursors, such as free fatty acids, glycerol and carbohydrates (Gotto et al. 1986). VLDL contains ApoB-100, E and C species (Table 1.6.1.).

Intermediate density lipoprotein (IDL)

IDL is partly depleted of triglyceride and is a short-lived intermediate lipoprotein, which is formed following hydrolysis of VLDL by lipoprotein lipase (LPL). It contains about equal amounts of cholesterol and triglyceride. Its major apolipoproteins are ApoB and E.

Low density lipoprotein (LDL)

LDL is the major carrier of cholesterol and cholesteryl ester in the plasma. Approximately 60% of the cholesterol is transported as LDL in man and about three-quarters of this is esterified. High-affinity apoB LDL-receptor mediated endocytosis is a major pathway by which LDL is catabolised in hepatocytes and most other nucleated cells (Patsch and Gotto, 1996). Esterified cholesterol from the core is then hydrolysed, yielding free cholesterol for the synthesis of cell membranes. ApoB-100 is the sole apolipoprotein of LDL, and is responsible for most of the LDL catabolism via the high affinity apoB-LDL-receptor pathway.

High density lipoprotein (HDL)

HDL is rich in protein and contains about 50% protein and 50% lipid (30% phospholipid and 20% cholesterol). The apolipoproteins of HDL are secreted by liver and intestine and most of the lipids in HDL come from chylomicrons and VLDL during lipolysis. At least three well defined HDL subgroups have been studied. These include HDLc, HDL_2 and HDL_3 (Stein, 1986). The apolipoproteins in HDL are AI, AII,C,E and D, but AI and AII are the major apolipoproteins and constitute about 90% of total HDL proteins.

Table 1.6.1: Composition and properties of human plasma lipoproteins

Lipoprotein	Density	Electrophoretic mobility	Major lipid constituents	Apoprotein constituents
Chylomicrons	0.95	Origin	Triglyceride	ApoA-I,ApoA-II, ApoA-IV, ApoB-48
VLDL	0.95-1.006	Pre-beta	Triglyceride, Phospholipid	ApoB-100, ApoC-I,ApoC-II, ApoC-III,ApoE
IDL	1.006-1.019	Beta	Esterified cholesterol, phospholipid	ApoB-100, ApoE
LDL	1.019-1.063	Beta	Triglyceride, esterified cholesterol	ApoB-100
HDL	1.061-1.210	Alpha	Phospholipid, cholesterol	ApoA-I,ApoA-II, ApoC-II,ApoE

* Adopted from Oxford Textbook of Pathology, edited by McGee et al. 1992.

Hypercholesterolemia has been considered as one of the major risk factors associated with atherosclerosis. Increased in vitro oxidation of lipoproteins has been shown in hypercholesterolemic patients (Lavy et al. 1991). Familial hypercholesterolemia (FH) is the genetic disorder of lipoproteins, which is strongly related with atherosclerosis (Goldstein et al. 1995; Witztum, 1993). In this case plasma cholesterol and LDL (rich in free and esterified cholesterol) are excessively high due to the fact that these patients

either lack LDL receptors or have receptors that are incapable of binding or responding to LDL, which lead to development of disease with advanced lesions. There is a lot of evidence to indicate that a crucial step in the pathogenesis of atherosclerosis is the oxidative modification of LDL (Steinberg et al. 1989; Esterbauer et al. 1990; Chisolm, 1991; Carpenter et al. 1991; Esterbauer et al. 1992).

1.6.2. Evidence for in vivo oxidation of LDL

In vitro oxidation of LDL has been reported by different research groups (Esterbauer et al. 1992; Bruckdorfer, 1990; Gebicki et al. 1991; Jürgen et al. 1987; Steinbrecher et al. 1984). LDL can be oxidised with macrophages, endothelial cells, smooth muscle cells, lymphocytes or in cell-free systems in the presence of traces of transition metal ions (e.g., Cu^{++} and Fe^{++}). In recent years much evidence which indicates that LDL can oxidise and undergoes modification in vivo has accumulated. This includes the following:

(1) difference in many properties of circulatory LDL and LDL extracted from atherosclerotic lesions. The LDL from lesions has been found to be more electronegative than circulatory LDL and shows enhanced recognition by mouse peritoneal macrophages possibly as result of partial degradation of apo B in lesion LDL (Clevidence et al. 1984; Goldstein et al. 1981);

(2) similarity between lesion LDL and oxidised LDL including recognition by the scavenger receptor on macrophages (Palinsiki et al. 1989; Yla-Herttuala et al. 1990);

(3) lesion LDL like oxidised LDL is chemotactic for monocytes, while native LDL lacks this property (Ylä-Herttuala et al. 1989);

(4) existence of antibody against oxidised LDL (Orekhov et al. 1991; Salonen et al. 1992) despite the fact that native LDL is not antigenic;

(5) at least a small proportion of the LDL particles in the circulation shows some degree of oxidative modification (Avogaro et al. 1988);

(6) MDA adducts can be found modifying the protein part of LDL during early and late stages of lesion formation (Haberland et al. 1988);

(7) studies on experimental animals have shown that antioxidants can inhibit the progression of atherosclerosis (Kita et al. 1987).

These data support the idea that oxidation of LDL does, indeed, occur in vivo, probably in the artery wall (Steinberg, 1989).

1.6.3. Mechanism of LDL modification

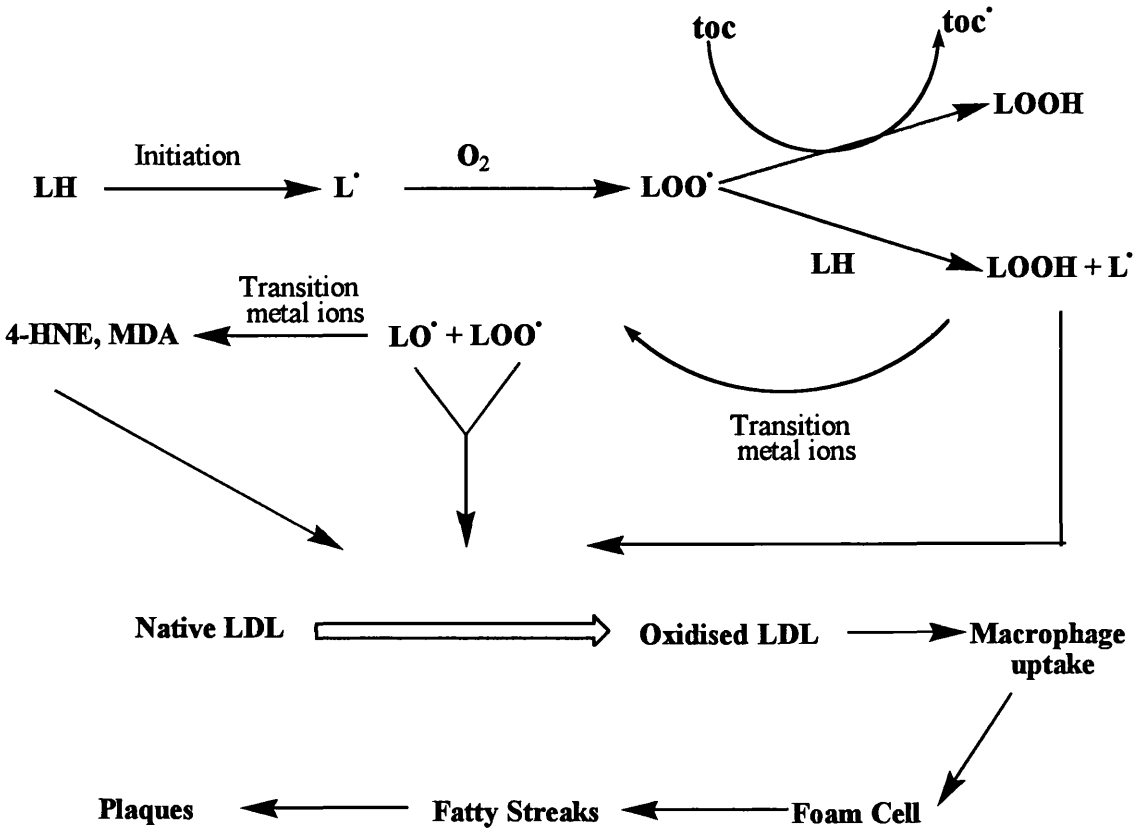
LDL is a large spherical particle with a hydrophobic central core containing cholesteryl ester and triglyceride molecules. A monolayer of phospholipid and free cholesterol surrounds the central core. A specific protein, which is called apoprotein B (apoB) binds to lipid at the outer surface of the particle and confers stability in aqueous solutions. LDL metabolism depends on the integrity of apoB. Uptake of LDL takes place via this protein that is recognised by the LDL receptor during the normal metabolic pathway. Oxidative modification of LDL decreases the affinity of apoB for LDL receptor and increases its affinity to a scavenger receptor. The LDL then binds to the scavenger receptor on macrophages, which internalise the LDL particles. It has been shown that the ε-amino groups of lysine and other amino acids are susceptible to oxidative modification (Steinbrecher, 1987).

LDL is not only rich in cholesterol but also in PUFAs, which makes it susceptible to oxidation. On the other hand, LDL is protected by the presence of several antioxidants (Esterbauer et al. 1991). The source of the radicals which initiate the peroxidation process is still not clear, though it has been suggested that lipooxygenases may be involved (Stienberg et al. 1989).

Certain cells such as macrophages and neutrophils generate large amounts of superoxide anions ($O_2^{\cdot -}$), as part of their biologic functions, during metabolic processes. Insufficient removal of these anions causes production of other radicals, which are more powerful than the original anions. LDL also contains small amounts of endogenous lipid peroxide (Bruckdorfer, 1990). A pro-oxidant effect of lipooxygenase derived peroxides on metal catalysed oxidation of LDL has been shown (O' Leary et al. 1992). Involvement of endogenous lipid hydroperoxides has been shown in the oxidation of LDL induced by copper ions or Azo-peroxyl radicals (Thomas and Jackson, 1991). The endogenous

peroxides possibly arise from lipooxygenases (Folcik et al. 1995), as a result of consumption of peroxidised food lipids of dietary origin (Wolff and Nourooz-Zadeh, 1996; Darley-Usmar and Halliwell, 1996). Peroxynitrite may be involved. It has been shown that addition of peroxynitrite to LDL causes lipid peroxidation and the recognition of oxidised LDL by scavenger receptors (Graham et al. 1993). Trace amounts of transition metals can catalyse this peroxide to alkoxy and peroxy radicals. These radicals can abstract hydrogen atoms from adjacent PUFA forming lipid peroxide and other fatty acid radicals which can propagate the peroxidation process. In the absence of a proper antioxidant system, such as vitamin E, which terminates the peroxidation process, all of PUFAs in LDL could convert to lipid hydroperoxides. The oxidised LDL is taken up by scavenger receptors of macrophages and leads to formation of foam cells containing cholesterol and cholesteryl ester; this, in turn, leads to formation of fatty streaks and plaques (Fig 1.6.3.).

Figure 1.6.3: The proposed mechanism of oxidation of LDL in vivo



Where LH = unsaturated fatty acid, L· = allyl radical, LO· = alkoxyl radical, LOO· = peroxyl radical and LOOH = hydroperoxide.

1.6.4. Potential role for oxidised LDL in atherosclerosis

As described previously, oxidative modification causes LDL to lose its ability to be recognised by LDL receptor, instead its affinity to other scavenger receptors increases (Steinbrecher et al. 1984; Freeman et al. 1991). Oxidised LDL contains highly cytotoxic lipid peroxidation products (Chisolm, 1991; Bruckdofer, 1990; Guyton et al. 1995), which may alter and impair cell function. Endothelial cells appear to be susceptible to the effects of oxidised LDL, and this leads to the development of the atherogenic lesion. Relaxation of arteries is impaired by oxidised LDL (Shimokawa and Wanhoutte, 1989; Jacobs et al. 1990; Schmidt et al. 1990; Chin et al. 1992). It has been shown that oxidised LDL causes changes in physico-chemical properties of endothelial cells and regulation of intracellular calcium (Thorin et al. 1995). Moreover, oxidised LDL provokes an immune response (Parums et al. 1990; Orekhov, 1991; Salonen et al 1992). Minimally-oxidised LDL also stimulates endothelial cells to release a number of biologically-active factors, such as monocyte-chemotactic proteins, endothelial-leucocyte adhesion molecules (ELAMS) and growth factors for monocytes (Rajavashisth et al. 1990; Leake, 1991). The term 'minimally oxidised LDL' refers to LDL which is prepared by prolonged storage (3-6 months at 4 °C) or by incubation with a low concentration of ($FeSO_4$, 1 μM) for 2-3 days. Unlike highly oxidised LDL, minimally-oxidised LDL is taken up by the classical LDL receptor rather than by the scavenger receptor on macrophages (Berliner et al. 1990). Oxidised LDL may also cause disturbances of eicosanoid homeostasis and aggregation of platelets. All these data strongly support the hypothesis that oxidised LDL is atherogenic.

1.7. Analytical techniques for detection of lipid peroxidation products

In the presence of initiator, PUFAs of cell membranes undergo reaction with molecular oxygen to yield lipid hydroperoxides (LOOH) as a primary product of lipid peroxidation. The LOOH can decompose to form other secondary products including malondialdehyde (MDA) alkanals, alkenals, hydroxyalkenals, (Halliwell and Gutteridge, 1991).
Because of the adverse biological activity of oxidised lipids, accurate measurement of these products has been the focus of pharmacological and analytical research. A vast variety of analytical techniques has been developed to measure lipid peroxidation, although every technique has its own limitation and not all of them are applicable to the in vivo studies (Cheeseman, 1993).

1.7.1. Uptake of oxygen

Rate of oxygen uptake can be used as an index of the peroxidation process during continuous measurement of peroxidation. Consumption of oxygen shows a lag and propagation phase during oxidation. However, it requires a large amount of sample to measure this process (Puhl et al. 1994). Most oxygen electrodes are Clark-type arrangements which measure dissolved oxygen. Calibration is carried out by exposing the electrode to known concentrations of dissolved oxygen, although it should be emphasised that the electrode measures the 'activity' of oxygen, not the concentration. It is usually assumed that air-saturated pure water has 0.258 μmol/ml of dissolved oxygen at 25°C and one atmosphere air pressure, although the presence of dissolved solutes will decrease this solubility slightly (Halliwell and Gutteridge, 1991).

1.7.2. Conjugated dienes (CD)

The oxidation of PUFAs by abstraction of allylic hydrogen results in a lipid free radical intermediate, which undergoes molecular rearrangement to form conjugated dienes (Fig 1.4.2.). The CD moiety in a fatty acid molecule is a strong chromophore which absorbs ultraviolet (UV) light in the wavelength region of 230-235 nm. Measurement of CD is a useful index of peroxidation in pure lipid systems. Determination of CD,

however, is difficult in biological systems because of the presence of many of the other substances (e.g., haem proteins) that have an absorbance at the same wavelength and create a high background. Extraction of conjugated dienes into an organic solvent decreases this high absorbance. However, PUFA themselves, and some products from the breakdown of LOOH, absorb UV light strongly at about 210 nm so that the conjugated diene absorbance appears as a shoulder on the PUFA absorbance spectrum (Slater, 1984; Recknagel and Glende, 1984). A second derivative spectroscopy method (Corongiu and Banni, 1994) allows greater sensitivity, since the conjugated diene shoulder that appears in the ordinary spectrum translates into a sharp minimum peak that is more easily measurable. The increased resolution of this technique may allow discrimination between the different conjugated diene moieties present in the sample. However, most (90%) of conjugated diene in human plasma is the non-oxygen-containing isomer of linoleic acid (9, 11-octadecadienoic acid) that can be assayed specifically by HPLC (Dormandy and Wickens, 1987; Britton et al. 1992; Jack et al. 1994). This product is not found in the plasma of animals subjected to oxidative stress and may be of dietary origin or produced by metabolism of gut bacteria (Thompson and Smith, 1985). Thus, conjugated diene methods probably do not measure lipid peroxidation in human body fluids, and is not recommended for human studies.

1.7.3. Thiobarbituric acid reactive materials and malondialdehyde

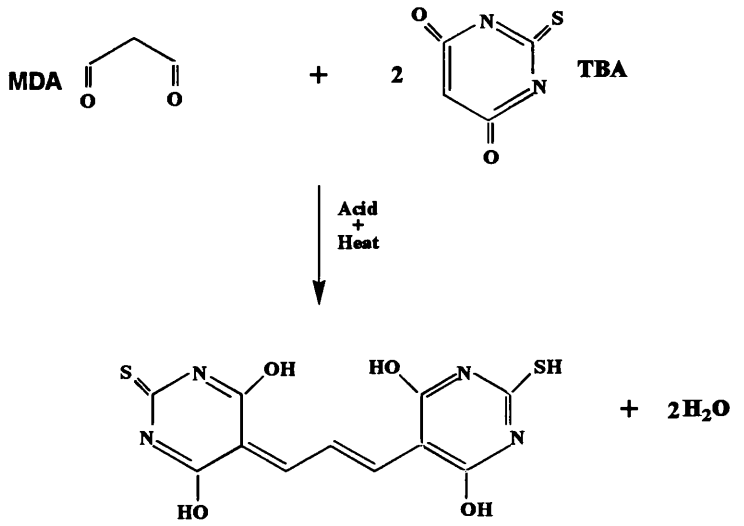
Malondialdehyde (MDA) is a low molecular weight end product, that results from peroxidation of PUFAs. Measurement of MDA has been used as an indicator of lipid peroxidation and free radical activity in biologic systems. Determination of MDA with thiobarbituric acid (TBA) is one of the popular methods in lipid peroxidation studies. The assay is based on the reaction of one molecule of MDA with two molecules of TBA to form a coloured complex (TBA-MDA) at high temperature (80-100 °C) under acidic conditions (Fig 1.7.3.). This complex is measured spectrophotometrically at 532 nm (Esterbauer et al. 1991; Janero, 1990). Extraction of samples with organic solvents such as butanol increases the sensitivity and the complex can be measured fluorimetrically at 553 nm emission with 515 nm excitation (Yagi, 1984). Spectrophotometric determination of MDA with TBA in biological systems is not specific for MDA, because

of the presence of other substances which react with TBA and the term "thiobarbituric acid reactive materials" (TBARMs) is more suitable.

For a more specific estimation of the free MDA in biologic systems various HPLC methods coupled with UV spectrophotometry or fluorimetric detector have been developed (Chirico et al. 1993; Li and Chow, 1994; Draper et al. 1993; Young and Trimble, 1991; Fukunaga et al. 1993; Guichardant et al. 1994; Esterbauer and Cheeseman, 1990; Lepage et al. 1991). These techniques require very careful handling of the samples. While these methods are specific for MDA, the discrepency in results is attributable to differences in sample preparation, TBA reaction condition and methods of purifying the reaction products.

However, determination of MDA in plasma is limited for the following reasons. The yield of MDA is varied and depends on the degree of unsaturation of fatty acids and is only formed from PUFAs with three or more double bonds (Frankel, 1987; Esterbauer et al. 1990). MDA is the side product of enzymatic and secondary-end product of non-enzymatic peroxidation of PUFAs (Kosugi et al. 1989); only certain lipid peroxidation products decompose to yield MDA (Frankel and Neff, 1983). MDA itself is a reactive compound and can decompose to other products such as CO_2 and H_2O. Mitochondrial aldehyde dehydrogenases can convert MDA to malonic acid semialdehyde, which spontaneously decarboxilates to acetaldehyde. Acetaldehyde is then further oxidised by aldehyde dehydrogenases to acetate and further still to CO_2 and H_2O (Siu and Draper, 1982); the formation of MDA precursors and their decomposition to MDA is influenced by the reaction environment (Gardner, 1989). Authentic MDA determination with HPLC enhances the specificity of the TBA test but does not eliminate all of the interfering factors (Chirico et al. 1993). Hence, even the HPLC-based TBA test is not always a precise index of lipid peroxidation in plasma.

Figure 1.7.3: Formation of TBA:MDA complex under acidic conditions



MDA : TBA Adduct

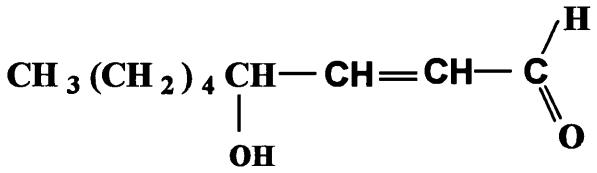
1.7.4. Aldehydes other than malondialdehyde

In addition to MDA, various classes of aldehydes including alkanals, hydroxyalkenal and alkenals are formed as decomposition products of PUFAs hydroperoxides in vivo. Although they are not radicals, they are very reactive and have numerous cytotoxic effects (Esterbauer et al. 1991). Currently, the method used most often for measurement of aldehydes is HPLC coupled with UV based on reaction of samples with 2,4 dinitrophenylhydrazine. In summary aldehydes react with dinitrophenylhydrazine to form dinitrophenylhydrazone (DNPH) derivatives, which are very stable products. The reaction mixture is extracted with organic solvent followed by preseparation of DNPH classes of different polarity with TLC. Finally individual classes of aldehydes are separated with HPLC (Esterbauer et al. 1991; Lang et al. 1990; Holley et al. 1993). Using GC-MS has the advantage of detection of lower concentration of aldehydes. The principle of preseparation of samples before detection with GC-MS is similar to the

HPLC method (Thomas et al. 1995). An alternative procedure involves HPLC separation of the fluorescent cyclohexanedione (CHD) derivatives of the aldehydes (Yoshino et al. 1986). These techniques are generally extremely time-consuming and are not convenient for routine measurements of lipid peroxidation.

Hydroxyalkenals, specially 4-hydroxynonenal (HNE), is probably the most important end product of lipid peroxidation process because of its cytotoxicity (Tsai and Sokoloski, 1995; Siems et al. 1996; Petras et al. 1995). Hence, in specific circumstances measurement of HNE can be important to assess the lipid peroxidation damage in biological systems. Chemical structure of 4-hydroxynonenal has been depicted in figure 1.7.4:

Figure 1.7.4: Chemical structure of 4-hydroxynonenal



1.7.5. Volatile hydrocarbons

Ethane and pentane are the main hydrocarbons released during peroxidation of lipids in biological tissues (Cailleux and Allain, 1993). They can be used as markers of lipid peroxidation in in vitro and in vivo studies. These hydrocarbons can be measured with GC alone or with GC-MS (Kohlmüller and Kochen, 1993; Mendis et al. 1995). In principle expired air is passed through adsorbent below -100 °C to adsorb and concentrate the alkanes, which can be measured by GC or GC-MS. This method is non-invasive and can be repeated in a single subject. There are ,however, factors that limit

the application of this method for routine procedures (Kneepkens et al. 1994). These include:

(1) hydrocarbons are the minor product of lipid peroxidation in biological systems;

(2) their production depends on the availability of iron ions and diet source;

(3) measurement is altered in smokers, because of increased concentration of pentane and ethane in the breath of smoker;

(4) the concentration of pentane and ethane in human breath is very low and similar to that in the surrounding air, which needs the preparation of subjects with hydrocarbon free air;

(5) the procedure is very time consuming, needs expensive instruments and may be inconvenient for some patients.

However, it is a measurement of rate of oxidation, not just of a steady state which depends on balance between production and removal. As such, it could be very useful if the method were to become simpler.

1.7.6. F_2-isoprostanes

A series of prostaglandin F_2 (PGF_2)-like compounds are formed in vivo during peroxidation of arachidonic acid (Fig 1.4.2.) by a mechanism independent of the cyclooxygenase pathway (Morrow et al. 1992). High levels of these compounds have been shown during free radical injury and lipid peroxidation compared to controls (Morrow et al. 1990; Morrow et al. 1992).

Of these the 8-epi $PGF_2\alpha$ is the major component. The 8-epi $PGF_2\alpha$ is also shown to be a potent and selective vasoconstrictor of renal and pulmonary arteries (Morrow et al. 1992). F_2-isoprostanes are initially formed esterified to phospholipids and are released into the circulation by the action of phospholipase A_2 to become biologically active (Morrow et al. 1992). The non-cyclooxygenase derived PGF_2-like compounds are collectively called F_2-isoprostanes. The procedure for the isolation of F_2-isoprostanes in plasma involves chromatography on a C_{18} silica Sep-Pak column which involves washing the column with different solvents in several steps to remove polar impurities and elution of samples. The solvent eluate is evaporated under N_2 gas and residue

separated by thin layer chromatography (TLC) on a silica plate. Quantitative analysis is carried out by GC-MS using negative chemical ionisation (Wendelborn et al. 1990; Morrow and Jackson-Robert, 1994). The disadvantages of the method are: (a) loss of nearly 80% of labelled PGF_2 during separation with TLC; (b) risk of artefact formation during the isolation procedure; (c) time consuming. An improved method has been developed based on clean-up procedure using C_{18} column followed by an aminopropyl (NH_2) cartridge. The NH_2 sorbent selectively binds to free fatty acid by an ion-exchange mechanism (Nourooz-Zadeh et al. 1995).

1.7.7. Fluorescent products

A variety of fluorescent products are formed during reaction of the secondary-end product of lipid peroxidation, such as aldehydes with amino acids (Shimasaki, 1994). Reaction of MDA with amino acids yields fluorescent chromophores, which are called aminoiminopropene Schiff bases and have following structure.

$$OHC{-}CH_2{-}CHO + 2\ R{-}NH_2 \longrightarrow R{-}N{=}CH{-}CH{=}CH{-}NHR$$

Malondealdehyde Amino acids 1-amino-3-iminopropene

Fluorescent products can also be produced by polymerisation of aldehydes including MDA. Lipofuscin is a well known fluorescent compound which has been regarded as an "age-pigment". It results from the reaction of end-products of the oxidative decomposition of lipids, and their cross-linking with proteins and other compounds with amino groups. These fluorescent chromophore products can be measured at 360 nm excitation and 430 nm emission in lipid extracts. Overall recovery of these products from organic solution is low because of their covalent binding to proteins (Shimasaki et al. 1982). Furthermore, it is difficult to discriminate between lipofuscin and

other fluorescent compounds which have fluorescence spectra identical with that of lipofuscin (Tsuchida et al. 1987).

1.7.8. Hydroperoxides

Hydroperoxides are initial products of lipid peroxidation and can be measured in biologic systems by a variety of techniques. Assay of hydroperoxides is the direct assessment of lipid peroxidation and a useful index of early oxidation damage (Pryor, 1987). Hydroperoxides, however, are unstable and undergo enzymatic and non-enzymatic degradation to produce a variety of secondary end products. Hence, it is advisable that samples be analysed freshly. When storage is required, it is necessary to prevent oxidation or decomposition, and it has been suggested that plasma samples must be treated with antioxidants and metal chelating agents. Methods for measuring hydroperoxides can be classified into two main groups. (a) techniques measuring total hydroperoxide and (b) techniques which measure individual classes of hydroperoxides using HPLC techniques.

1.7.8.1. Total hydroperoxides

Iodometric

One of the classic methods for hydroperoxide determination is the iodometric assay (Jessup et al. 1994.) The assay is based on the ability of iodide to reduce hydroperoxides to their corresponding alcohols while being converted to iodine itself. The liberated iodine subsequently reacts in the presence of excess iodide to form triiodide anion (I_3^-) in the following reaction:

$$ROOH + 2H^+ + 2I^- \longrightarrow ROH + H_2O + I_2$$

$$I^- + I_2 \longleftrightarrow I_3^-$$

Triiodide (I_3^-) can either be titrated with thiosulfate or monitored spectrophotometrically at 290 nm with molar extinction coefficient of [$E(H_2O_2)= 4.4 \times 10^4\ M^{-1}\ cm^{-1}$] or 360 nm [$E(H_2O_2)= 2.8 \times 10^4\ M^{-1}\ cm^{-1}$] (Hicks and Gebicki, 1979). Triiodide, can also be quantified with HPLC using cadmium acetate (Gebicki and Guille, 1989). Some modified versions of the basic assay are: (a) using high temperature (50 °C) to accelerate the reaction of less-reactive hydroperoxides (Darrow and Organisciak, 1994); (b) Automated Continuous-Flow Assay, which allows measurement of lipid and non-lipid hydroperoxides with the facility for assaying large number of samples (Thomas et al. 1989). The precise measurement of hydroperoxides in an iodometric assay is limited by interfering factors such as:

(a) Sensitivity to presence of molecular oxygen due to liberation of iodine by air oxidation of iodide.

(b) Transition metal ions and oxidising substances affect the assay by reaction with hydroperoxides or intermediate formed during the reaction.

(c) Coloured substances absorbing light in the region between 290 and 360 nm.

(d) Kinetics of the reaction of iodide are influenced by reactivity of different hydroperoxides which make standardisation difficult (Pryor and Castle, 1984; Jessup et al. 1994).

Moreover, iodometric determination of plasma hydroperoxides requires lipid extraction, which leads to a significant loss of hydroperoxides due to decomposition of hydroperoxide or incomplete recovery. Also correction for non-hydroperoxide oxidising chromophores has to be carried out by titration with sodium thiosulfate (Cramer et al. 1991). The use of a commercially available kit (El-Saadani et al. 1989) for cholesterol determination with molar extinction coefficient of [$(EH_2O_2)= 2.45\times10^4\ M^{-1}\ cm^{-1}$] improved some short-comings of original iodometric technique. The kit contains potassium iodide together with several other stabilising components which allow the assay to be carried out in an open cuvette.

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Cyclooxygenase

Another method for measurement of LOOH is the cyclooxygenase assay. The method is based on the ability of LOOH to activate the cyclooxygenase reaction catalysed by activation of prostaglandin endoperoxide synthase. Cyclooxygenase activity is measured using an oxygen electrode (Marshall et al. 1985; Kulmacz et al. 1990). The activation of cyclooxygenase by hydroperoxides is sensitive, but requires isolation of PGH synthase. Compounds such as ethanol and acetone, also, may activate cyclooxygenase. Since the method is based on the measurement of oxygen consumption for the determination of enzyme activity, it is affected by parameters such as pH, temperature and partial pressure which control oxygen solubility. In addition, the effect of esterified fatty acid hydroperoxides on activation of cyclooxygenase is not clear. According to Marshall et al (1985) fatty acid peroxides stimulate cyclooxygenase activity regardless of whether they are in free or esterified form. It has been reported that esterified fatty acid hydroperoxides are very poor activators of cyclooxygenase and any esterified fatty acid hydroperoxides must be treated with esterase before assay with the cyclooxygenase method (Kulmacz et al. 1990).

Haem degradation

This method is based on the oxidation of colourless compounds such as leucomethylene blue [LMB] (Ohishi et al. 1985; Auerbach et al. 1992) or tetramethylbenzidine [TMB] (Thomas and Poznansky, 1990) to coloured compounds such as methylene blue. The oxidation reaction, which is catalysed by haemoglobin, involves hydroperoxides as oxidants for the oxidation LMB or TMB to MB, a compound which absorbs strongly at 660 nm. The overall reaction has been illustrated below:

$$\text{ROOH} + \text{LMB} \xrightarrow{\text{Haemoglobin}} \text{MB (colour)} + \text{ROH}$$

The method is simple and commercially available. However, the application only suitable for pure lipid systems. The advantage of the LMB method is the stability of the MB produced to temperature and ambient light.

Dichlorofluoroscein (DCF)

This assay has been developed by Cathcart et al (1983). It is based on the ion-catalysed decomposition of hydroperoxides to reactive intermediates. The latter in turn participate in the oxidation of dichlorofluoroscin "marker substrate" to the fluorescent dichlorofluoroscein (DCF) which can be monitored fluorometrically. Although the assay shows a high specificity for hydroperoxides it is not suitable for the direct measurement of plasma hydroperoxides because: (a) it has to be carried out on total lipid extract and (b) vitamin E present in total lipid extract interferes with the assay.

Triphenylphosphine oxide (TPPO)

This assay is based on the oxidation of triphenylphosphine (TPP) to triphenylphosphine oxide (TPPO) in the presence of hydroperoxides. After reduction of hydroperoxides with TPP in cyclohexane the amount of TPPO produced is determined by HPLC in combination with UV detector at 220 or 260 nm (Nakamura and Maeda, 1991). The assay is sensitive and the total class of hydroperoxides can be measured. However, lipids from biological sources can contain substances with absorption at the UV region. Hence, the peaks from other compounds that might overlap with TPPO must be excluded. In addition like other methods, a lipid extraction procedure is necessary, which limits the usefulness of the assay.

Chemiluminescence (CL)

A method for measuring hydroperoxides directly without need for any extraction steps based on single photon counting of LOOH has been developed by Zamburlini et al (1995). In summary, at alkaline pH luminol reacts with hydroperoxides and emits light. This is accelerated by the addition of hemin to the reaction mixture. The exponential

decay of photon emission is integrated and plotted against the amount of internal standard that has been added. This method has the advantage of direct determination of hydroperoxide in plasma but water soluble antioxidants in plasma interfere with the chemiluminescent procedure and must be isolated before hydroperoxide measurement. The method is sophisticated and internal standard hydroperoxides are necessary for calibration.

Ferrous Oxidation with Xylenol Orange (FOX)

This method is based on the oxidation of the ferrous ions to ferric ions in the presence of hydroperoxides. The ferrous ion is relatively resistant to autoxidation in acidic conditions. However, in the presence of hydroperoxides it can be oxidised to yield ferric ion (Fe^{3+}) and oxyl ($RO^{\bullet}$) radicals (reaction A). The resultant ferric ions can be measured as indirect measurements of hydroperoxides using xylenol orange, which binds specifically to ferric ions and produces a colour (blue-purple) with an absorbance maximum at 560 nm (reaction B).

$$Fe^{2+} + ROOH \xrightarrow{\text{Acidic pH}} Fe^{3+} + RO^{\bullet} + OH^{-} \qquad (A)$$

$$Fe^{3+} + XO \longrightarrow \text{blue-purple complex (560 nm)} \qquad (B)$$

Two versions of the assay have been applied for measurement of hydroperoxides: FOX1 and FOX2.

FOX1

This version of the assay is suitable for determination of small amounts of hydrogen peroxide and other water soluble hydroperoxides. The oxidation of ferrous to ferric ion

is a consequence of one-electron reduction of ROOH, followed by homolytic cleavage of ROOH (reaction 1).

$$Fe^{2+} + ROOH \longrightarrow Fe^{3+} + RO^{\bullet} + OH^{-} \qquad (1)$$

Oxyl radicals ($RO^{\bullet}$) generated as a consequence of reduction of hydroperoxides by ferrous ions (reaction 1) are very reactive and capable of reacting further with another ferrous ion, solvent molecule (RH) and ROOH (reaction 2,3 and 4):

$$RO^{\bullet} + Fe^{2+} + H^{+} \longrightarrow ROH + Fe^{3+} \qquad (2)$$

$$RO^{\bullet} + RH \longrightarrow ROH + R^{\bullet} \qquad (3)$$

$$RO^{\bullet} + ROOH \longrightarrow ROH + ROO^{\bullet} \qquad (4)$$

The nature of the radical $R^{\bullet}$ and its subsequent reactions depend on the solvent which is used in the assay. In FOX1 the oxyl radical can react with sorbitol to give a carbon centred radical. In the presence of oxygen the carbon centred radical is oxidised to give a hydroperoxy radical, which can further oxidise ferrous ions (Wolff, 1994). Hence, the apparent extinction coefficient for H_2O_2 in the reaction mixture contains 100 µM xylenol orange, 250 µM ammonium ferrous sulphate, 100 mM sorbitol, and 25 mM H_2SO_4 is $2.2\times 10^5\ M^{-1}\ cm^{-1}$, i.e. more than one Fe^{+3} is generated per molecule of H_2O_2.

FOX2

FOX2 assay is suitable for measuring of a wide range of non-H_2O_2 and lipid soluble hydroperoxides. In the FOX2 version sorbitol has been replaced with methanol in order to solubilise lipids, and butylated hydroxytoluene (BHT) is used as a chain-breaking antioxidant. It has been proposed that reaction of oxyl radical ($RO^{\bullet}$) with methanol gives

a reducing hydroxymethyl radical (reaction 5)(Paul et al. 1978). The $^{\bullet}CH_2OH$ in turn may decrease the amount of already formed ferric ions (reaction 6)(Grodkowski et al. 1985):

$$RO^{\bullet} + CH_3OH \longrightarrow ROH + {}^{\bullet}CH_2OH \qquad (5)$$

$$^{\bullet}CH_2OH + Fe^{3+} \longrightarrow HCHO + Fe^{2+} + H^{+} \qquad (6)$$

Therefore, the final amount of the ferric ion in the reaction medium depends on the nature of the solvent, ROOH and pH, which subsequently determines the apparent extinction coefficient in the assay. In the FOX2 reaction medium which contains 90% methanol and 4 mM BHT apparent extinction coefficient of 4.5×10^4 M^{-1} cm^{-1} has been found for H_2O_2 or authentic lipid hydroperoxide. The actual extinction coefficient of the Fe^{3+}-xylenol orange complex at 560 nm, using freshly made acidic solutions of ferric chloride, was found to be 1.5×10^4 $M^{-1}cm^{-1}$, indicating that the composition of the reaction mixture in the assay is the source of its different extinction coefficients.

Detection of organic hydroperoxides using the FOX2 assay is superior to other spectrophotometric methods because: (a) kinetics for the oxidation of ferrous ion by hydroperoxides is independent of chemical structure and thus formation of steady -state levels of ferric ion-xylenol orange complex are complete within 30 minutes using a variety of hydroperoxides (see table 3.2.5); (b) no extraction step is normally needed ,which is one of the intrinsic limitation of other methods, for analysis of liposomes and lipoprotein suspensions because the use of the 90% methanol/25mM H_2SO_4 environment in which the assay is performed denatures proteins sufficiently to allow access of the ferrous ions to available hydroperoxides; and (c) there is no need for sophisticated instruments. The simplicity of the assay has the advantage of allowing measurement of large number of biological samples in a day.

1.7.8.2. Individual classes of hydroperoxides

Various methods have been used to determine the different classes of LOOH (Yamamoto et al. 1987; Frei et al. 1988; Yamamoto and Niki, 1989; Miazawa, 1989; Holley and Slater, 1991; Coghlan et al. 1991). In principle hydroperoxides react with microperoxidase and oxidise luminol/isoluminol via intermediate radicals to emit chemiluminescence under alkaline condition. Hydroperoxides are identified by comparison with authentic standards. Microperoxidase, a peptide formed by proteolytic degradation of cytochrome c, has been shown to be the most effective catalyst for this assay (Olsson, 1982). The mechanism of the whole reaction has been figured below:

$$LOOH \xrightarrow{\text{microperoxidase}} LO^{\bullet}$$

$$LO^{\bullet} + (QH^{-}) \longrightarrow LOH + Q^{\bullet -}$$

$$Q^{\bullet -} + O_2 \longrightarrow Q + O_2^{\bullet -}$$

$$Q^{\bullet -} + O_2^{\bullet -} \longrightarrow \text{photon emission (430 nm)}$$

Where LOOH = lipid hydroperoxide, $LO^{\bullet}$ = alkoxyl radical, QH^{-} = luminol/isoluminol, $Q^{\bullet -}$ = semiquinone radical, $O_2^{\bullet -}$ = superoxide radical and Q = quinone.

These methods are sensitive and different classes of hydroperoxides are detected at picomol levels. Nevertheless, these techniques tend to give positive results as a consequence of interfering factors (Frei et al. 1988). Zhang et al (1995) have reported the detection of PC-OOH in biological systems using HPLC-CL, while Yamamoto et al (1987) were unable to show presence of PC-OOH in plasma. Recovery of hydroperoxides was found to be affected by different organic solvents during the extraction procedure. Furthermore, these techniques are time consuming and are best used when determination of the specific class of hydroperoxides is needed.

1.8. Aim

There has been an increasing demand for techniques for quantitative determination of plasma levels of lipid hydroperoxide (the initial products of the reaction of free radicals with lipids in biological systems). This is a consequence of the greater understanding of the role of free radicals in the pathology of a variety of diseases. Hence, using simple and sensitive methods for determination of hydroperoxides will increase our understanding of the role of free radicals in the pathology of those diseases which are related to oxidative stress.

For this purpose a new simple and sensitive method (FOX2) for direct measurement of lipid hydroperoxides, without the need for any extraction procedure and using a specific hydroperoxide reductant "TPP", was developed for use in plasma samples.

Location and distribution of hydroperoxides in plasma was analysed after fractionating of plasma into its major lipoprotein classes. Furthermore, the method was applied for measurement of LOOH in non-insulin dependent diabetes mellitus (NIDDM) as an example of a disease which is associated with oxidative stress.

CHAPTER II MATERIALS & METHODS

2.1. Chemicals

Hydrogen peroxide (H_2O_2), tert-butyl hydroperoxide (TB-OOH), cumene hydroperoxide (CU-OOH), butylated hydroxytoluene (BHT), thiobarbituric acid (TBA), trichloro acetic acid (TCA), cholesterol, arachidonic acid (AA), linolenic acid (LN), linoleic acid (LA), oleic acid, potassium iodide, catalase, glutathione (reduced form, GSH), glutathione peroxidase (G/PX), phospholipase A_2 (PA_2), phosphatidylcholine, xylenol orange [o-cresolsulfonphthalein-3,3-bis (methyliminodiacetic acid) sodium salt], α-tocopherol, γ-tocopherol, ascorbic acid (AH), boron trifluoride-methanol, 2-mercaptoethanol (M.E), holo-transferrin, apo-transferrin, folins reagent, standard mixture of fatty acids, bovine serum albumin, were obtained from Sigma Chemical Co. Ammonium ferrous sulphate, diethylenetriaminepenta-acetic acid (DETPAC), triphenylphosphine (TPP), were purchased from Aldrich. 2,2-Azobis (2-amidinopropane) hydrochloride (AAPH) was obtained from Polysciences. Sodium chloride, potassium dihydrogen phosphate, sodium carbonate, potassium sodium tartarate, ethylenediaminetetra-acetic acid, copper sulphate pentahydrate, ferric chloride, methanol, ethanol, propan-1-ol, ethylacetate, hexane, acetonitrile, tetrahydrofuran, sulphuric acid were obtained from BDH. [5(S),6E,8Z,11Z,14Z]-5-Hydroperoxyeicosatetraenoic acid (5-HPETE), [9(S), 10E, 12Z]-9-Hydroperoxyoctadeca dien-1-oic acid (9-HPODE) and [9Z, 11E, 13(S)]-13-Hydroperoxyoctadecadien-1-oic acid (13-HPODE) were obtain from CASCADE BIOCHEM, Reading, Berkshire, UK.

2.2. Reagents

Ferrous Oxidation in Xylenol Orange (FOX) version 2

FOX2 reagent was prepared by dissolving xylenol orange (7.6 mg) and ammonium ferrous sulphate (9.8 mg) in 10 ml H_2SO_4 (250 mM) to a final concentration of 1 and 2.5 mM, respectively. One volume of this concentrated reagent was added to nine volumes of HPLC-grade methanol containing (80 mg) BHT to make the working reagent, which comprised 250 μM ammonium ferrous sulphate, 100 μM xylenol orange, 25 mM H_2SO_4, and 4 mM BHT in 90% (v/v) methanol. This working reagent was routinely calibrated against solution of H_2O_2 or authentic lipid hydroperoxides of known concentration.

Protein assay solutions (Lowry)

Solution A consisted of 10 volumes of 2% Na_2CO_3 in 0.4% NaOH, 0.1 volumes of 1% $CuSO_4.H_2O$, and 0.1 volumes of 2% Potassium Sodium tartarate respectively. Solution B consisted of a 1:1 mixture of Folin's reagent and distilled water. The working solution was calibrated against bovine serum albumin (BSA).

Iodometric reagents

Classic version

Solution A consisted of a 1:1 mixture of HPLC-grade methanol and glacial acetic acid containing 1 mg/ml EDTA (disodium salt). Solution B consisted of HPLC-grade methanol containing of 20% (w/v) potassium iodide. Both solutions were degassed under a stream of nitrogen for 20 minutes before use.

Modified version

The reagent is available as a kit (supplied by Merck CO) consisting of potassium phosphate (pH 6.2) 0.2 M, potassium iodide 0.12 M, sodium azide 0.15 mM, polyethylenglycol mono[p-(1,1 ,3,3-tetramethyl-butyl)-phenyl]ether 2g/l, alkylbenzyldimethylammonium chloride 0.1g/l, and ammonium molybdate 10μM.

Thiobarbituric acid (TBA) reagents

Reagent A consisted of 0.67% thiobarbituric acid (TBA) and reagent B consisted of 20% trichloroacetic acid (TCA) in distilled water.

2.3. Methods

2.3.1. Plasma preparation

Plasma samples were obtained from patients with well-characterised non insulin-dependent diabetes mellitus (NIDDM) after permission had been granted by the local ethics committee and consent of the patients attending at the University College London Hospitals Diabetic department. Patients were subdivided depending on whether or not they had complications such as retinopathy, neuropathy and or nephropathy and or macroangiopathy. Smokers were not excluded from the study. Non-smokers were defined as those individuals who claimed they had not smoked during the three-year period prior to plasma sampling for the study. Patients were treated by diet alone, by diet plus oral hypoglycaemics (metformin and tolbutamide) or by diet, oral hypoglycaemics and injected insulin. Control subjects were recruited from staff at the Department of Medicine, University College London Medical School. None of the control subjects was on any kind of special diet or medication related to diabetes. 10 ml blood was taken from each participant by venepuncture and put into sampling vials containing heparin. Platelet-depleted plasma was prepared by centrifugation at 2500xg for 10 minutes at room temperature.

2.3.2. Isolation of major classes of plasma lipoproteins

Lipoproteins were isolated sequentially from platelet-depleted plasma. In brief, platelet-depleted plasma was prepared by centrifugation at 2500xg for 10 minutes at room temperature in the presence of 1mg/ml (EDTA) to prevent oxidation. After centrifugation for 18 hours at 100 000xg, the supernatant (density < 1.006), containing very low density lipoprotein (VLDL) and chylomicrons, was removed. The density of infranatant was adjusted to 1.063 g/ml by addition of solid potassium bromide and was recentrifuged as before. The LDL, which floats at a relative density of 1.006-1.063, was collected. The remaining infranatant (1.063-1.210), which contained high density lipoprotein (HDL) with other lipoproteins, was dialysed against phosphate-buffered

saline (PBS, pH 7.4) overnight. The hydroperoxide content in the various lipoprotein fractions was determined as a function of protein content by the Lowry assay.

2.3.3. Preparation of liposomes

Two ml of phosphatidylcholine (100 mg/ml in chloroform) was dried in a glass tube under nitrogen and then hydrated in 10 ml of phosphate-buffered saline (10 mM H_2NaPO_4 in 150 mM NaCl, pH 7.4; PBS) and kept in the dark at 4°C for 1 hour. The resulting dispersion was ultrasonicated for three minutes while cooling on ice. The small liposomes were then diluted with PBS at a final concentration of a 10 mg/ml.

2.3.4. Preparation of oxidised samples

Oxidation of liposomes (10 mg/ml in PBS) was carried out by incubation in the presence of copper sulphate and [2'2'Azobis (2-Amidinopropane hydrochloride)] (AAPH) (final concentration, 10 μM and 1000 μM, respectively) at 37 °C. Cholesterol (1mg/ml in ethanol) was oxidised as above. Lipoprotein fractions were diluted with PBS to a final concentration of 200 μg/ml and then incubated with copper sulphate and AAPH as described for liposomes. Samples (100 μl) were removed at defined time intervals and assayed for different analyses.

Plasma samples were incubated in the presence and absence of 10 mM AA-PH at 37 °C for 6 hours. Samples were removed at defined time intervals and assayed for hydroperoxides and TBARMs respectively.

2.3.5. Extraction of total lipids

Plasma samples (200 μl) were transferred to a glass test tube. Water (300 μl) and methanol (250 μl) were added. The mixture was vortexed for approximately 20 seconds. Ethyl acetate (500 μl) was then added and the mixture was vortexed again. The suspension was centrifuged at 3000g for 5 minutes. The organic (upper) layer was then transferred to a 1.5 ml microcentrifuge vial. Ethyl acetate (500 μl) was added to the residual aqueous phase. This was vortexed and centrifuged as described above. The

organic layers were pooled and concentrated under a stream of nitrogen by evaporation to a final volume of approximately 100 µl.

2.4. Biochemical analyses

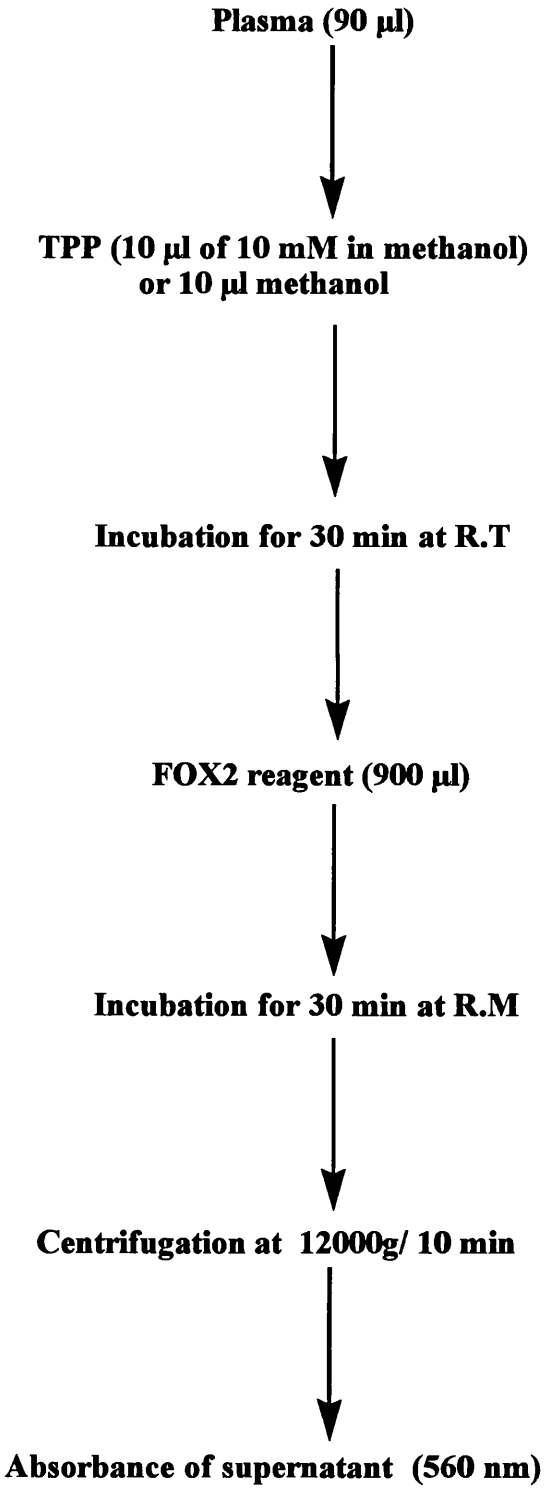
2.4.1. Measurement of hydroperoxides

Ferrous Oxidation with Xylenol Orange (FOX2 assay)

Samples (90 µl) were transferred into 6 (1.5 ml) microcentrifuge vials. TPP (10 µl of 10 mM) in methanol was added to 3 of the vials to remove hydroperoxides. Methanol (10 µl) was added to the remaining 3 vials. This generated blank and test samples respectively. All vials were then vortexed and incubated at room temperature for 30 minutes prior to the addition of 900 µl FOX2 reagent. After incubation at room temperature for a further 30 minutes the vials were centrifuged at 12000xg for 10 minutes. Absorbance of the supernatant was measured at 560 nm.

The hydroperoxide content in the samples was determined as a function of the difference between the means of the absorbance of samples, with and without elimination of ROOH by TPP, using the molar extinction coefficient of 4.5×10^4 M^{-1} cm^{-1} at 560 nm. The standard deviation was taken as the larger of the standard deviations of the measurements obtained with or without TPP treatment. The coefficient of variation for individual samples using the method was typically less than 5%. The schematically modified version of the assay for measurement of hydroperoxides in plasma has been depicted below.

Schematic procedure for measurement of plasma hydroperoxides using FOX2:



Iodometric assay

Classic version

The hydroperoxide content of lipids extracted from the samples and synthetic hydroperoxides was also determined by the classic iodometric method. Solution A consisted of a mixture of HPLC-grade methanol and glacial acetic acid (1:1, v/v) containing 1 mg/ml of EDTA (disodium salt) degassed with a stream of nitrogen for 20 minutes. Solution B consisted of HPLC-grade methanol degassed with N_2 for 20 minutes followed by addition of 20% w/v potassium iodide, and degassed for a further 20 minutes.

1 ml of solution A was added to 1.5 ml of solution B in a 3 ml cuvette adapted to permit degassing with nitrogen as well as the addition of small volumes of fluid under anoxic conditions. The contents of the cuvette were treated with nitrogen for 5 min and the cuvette was sealed. Absorbance was monitored at 290 nm for 4 min. The solution was discarded if absorbance increased by more than 0.005 AU. A small volume (1-10 µl) of an N_2 treated standard hydroperoxide solution or other extracted lipids was then injected. The cuvette contents were mixed by gentle agitation. Absorbance at 290 nm was monitored for 5 minutes, after which time no further absorbance change was observed. A standard curve generated with H_2O_2 gave an extinction coefficient for tri-iodide at 290 nm of 3.9×10^4 M^{-1} cm^{-1}.

Modified version

Authentic hydroperoxides (20 µl) were mixed with 2 ml of CHOD-iodide reagent in a 3 ml cuvette at room temperature. The absorbance at 290 nm was continuously monitored for 15 min and calibrated against standardised hydrogen peroxide solutions.

2.4.2. Measurement of conjugated dienes

Conjugated diene, absorbs maximally at 234 nm wavelength and was detected by UV spectrophotometry. Its concentration was calculated using an extinction coefficient of

2.8×10^4 M^{-1} cm^{-1} (Pryor & Castle 1984). 100 µl of lipoprotein (200 µg/ml protein), liposome (10 mg/ml phosphathidylcholine in PBS) or lipid extract were added to 900 µl pure methanol in an eppendorff tube and centrifuged at 12000xg for 3 minutes. The supernatant was monitored at 234 nm spectrophotometrically.

2.4.3. Measurement of thiobarbituric acid reactive materials (TBARMs)

Samples (100 µl) were mixed with 20% TCA (500 µl) and 0.67% TBA (1000 µl) followed by incubation at 100 °C for 20 minutes. Absorbance of supernatant was monitored at 532 nm after centrifugation at 12,000g for 5 minutes. The concentration of lipid peroxidation products was calculated as TBARMs using the extinction coefficient for the malondialdehyde-thiobarbituric acid complex of 1.56×10^5 M^{-1} cm^{-1} at 532 nm.

2.4.4. Analysis of vitamin E (α–tocopherol)

Samples (100 µl) were mixed with 500 µl ethanol (containing 200 ng/ml of γ-tocopherol as internal standard), 500 µl hexane and 400 µl water. After centrifugation at 3000xg for 5 minutes, the hexane (upper) layer was transferred to a glass-vial. Hexane 500 µl was added to the residual aqueous phase. This was vortexed and centrifuged as described above. The hexane layers were pooled. The solvent was dried under a stream of nitrogen. The residue was then redissolved in 100 µl acetonitrile. HPLC separation was performed on a hypersil-ODS column (10 cm X 5 mm, particle size 5 µM; Chrompack, the Netherlands) using acetonitrile: tetrahydrofuran: water (in ratios 80: 14: 6 by volume) at a flow rate of 0.7 ml/min. Tocopherols were monitored fluorometrically (Emission: 295 nm; Excitation: 340 nm).

2.4.5. Fatty acid methyl ester analysis (FAME)

Samples (100 µl) were mixed with 250 µl ethanol (containing 200 µg/ml heptadecanoic acid) as an internal standard. Ethyl acetate (500µl) and water (300 µl) were then added and vigorously mixed with the other ingredients. The samples were then centrifuged at 3000xg for 5 minutes. The organic (upper) layer was then transferred to a glass vial.

Ethyl acetate (500 µl) was added to the remaining aqueous layer with vortexing followed by centrifugation at 3000xg. The organic layers from the first and second extractions were pooled and dried under a nitrogen stream. Boron trifluoride-methanol (14%) solution (500 µl) was then added and the vials were heated at 60 °C for 30 minutes. Water (200 µl) and of hexane (500 µl) were added and centrifuged at 3000xg for 5 minutes. The organic layers were transferred to screw cap glass-vials. Hexane (500 µl) was added to the remaining aqueous residue with vortexing followed by centrifugation at 3000xg. The organic layer was pooled and dried under a stream of nitrogen. The residue was redissolved in hexane (100 µl) of which 1 µl was injected onto a Stabilwax column (30 m X 0.53 mm, film thickness 1.0 µM) using a temperature gradient of 120 °C to 220 °C at 4 °C/min. The signal was detected by a flame ionization detector (FID).

2.5. Protein determination

The protein content of plasma lipoproteins was estimated according to the method by Lowry et al (1951). The method involves treatment of proteins with copper reagent which reacts with phenolic amino acids to generate a colour which can be measured spectrophotometrically at 650 nm. The working solutions consisted of the following:
Solution A: 10 volumes of 2% Na_2CO_3 in 0.4% NaOH, 0.1 volume of 1% $CuSO_4.H_2O$, and 0.1 volume of 2% Potassium Sodium tartarate respectively.
Solution B: 1:1 mixture of Folin's reagent and distilled water. The working solution was calibrated against bovine serum albumin (BSA).
Purified lipoproteins were diluted with 10 fold PBS. 1 ml of solution A was added to different volumes (0.01, 0.02, 0.03 and 0.04 ml) of diluted lipoproteins in 1.5 ml microcentrifuge vials . After vortexing, samples were kept for 10 minutes at room temperature. 0.1 ml of solution B was then added and immediately mixed. Subsequently all samples were adjusted to a final volume of 1.5 ml with PBS. Samples were vortexed and incubated for a further 30 minutes at room temperature. Absorbance of the blue colour formed was measured at 650 nm. The amount of protein per sample in triplicate analysis was determined from a standard curve using bovine serum albumin (BSA), in the range of 0-40 µg/assay.

2.6. Clinical laboratory measurements

Glycaemic control was measured by fasting plasma glucose by a glucose oxidase method. Haemoglobin A1 was measured by agar gel electrophoresis. Total plasma cholesterol was measured using the Cholesterol-C high performance CHOD-PAP method (Boehringer Mannheim GmbH Diagnostica). Total triglyceride was measured using the GPO-PAP high-performance enzymatic colorimetric test (Boehringer Mannheim GmbH Diagnostica). High-density lipoprotein cholesterol was measured following selective precipitation of lipoproteins containing apoprotein-B with heparin manganous sulphate. Low density lipoprotein cholesterol was calculated according to the Friedewald formula.

2.7. Statistical analysis

Correlation coefficients and statistical analyses were obtained using scientific software by Sigmastat Ltd, Jandel, (Germany).

CHAPTER III RESULTS

Part 1

3.1. Effect of interfering factors on the FOX2 assay

The principle of the FOX assay is based on the oxidation of ferrous to ferric ions in the presence of hydroperoxides. The resultant ferric ions can be measured spectrophotometrically using the ferric sensitive dye, xylenol orange. During measurement of hydroperoxide in biological samples, it is possible that iron ions in the FOX reagent interact with interfering factors. In order to implement the assay, for measurement of hydroperoxides in complex systems such as plasma the effect of interfering factors on the FOX2 reagent and on plasma hydroperoxide was investigated.

3.1.1. Presence of ferric ions

The distribution of iron in normal adults is shown in Table 3.1.1. Circulatory iron in plasma is generally bound to the protein transferrin, which functions as a carrier molecule. Transferrin is a glycoprotein and each molecule has two separate binding sites to which Fe^{3+} attaches extremely tightly at physiological pH. Under normal conditions an average of only 30% of transferrin is loaded with iron, which indicates that there is essentially no free iron in plasma at physiological conditions. Iron from transferrin enters the cytoplasmic vacuoles in various cells via receptor-mediated endocytosis for the synthesis of iron-containing enzymes and proteins. In the acidic condition of intracellular vacuoles Fe^{3+} is released from transferrin and is consumed for various purposes.

Table 3.1.1: Iron distribution in normal adults*

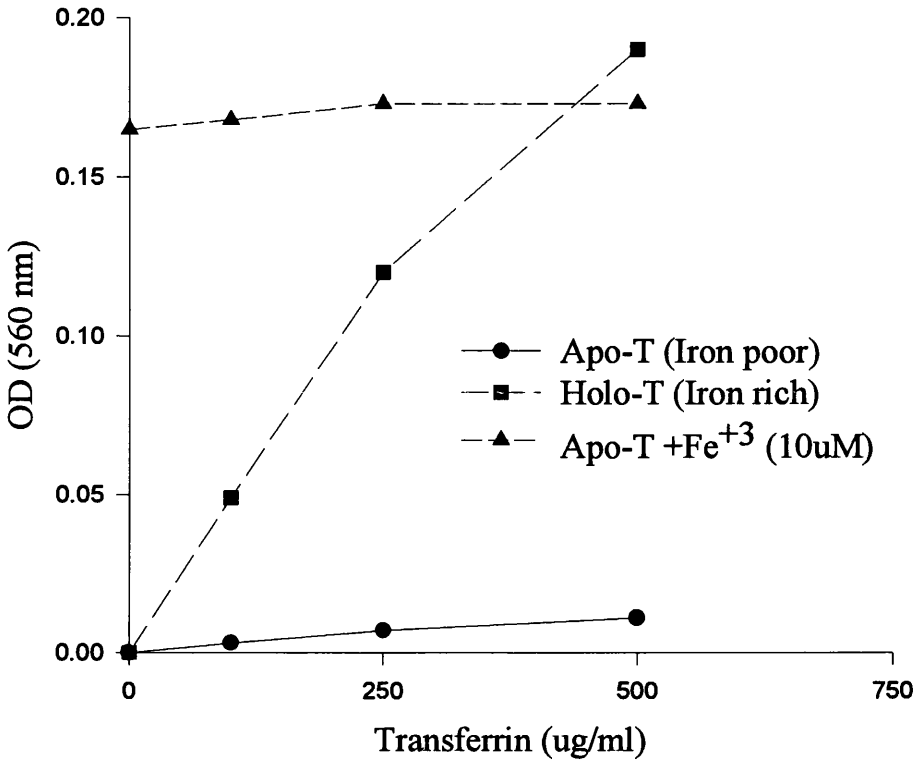
	Iron content (mg)	
	Men	Women
Haemoglobin	3050	1700
Myoglobin	430	300
Enzymes	10	8
Transport (transferrin)	8	6
Storage (ferritin & haemosiderin)	750	300
Total	4248	2314

* Adapted from Basic and Clinical Pharmacology, edited by Katzung, 1995.

In order to examine possible interactions of transferrin containing ferric ions with the FOX2 assay different concentrations of Apo-transferrin (iron poor) and Holo-transferrin (iron-rich) were added to the FOX2 reagent. At the same time fixed amounts of ferric ions were added to increasing concentrations of Apo-transferrin. As indicated in figure 3.1.1.1. the signal rose consistently with increased concentration of Holo-T. The signal from added Fe^{3+} remained unchanged in the presence of Apo-T. Thus, ferric ions from transferrin interfere with FOX2 reagent and give a high signal during measurement of hydroperoxides in samples containing ferric ions.

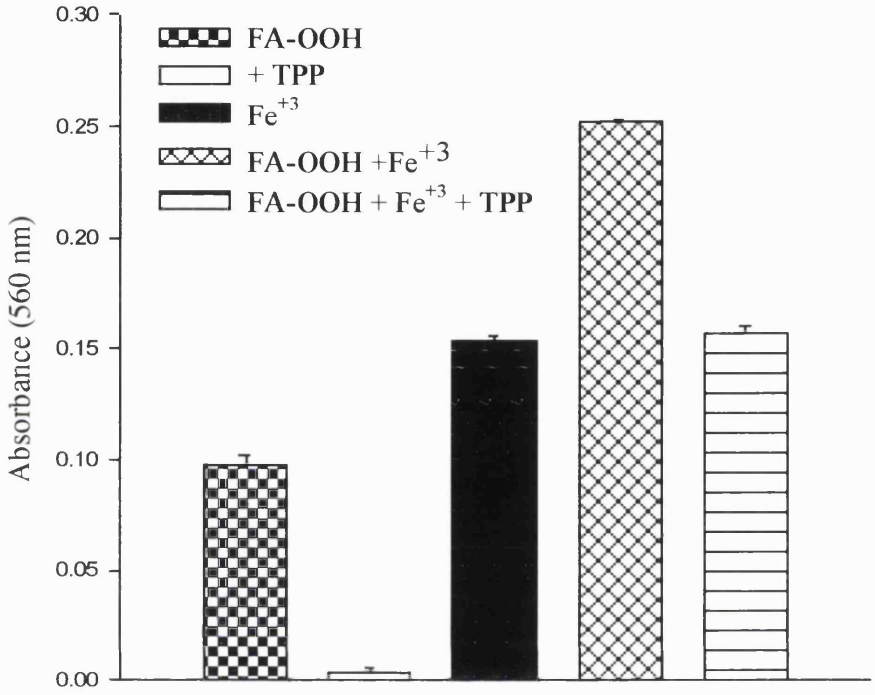
In a further experiment, samples of authentic hydroperoxide (20 μM) and ferric ions (100 μM) alone or in combination were pre-treated with 10 μl of 1mM TPP, a selective hydroperoxide reductant, or PBS. After incubation for 30 minutes at room temperature, FOX2 reagent (900 μl) was added and the samples were incubated for another 30 minutes at room temperature. Figure 3.1.1.2. shows TPP selectively reduced hydroperoxides, while it had no effect on ferric ions. It was also found that in samples containing a mixture of hydroperoxide and ferric ions, TPP selectively destroyed hydroperoxide related signal, while absorbance from ferric ions remained unchanged. Hence, this manoeuvre with TPP is an effective step for measurement of hydroperoxides in biological samples which contain ferric ions.

Figure 3.1.1.1: Interaction of iron transfer proteins and ferric ions with FOX2 reagent



Different concentrations of Apo-T and Holo-T (equivalent approximately to 1.5-10 μM ferric ions in one ml reagent) were added to FOX2 reagent. At the same time Apo-T was incubated with 10 μM ferric ions (final concentration in one ml reagent). After incubation for 30 minutes at room temperature following centrifugation at 12000g for 5 minutes absorbance of samples were read at 560nm. Data represent means ± SD of triplicate samples. Error bars lie within the mean data points.
* Apo-T and Holo-T were obtained from Sigma.

Figure 3.1.1.2: Reduction of hydroperoxides with TPP



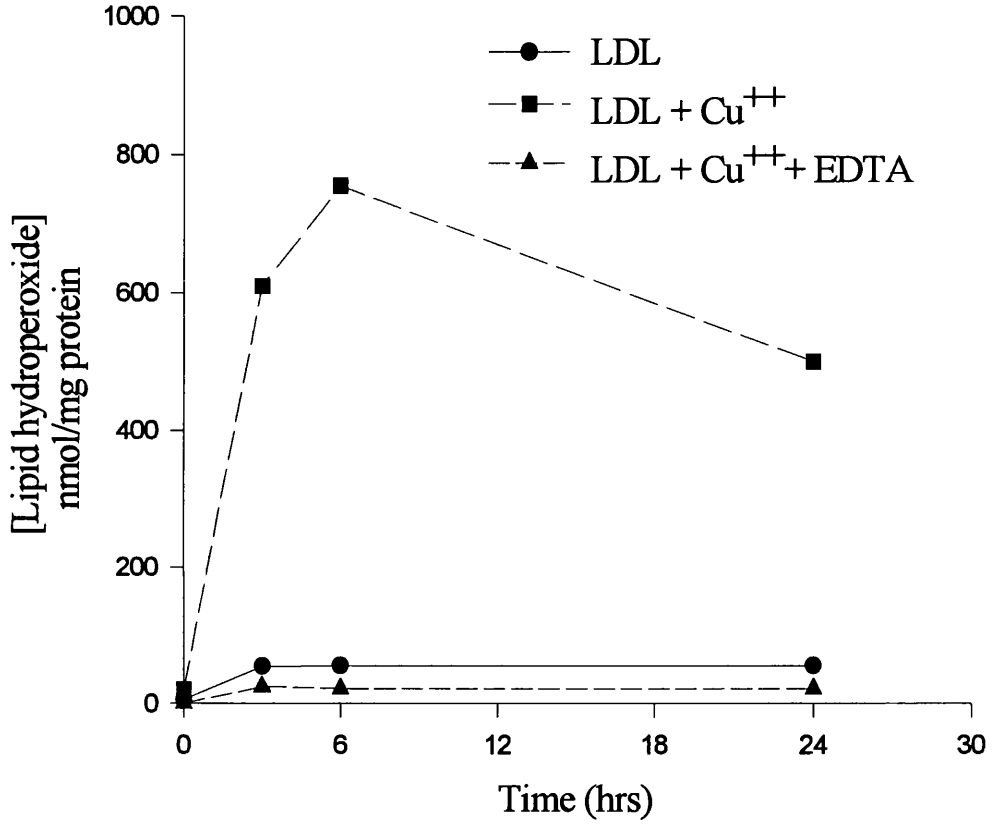
5-hydroperoxyeicosatetraenoic acid (5-HPETE) [obtained from CASCADE] was diluted in PBS. Samples of (90 µl) of FA-OOH and ferric ions (at final concentrations of 20 µM and 100 µM respectively) were incubated, alone or in combination, with 10 µl of 10 mM TPP at final concentration of 1mM or PBS for 30 minutes at room temperature prior to the addition of FOX2 reagent. Absorbance of samples was measured at 560 nm after further incubation for 30 minutes at room temperature. Data represent means ± SD of triplicate analyses.

3.1.2. Effect of chelating agents

EDTA and DETAPAC are often used as metal chelating agents during the collection and preparation of samples for hydroperoxide measurement. The main effect of these chelating agents is the prevention of transition metals ions for oxidation processes. However, since FOX2 reagent contains Fe^{2+} ions, plasma samples must be collected in heparinised or citrate buffered tube. As a result, plasma lipoprotein fractions must be dialysed against PBS after preparation with EDTA. If samples are deliberately oxidised using metal ions such as copper, any remaining EDTA in the samples will chelate copper as well as the Fe^{2+} in the FOX2 reagent.

To assess the chelating effect of EDTA on metal ions and consequently on hydroperoxide levels, LDL (200μg/ml in PBS) was incubated with or without copper ions (10 μM) in the presence or absence of 1 mM EDTA at 37 °C. Samples were analysed at different intervals for hydroperoxide content. Figure 3.1.2. shows that EDTA completely prevented the oxidation of LDL in the presence of copper, presumably because of chelation of copper ions.

Figure 3.1.2: Effect of chelation by EDTA during time course oxidation of LDL in the presence of copper



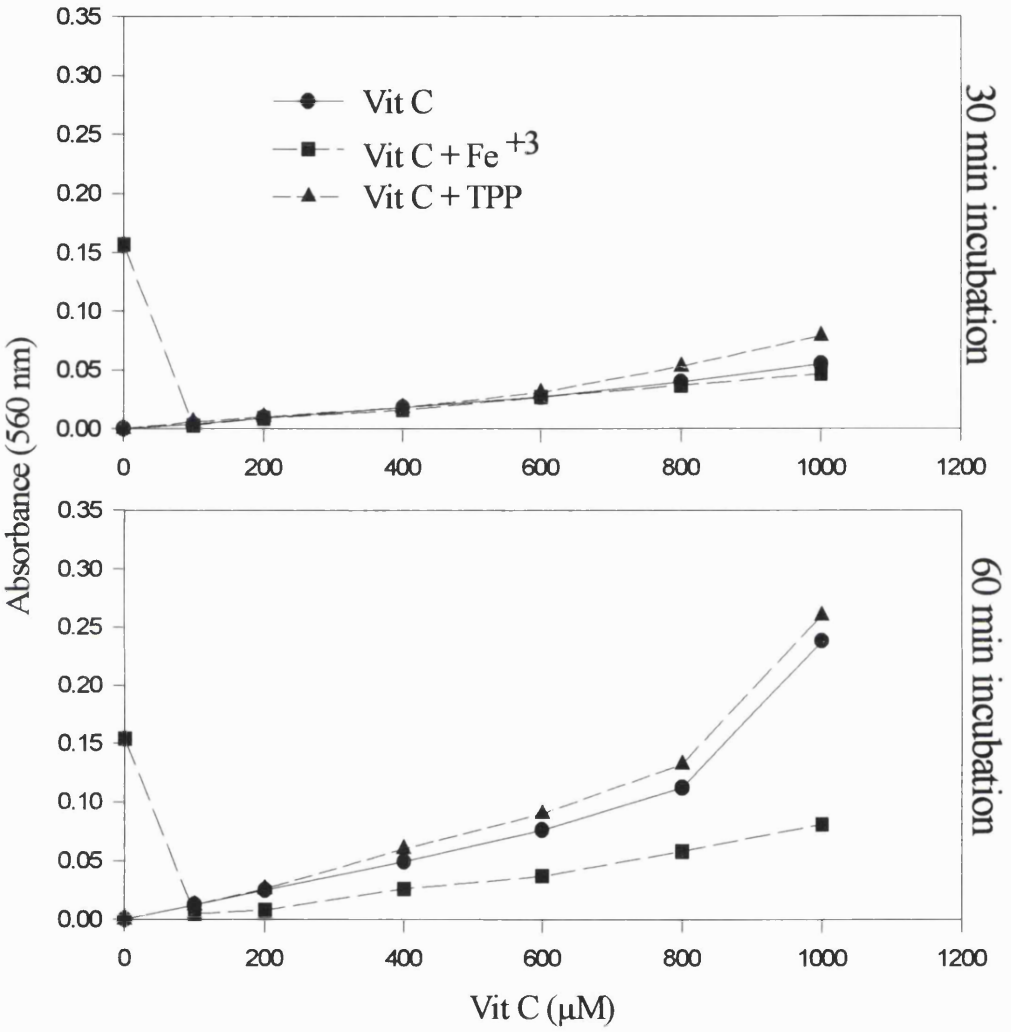
LDL from the plasma of healthy subjects was diluted in PBS at a concentration of 200 μg/ml. Samples (90 μl) were incubated with or without copper ions (10 μM) in the presence or absence of EDTA (1 mM) at 37 °C. Hydroperoxide content was determined after dialysing at different intervals using an molar absorption coefficient of 4.5×10^4 M^{-1}. cm^{-1} or by reference to a H_2O_2 standard curve. Data represent means ± SD of triplicate analyses. Error bars lie within the mean data points.

3.1.3. Ascorbate interaction

Ascorbic acid (vitamin C) is a water soluble vitamin, which is present at the range of 2-20 μg/ml (12-120 μM) in plasma. The most important chemical property of ascorbate is its antioxidant effect as a reducing agent. However, in the presence of oxygen and transition metals (e.g. iron and copper ions) it is oxidised and produces H_2O_2 and hydroxyl radicals. To test any interaction of ascorbate with FOX2 assay, increasing concentrations of ascorbate in the presence and absence of Fe^{3+} were analysed in the FOX2 reagent. Different concentrations of ascorbate (50-1000 μM) in PBS were incubated with FOX2 reagent with or without Fe^{3+} (100 μM) for 30 and 60 min at room temperature. Samples were pre-treated with TPP (1mM) for 30 min before incubation with FOX2 reagent. Figure 3.1.3.1. shows that the addition of ascorbate at range of 50-100 μM to the FOX2 reagent had no effect on signal, while increasing the concentration to more than 200 μM caused a rise in signal. Figure 3.1.3.1. also shows the reduction of exogenously added Fe^{3+} to Fe^{2+} by ascorbate in the FOX2 assay. It also shows production of H_2O_2 at increased concentrations of ascorbate as a result of oxidation in the presence of Fe^{2+} ions, which did not stop after 30 min. There was no difference between the samples which had been pre-treated with TPP and those which had not, suggesting that the generated signal was due to H_2O_2 formation in the reaction mixture (see 3.2.1.1.).

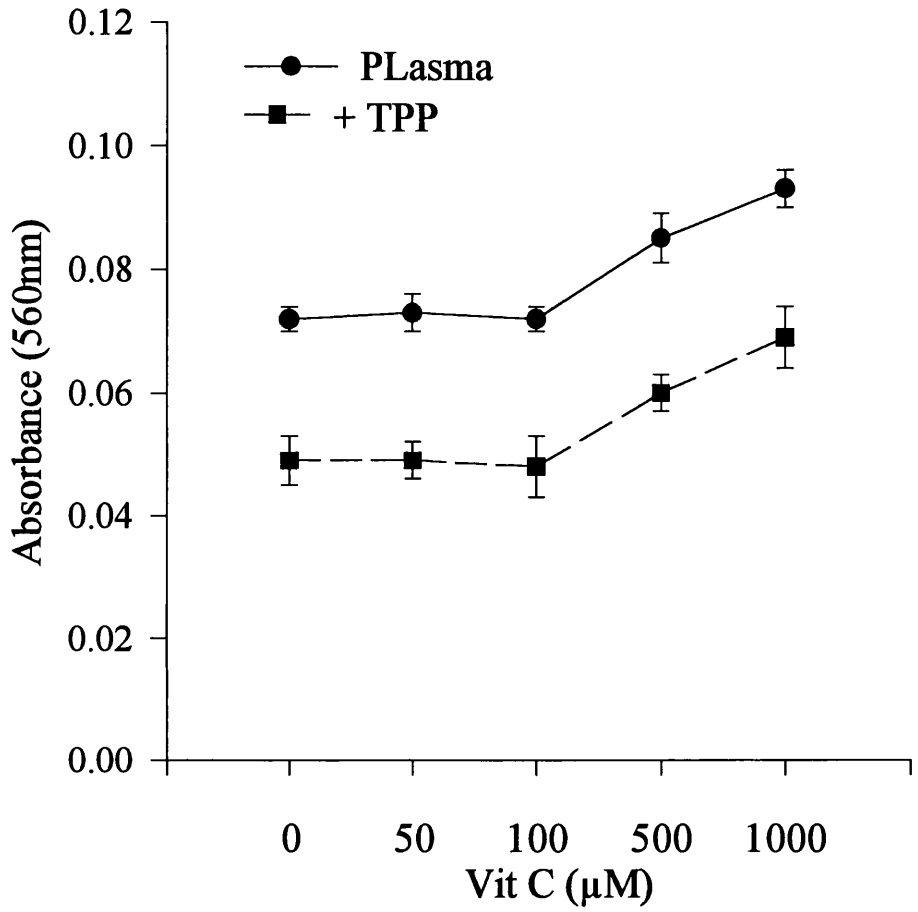
The effect of different concentrations of ascorbate on plasma hydroperoxide levels was also analysed. Ascorbate in the concentration range of 10-100 μM did not affect recovery of hydroperoxides. However, the addition of a high concentration (500-1000 μM) caused oxidation of ascorbate in the presence of ferrous ions and production of H_2O_2. Pre-treatment with TPP only reduced the signal to control level (Fig 3.1.3.2.) as it has no effect on H_2O_2 (see 3.2.1.1.).

Figure 3.1.3.1: Interaction of iron ions with ascorbate in the FOX2 assay



Different concentrations of ascorbate (50-1000 μM) in PBS were incubated with FOX2 reagent with or without Fe^{+3} (100 μM) for 30 and 60 min at room temperature. Samples were pre-treated with TPP (1mM) for 30 min before incubation with reagent. Absorbance was measured at 560 nm. Data represent means ± SD of triplicate analyses. Error bars lie within the mean data points.

Figure 3.1.3.2: Effect of increasing concentration of ascorbate on plasma hydroperoxide

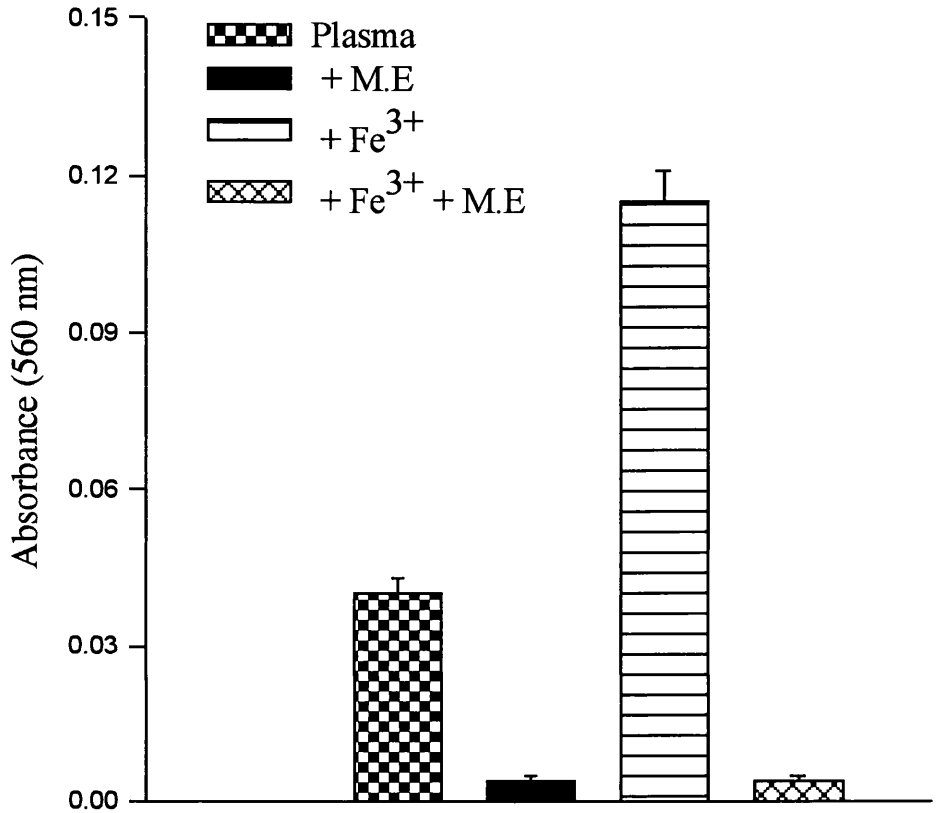


Plasma samples were incubated with different concentrations of ascorbate with or without TPP (1mM) for 30 minutes at room temperature. After addition of FOX2 reagent samples were further incubated for 30 minutes at room temperature. Absorbance was measured following centrifugation of samples at 12000g for 10 minutes. Data represent means ± SD of triplicate samples.

3.1.4. Effect of organic reducing agents

Presence of iron from haemoglobin in plasma is one of the factors that may cause high background absorbance as a consequence of haemolysis during sample preparation. Furthermore, release of Fe^{3+} ions from transferrin takes place in acidic conditions. In order to examine the colour signal observed with plasma in the xylenol orange system, plasma samples were treated with 2-mercaptoethanol (2-ME) which reduces Fe^{3+} to Fe^{2+} ions. Figure 3.1.4. shows that pre-treatment of plasma with 2-ME destroyed the signal. Addition of ferric ions to the plasma also led to an increase in signal which was quenched by preincubation of the samples with 2-ME. Thus a high proportion of the signal generated by direct addition of plasma to the FOX2 reagent was attributable to ferric ion in the plasma. ME can also be used as a diagnostic tool to confirm presence of Fe^{3+} in FOX reagent, that may occur as a contamination or as a result of oxidation during long storage.

Figure 3.1.4: Effect of 2-ME on ferric ions in plasma



Plasma samples , in the presence and absence of added ferric chloride (final concentration in plasma, 100 μM), were mixed with 10 μl 1 M mercaptoethanol and heated for 30 seconds at 70 °C. Samples were mixed with 900 μl FOX2 reagent and incubated for 30 minutes at room temperature. Absorbance of supernatant was measured at 560 nm following centrifugation at 12000g for 10 minutes. Data represent means ± SD of triplicate analyses.

3.1.5. Effect of uric acid

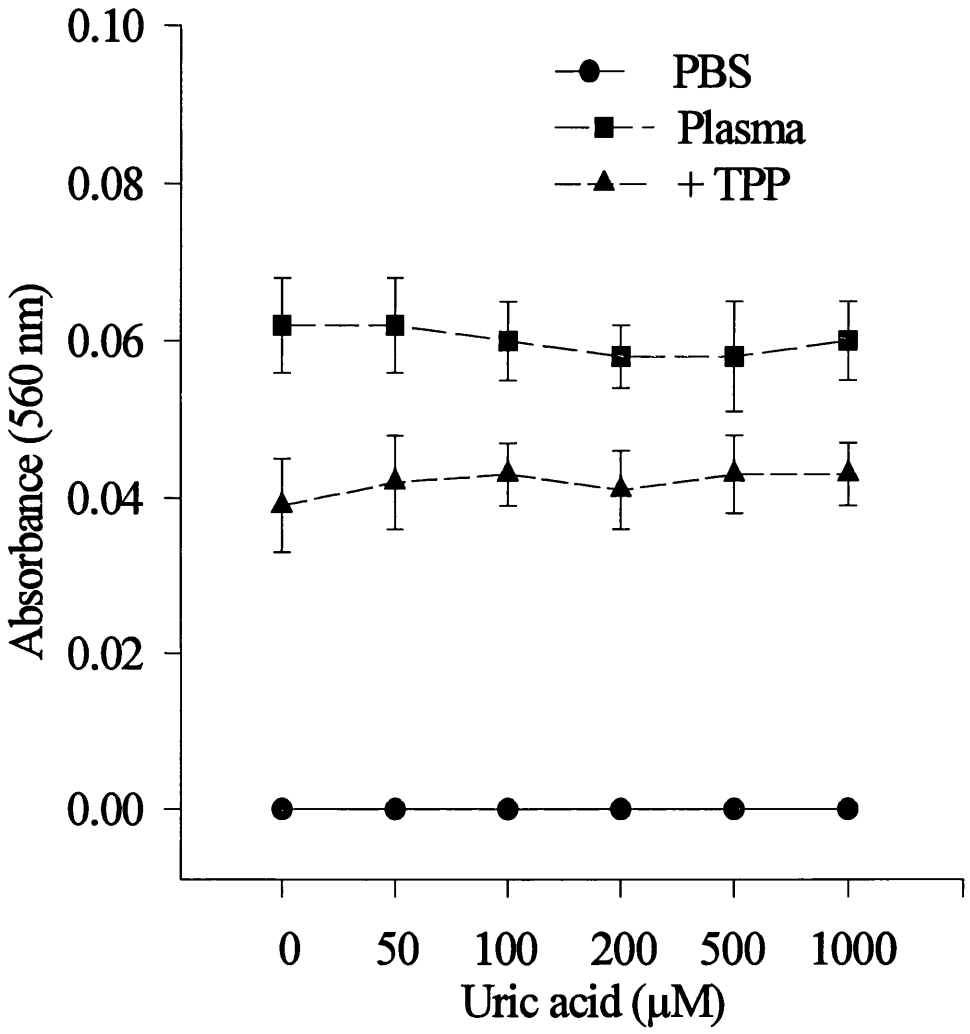
Uric aid is a poorly soluble substance, a major end product of purine metabolism. In most mammals, uricase converts uric acid to the more soluble allantoin; this enzyme is absent in human. The normal concentration of uric acid in human plasma is 250-450 μM. It has been shown that uric acid is a powerful scavenger of singlet oxygen (O^1_2), peroxyl radicals ($ROO^{\bullet}$), and hydroxyl radicals ($OH^{\bullet}$), and it has been suggested that uric acid functions as an antioxidant in vivo (Ames et al. 1981).

To determine any interaction or effect of uric acid with FOX2 reagent or hydroperoxide in plasma, PBS solution and plasma samples were incubated with different concentrations of uric acid (50,100,200,500 and 1000 μM). There was no change in absorbance of FOX2 or apparent hydroperoxide level in plasma , indicating that there was no in vitro effect of uric acid on hydroperoxides as shown in figure 3.1.5.

3.1.6. Effect of vitamin E

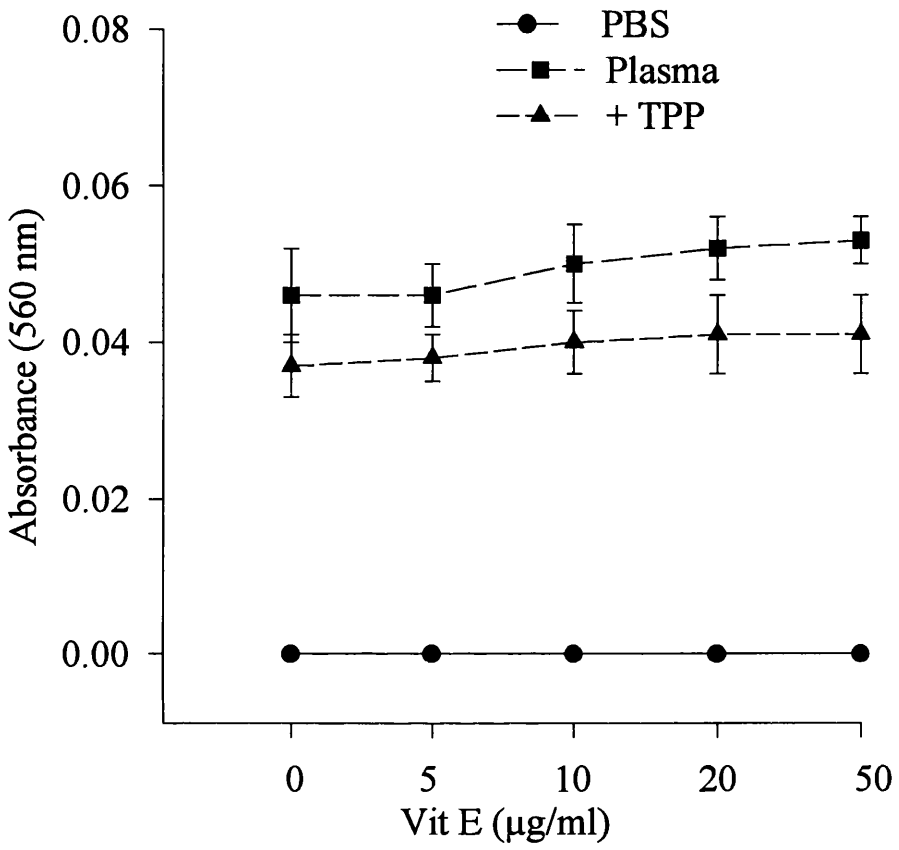
Vitamin E is the major peroxyl radical-trapping, chain-breaking antioxidant in human blood, and it thereby reduces the length of the oxidation chains. In order to examine the possible interaction of vitamin E with FOX2 reagent and also to analyse any direct effect on hydroperoxide levels in plasma, different concentrations of vitamin E (0-50 μg/ml., 0-115 μM) were incubated with plasma and PBS. As indicated in figure 3.1.6., there was no interaction between FOX2 reagent and vitamin E. Figure 3.1.6. also shows that vitamin E had no direct reducing effect on previously formed LOOH.

Figure 3.1.5: Lack of interference between uric acid and plasma hydroperoxides



Plasma samples and PBS (5 mM phosphate buffer in 150 mM sodium chloride) solutions were incubated with different concentrations of uric acid in the presence or absence of 1mM TPP for 30 minute at room temperature. Samples were mixed with FOX2 reagent and incubated for a further 30 minutes at room temperature. Absorbance of supernatant was measured at 560 nm following centrifugation of samples at 12000g for 10 minutes. Data represent means ± SD of triplicate analyses.

Figure 3.1.6: Effect of vitamin E on plasma hydroperoxide



Plasma samples and PBS solutions were incubated with different concentrations of Vit E (0-50 µg/ml., 0-115 µM) in the presence or absence of 1mM TPP for 30 minutes at room temperature. Samples were mixed with FOX2 reagent and incubated for a further 30 minutes at room temperature. Absorbance of supernatant was measured at 560 nm following centrifugation of samples at 12000g for 10 minutes. Data represent means ± SD of triplicate analyses.

Part 2

3.2. Application of the FOX2 assay for measurement of plasma hydroperoxides

The FOX2 assay measures total hydroperoxides in plasma. In order to implement the FOX2 assay for direct measurement of plasma hydroperoxides the effect of different hydroperoxide reducing agents was studied. Among these agents, it was found that triphenylphosphine (TPP) was effective in destroying all classes of hydroperoxides except H_2O_2 without interfering with FOX2. After adapting the assay to measure of hydroperoxides in plasma with the TPP strategy, hydroperoxide levels in 23 healthy subjects were analysed. Hydroperoxide levels were also compared before and after extraction of lipids.

3.2.1. Reduction of hydroperoxides with triphenylphosphine (TPP)

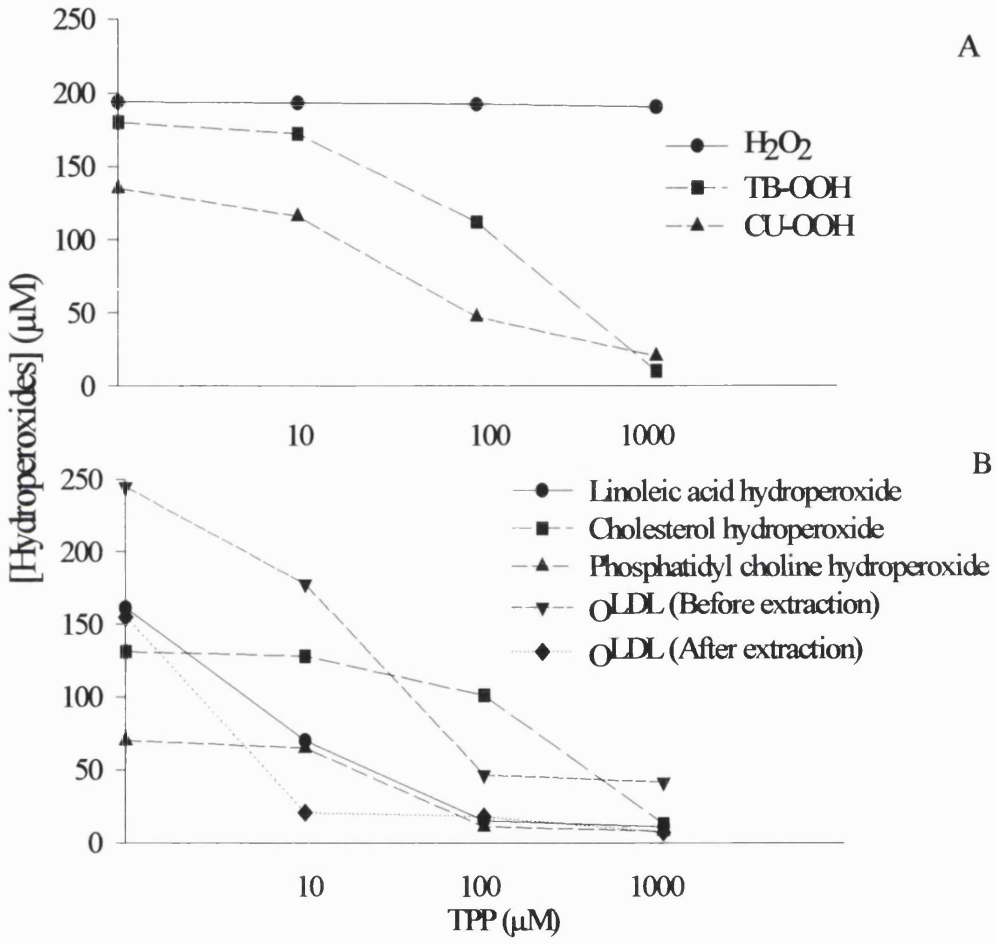
TPP has been used as a specific hydroperoxide reducing agent. It reduces hydroperoxides to their corresponding alcohols, while being quantitatively oxidised to triphenylphosphine oxide (TPPO) itself (Nakamura and Maeda, 1991). To demonstrate that colour development in the FOX2 assay was due to the presence of hydroperoxides and not because of non-peroxide artefacts, different hydroperoxides were incubated with TPP to assess the effectiveness of TPP in removing them. Stock solutions of hydrogen peroxide (H_2O_2) and tert-butyl hydroperoxide (TB-OOH) were diluted in distilled water at a final concentration of 1 mM. In the case of cumene hydroperoxide (CU-OOH) dilution was carried out with ethyl acetate, since it is lipophilic. Diluted samples of hydroperoxides approximately, 150-200 µM, were treated with 10 µl of TPP at various concentrations (0.1, 1 and 10 mM in methanol) for 30 minutes at room temperature. Figure 3.2.1.1.a., shows the effect of increasing concentrations of TPP on fixed amounts of CU-OOH and TB-OOH, and H_2O_2. CU-OOH and TB-OOH were removed effectively by TPP at a concentration of 1mM. However, TPP had no effect on H_2O_2 even at 10-fold stoichiometric excess. Hence TPP can be used as an efficient tool for distinguishing between H_2O_2 and non-H_2O_2 hydroperoxides.

Subsequently the effect of TPP on hydroperoxides of linoleic acid, phosphatidylcholine, and cholesterol, as well as copper-oxidised LDL, were analysed. Linoleic acid and LDL were diluted at a final concentration of 200 µg/ml in PBS. Cholesterol was dissolved in ethanol at a final concentration of 1mg/ml. Liposomes of phosphatidylcholine were prepared in PBS at a final concentration of 10 mg/ml (see 2.3.3.). Oxidation of the samples was carried out by incubation with Cu^{++} (10 µM) or AA-PH (1mM) for six hours at 37 °C. Peroxidised samples containing approximately 50-250 µM hydroperoxide were treated with 10 µl of different concentrations of TPP (0.1, 1 and 10 mM in methanol) for 30 minutes at room temperature. Figure 3.2.1.1.b. shows that when TPP was incubated with hydroperoxides of linoleic acid, phosphatidylcholine and cholesterol, as well as copper-oxidised LDL, it was effective in destroying more than 90% of linoleic acid, phosphatidylcholine, and cholesterol hydroperoxides. TPP in concentration of 1 mM also removed more than 80% of the hydroperoxides in copper-oxidised LDL when it was added to LDL directly in aqueous solution, while nearly all of the hydroperoxides were destroyed by TPP after ethyl acetate extraction. However, in the direct measurement system (without extraction) a proportion (between 5 and 15%) of the FOX2-generated signal could not be eradicated by TPP. The residual signal could also not be removed by pre-exposure to catalase (100 IU/ml), indicating that it was not due to H_2O_2, and seemed to result from unknown chromophores in the original sample. Data also indicates that TPP at concentration of 1mM destroys non-H_2O_2 hydroperoxide in all samples.

The assay was subsequently adapted for hydroperoxide measurement in plasma using TPP. Figure 3.2.1.2. shows the effect of TPP at final concentration of 1 mM on hydroperoxide levels in oxidised plasma , which had been incubated at 37 °C for 3 days. As is shown in figure 3.2.1.2, the hydroperoxide signals are removed at each time point by pre-incubation with TPP. The remaining signal which cannot be demolished by TPP, possibly originates from iron proteins, especially transferrin which releases ferric ions in acidic conditions. This signal can be destroyed by pre-incubation of plasma samples with 2-mercaptoethanol (ME) (see 3.1.5). The spectral characteristics of plasma hydroperoxides using the FOX2 assay are shown in figure 3.2.1.3. Figure 3.2.1.3.b shows spectra including the difference spectrum of an AA-PH incubated plasma sample before and after TPP treatment. Similar spectral changes were observed for native

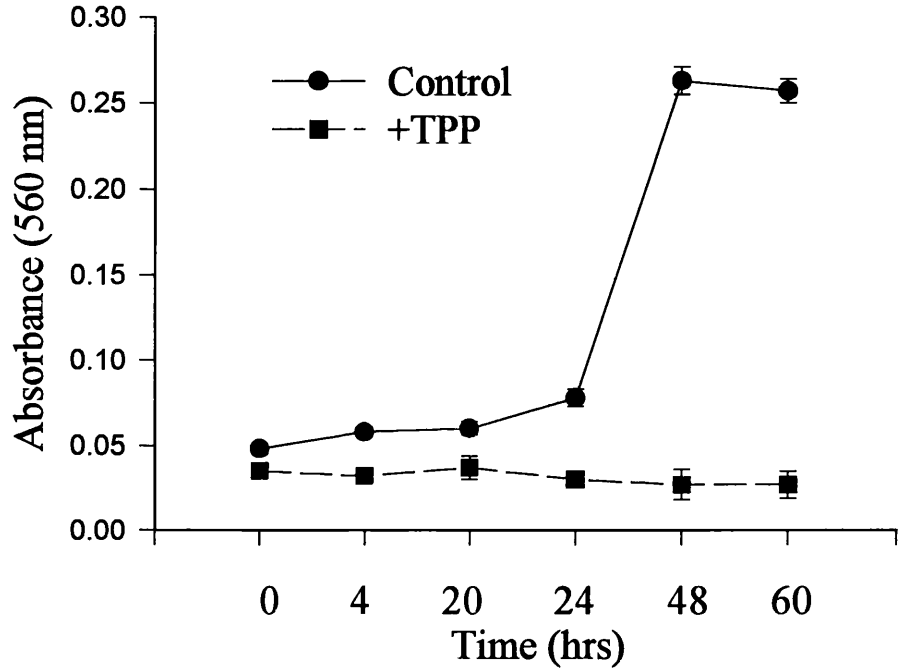
plasma (Fig 3.2.1.3.a), but in a smaller range. Plasma hydroperoxides can thus be determined directly by monitoring the 560 nm absorbance changes in the FOX2 assay before and after addition of TPP. This approach obviates the difficulty caused by the direct reaction of plasma ferric ion with xylenol orange.

Figure 3.2.1.1: Effect of increasing concentration of TPP on different peroxides



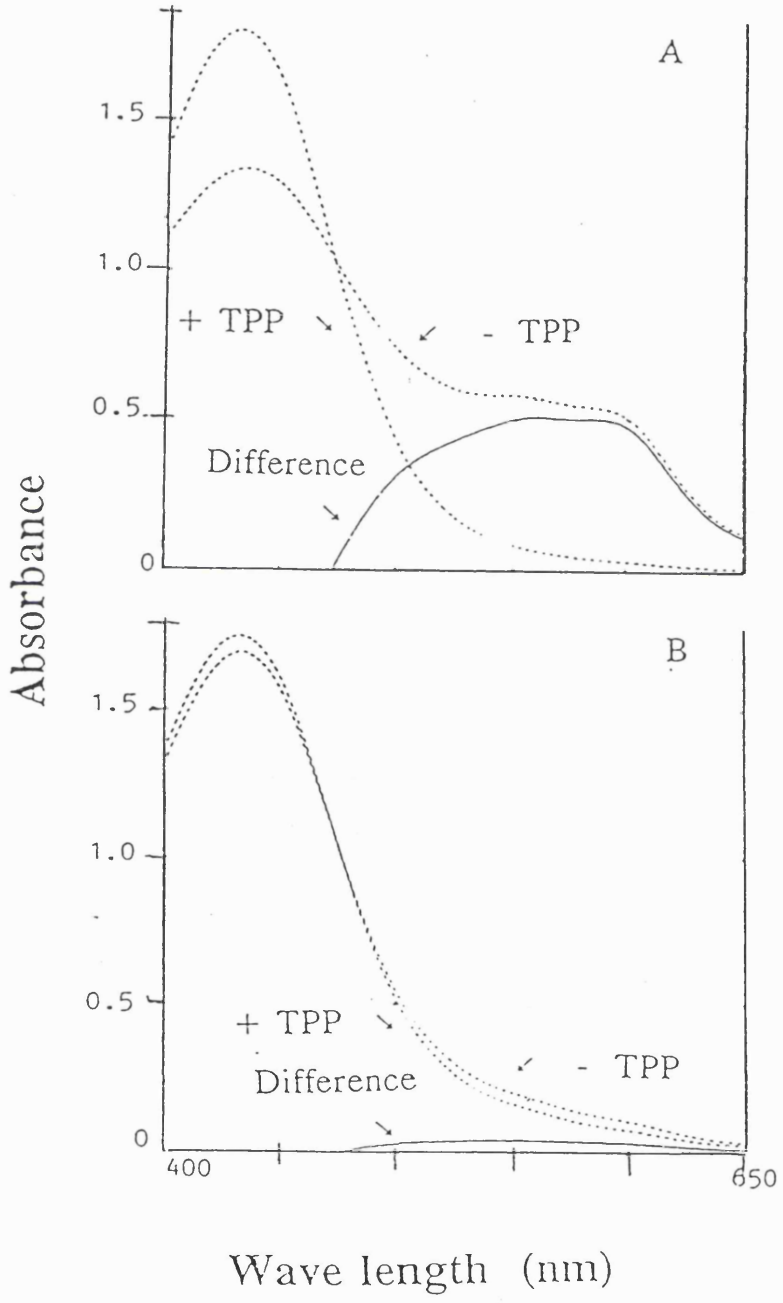
A: Samples (90 μl) of hydroperoxides in distilled water and ethyl acetate at a concentration of approximately 150-200 μM were incubated with 10 μl of 0.1, 1, and 10 mM of TPP in methanol for 30 minutes at room temperature. Samples were mixed with 900 μl FOX2 reagent and incubated for a further 30 minutes at room temperature. Absorbance was measured at 560 nm. Data represent means ± SD of triplicate analyses.
B: Lipids and lipoproteins were oxidised with Cu^{++} (10μM) and AA-PH (1mM) for 6 hours at 37 °C. Oxidised samples (90 μl) were incubated with 10 μl of 0.1, 1, and 10 mM of TPP in methanol for 30 minutes at room temperature (in the case of LDL, with and without ethyl acetate extraction). Samples were mixed with 900 μl FOX2 reagent and incubated for a further 30 minutes at room temperature. Absorbance of supernatant was measured at 560 nm following centrifugation of samples at 12000g for 5 minutes. Data represent means ± SD of triplicate analyses. Error bars lie within the mean data points.

Figure 3.2.1.2: Effect of TPP on hydroperoxides generated during peroxidation of whole plasma at 37 °C



Aliquots (90 µl) were removed at different time intervals and incubated with or without TPP (at final a concentration of 1mM) for 30 minutes at room temperature. Absorbance was measured at 560 nm after further incubation for 30 minutes and subsequent centrifugation at 12000g for 10 minutes to remove fluctuated protein. Data represent the mean of triplicate analyses ± SD.

Figure 3.2.1.3: Spectral characteristics of plasma hydroperoxides ± TPP using the FOX2 assay

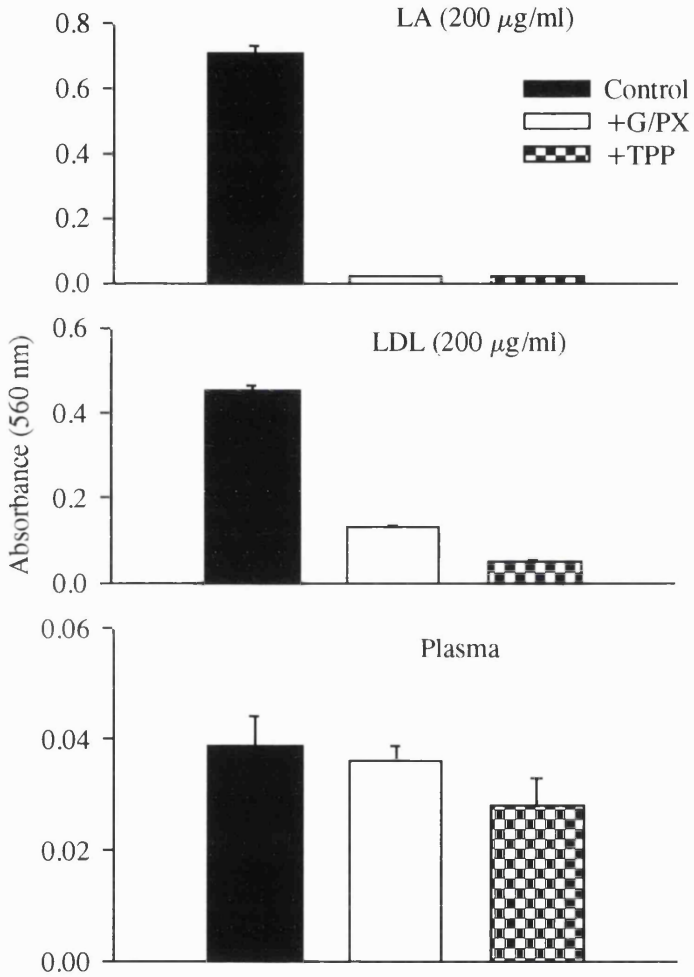


A: plasma incubated with AA-PH (1 mM for 12 hours at 37 °C).
B: plasma incubated in the absence of AA-PH. Samples (90µl) were incubated in the absence or presence of TPP (1mM) for 30 minutes at room temperature. FOX2 reagent (900 µl) was then added. Absorbance was scanned from 400 to 650 nm after 30 minutes incubation at room temperature and centrifugation at 12000g for 10 minutes.

3.2.2. Effect of glutathione peroxidase (G/PX)

G/PX is regarded as one of the major defences against oxidative stress, since it can reduce H_2O_2 and other organic hydroperoxides. Three types of enzyme have been recognised, and all of them contain selenium. The classical G/PX found in red blood cells, liver and other intracellular spaces (Flohe, 1982; Tappel et al. 1984) can reduce H_2O_2 and free fatty acid hydroperoxides but not PC-OOH (Ursini et al. 1982; Ursini et al. 1985). Reduction of PC-OOH has been shown by a second intracellular G/PX (Ursini et al. 1982). A third G/PX has been found in plasma (extracellular), which can also reduce H_2O_2 and free fatty acid hydroperoxides (Maddipati and Marnett, 1987). It has been shown that plasma G/PX can reduce PC-OOH but not CE-OOH (Yamamoto et al. 1993). The second substrate for the enzyme is the low-molecular-weight thiol compound glutathione, which is found in animals, plants and some bacteria. In order to examine the reducing effect of G/PX on hydroperoxides, samples of FA-OOH, oxidised LDL, and plasma were treated with G/PX or TPP. Figure 3.2.2. shows that G/PX and TPP significantly reduced hydroperoxides in oxidised linoleic acid. However, in oxidised LDL and plasma G/PX was less effective than TPP in destroying hydroperoxides. This is probably because of the presence of esterified hydroperoxides and also due to less penetration of G/PX inside of LDL particles. TPP reduced all hydroperoxides in the samples.

Figure 3.2.2: Effect of G/PX and TPP on lipid hydroperoxide levels in plasma, oxidised LA and LDL

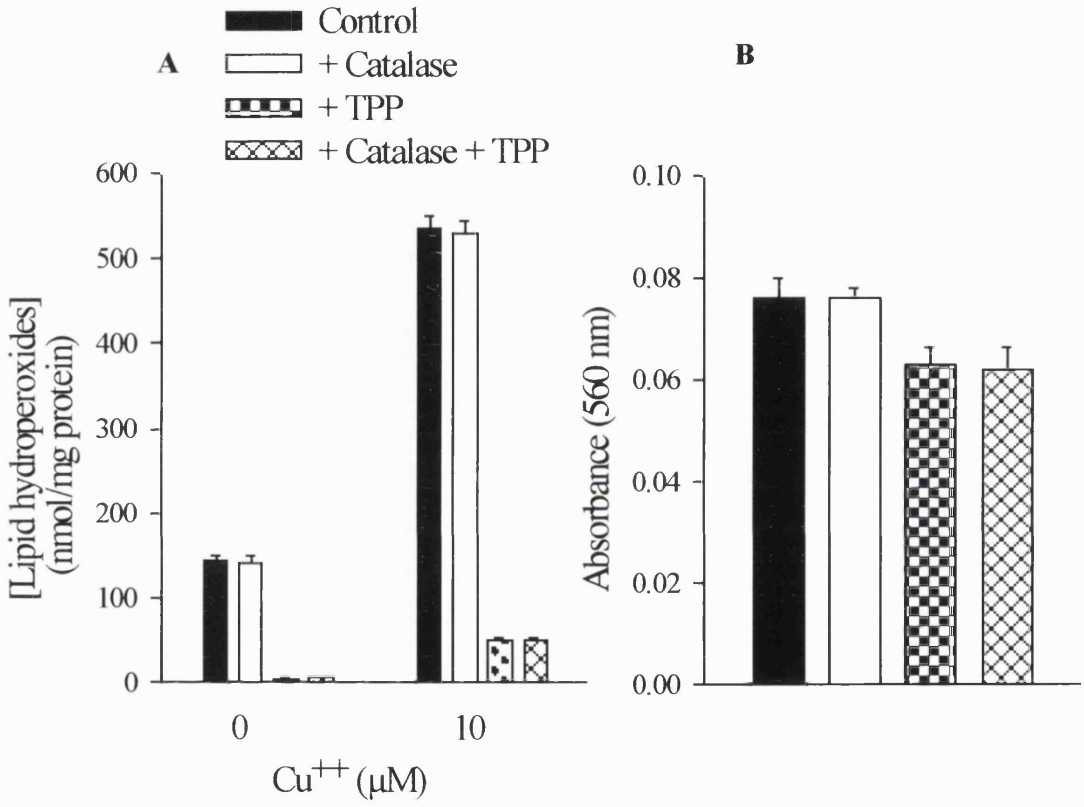


Plasma, LA and LDL (200 μg/ml in PBS) oxidised with Cu^{++} (10 μM) at 37 °C for 6 hours. All samples (90 μl) were subsequently incubated in the presence or absence of 10 μl of 1mM TPP or 5 μl of 100 U/ml G/PX (bovine erythrocyte) for 30 minutes at room temperature. GSH at a concentration of 1mM was used as a substrate for G/PX. Samples were mixed with FOX2 reagent and incubated for a further 30 minutes at room temperature. Absorbance of supernatant was measured at 560 nm following centrifugation of samples at 12000g for 10 minutes. Data represent means ± SD of triplicate analyses.

3.2.3. Effect of catalase

Catalase along with glutathione peroxidase is known as a rapid H_2O_2 removing enzyme. Catalase is present in all major body organs, especially liver and erythrocytes, although the activity of the enzyme varies between different organs. The effect of catalase was studied during measurement of lipid hydroperoxide in oxidised LDL and plasma along with TPP to distinguish H_2O_2 and non-H_2O_2 hydroperoxide signals. Figure 3.2.3.a. shows the effect of catalase and TPP on hydroperoxides in LDL which was incubated with or without copper (10 μM) for 20 hours at 37 °C. As demonstrated in figure 3.2.3.a, catalase had no effect on the hydroperoxide level, while TPP removed more than 90% of lipid hydroperoxides. The effect of catalase and TPP on plasma hydroperoxide is also shown in figure 3.2.3.b., which indicates that concentrations of H_2O_2 in plasma are below the level of detection.

Fig 3.2.3: Effect of Catalase and TPP on lipid hydroperoxide levels in LDL and plasma



A: LDL (200 μg/ml in PBS) was incubated with or without copper (10 μM) for 20 hours at 37 °C. Samples (90 μl) were incubated with 10 μl of 10 mM TPP and 10 μl of 1000 u/ml catalase or 10 μl PBS for 30 minutes at room temperature. The samples were then mixed with FOX2 reagent and incubated for another 30 minutes at room temperature. Absorbance was measured at 560 nm following centrifugation at 12000g for 10 minutes. B: Same procedure carried out for plasma except the peroxidation was with the copper only. Data represent means ± SD of triplicate analyses.

3.2.4. Extraction of plasma hydroperoxides

In order to validate the method using TPP, it was important to demonstrate that the signal detected by direct measurement of hydroperoxides in plasma samples by the method involving addition of TPP to whole plasma was well correlated with hydroperoxides measurable in the same samples following total extraction of lipids. First we used linoleic acid hydroperoxides and oxidised LDL. Samples (90 μl) of copper-oxidised linoleic acid (200 μg/ml) or copper-oxidised LDL (200 μg/ml) were assayed for hydroperoxide content by direct addition of FOX2 reagent (900 μl) with and without TPP (1mM) pre-treatment. These values were then compared with those obtained for the same samples after lipid extraction with ethylacetate (see Methods) followed by evaporation of the organic phase down to 100 μl prior to addition of FOX2 reagent. These analyses revealed that 87% of synthetic fatty acid hydroperoxide (5-HPETE), 72% of the linoleic acid hydroperoxide and 77% of the hydroperoxides present in LDL, as determined in aqueous phase by the method using TPP, were recovered after extraction of the lipid (Table 3.2.4.). The addition of BHT (25μM) to the organic phase prior to the evaporative step did not alter recovery, suggesting that further peroxidation reactions during evaporation do not contribute to final hydroperoxide values.

Plasma samples behaved in a way similar to that of the model lipid and lipoprotein. In a typical plasma sample, 63% of the signal detected by TPP pre-treatment of a whole plasma sample was recovered after ethyl acetate extraction. Recovery of hydroperoxide added to whole plasma was 77%. In all cases, TPP pre-treatment of the ethyl acetate extract resulted in a greater than 95% loss of signal with the FOX2 reagent.

These data indicate reasonable agreement between hydroperoxide concentrations determined in aqueous phases using TPP pre-treatment or post-extraction of total lipids.

Table 3.2.4: Comparison of lipid hydroperoxide levels before and after lipid extraction

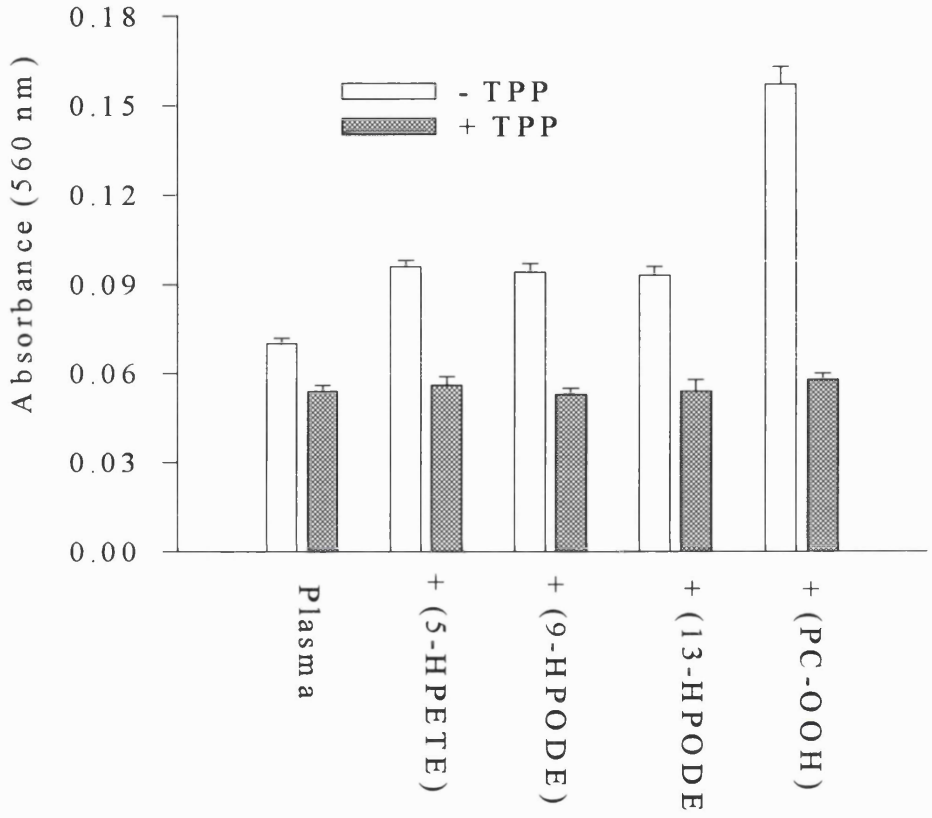
	[Hydroperoxide] (μM)		
Sample	Direct	Post-extraction	Recovery (%)
5-HPETE	200.00± 0.74	174.00± 1.33	87
Oxidized linoleic acid	36.00 ± 1.33	26.00 ± 2.67	72
Oxidized LDL	57.78 ± 1.33	44.44 ± 1.33	77
Plasma	2.44 ± 1.33	1.56 ± 0.22	63
Plasma + LA-OOH	12.44 ± 1.33	9.33 ± 0.44	77

* Data represent means ± SD of triplicate analyses.

3.2.5. Recovery of synthetic hydroperoxides from plasma

Since TPP selectively reduces various hydroperoxides except for H_2O_2 (see 3.2.1.), it eliminates the ROOH-derived absorbance in the FOX2 assay. In this way it generates a blank sample usable for correction for non-peroxide materials. This manoeuvre is necessary since plasma samples contain variable amounts of ferric ion detectable by xylenol orange. This contributes to high background absorbance (see 3.1.2), particularly if there is haemolysis. It was shown, however, that the amount of hydroperoxide detected using TPP in whole plasma corresponds very closely with the amounts of hydroperoxide measurable if total lipid in plasma is first separated from water-soluble material, including ferric ion, by extraction of plasma with ethyl acetate (see 3.2.4.). Figure 3.2.5.1. shows data for a plasma sample, before and after the addition of certain amounts of synthetic free FA-OOHs and PC-OOHs to the same sample before and after TPP treatment. The assay generated approximately equivalent signals with equal concentrations of exogenous FA-OOH. The signals were uniformly reduced to the background level by treatment with TPP. These data reconfirm that physiological L-OOH of varying structure generates the same signal in the FOX2 assay. This is reassuring since it is possible that different individuals may have different classes of FA-OOH present in their plasma. Table 3.2.5. compares the extinction coefficients generated for these LOOH in the FOX2 assay with the values obtained using the iodometric assay for LOOH described in Materials and Methods. Figure 3.2.5.2. shows standard curve for H_2O_2 and 5-HPETE using FOX2 assay.

Figure 3.2.5.1: Detection of authentic synthetic hydroperoxides in plasma



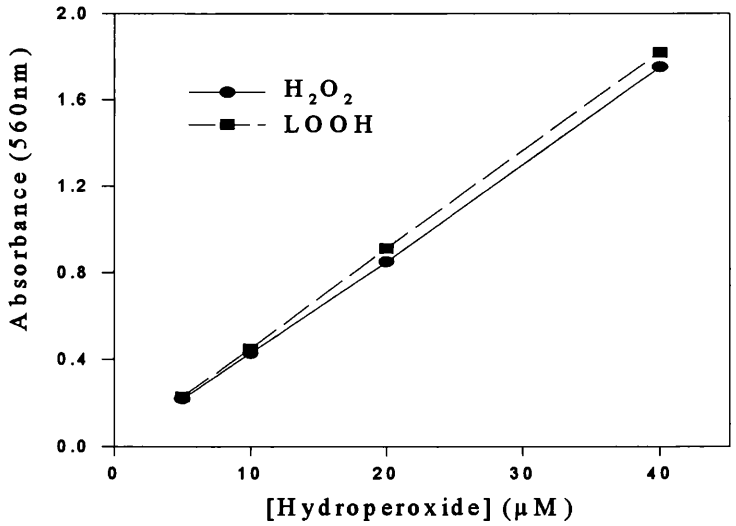
Plasma samples (80 µl) were spiked with 10 µl of 200 µM 5-HPETE, 9-HPODE, 13-HPODE and 10µl of peroxidised phosphatidylcholine (PC-OOH) at a concentration of approximately 260 µM or methanol. The samples were incubated with 10 µl of 10 mM TPP or methanol alone for 30 minutes at room temperature. After addition of 900 µl FOX2 reagent, samples were incubated for a further 30 minutes at room temperature. Absorbance of supernatant was measured following centrifugation of samples at 12000g for 10 minutes. Data represent means ± SD of triplicate analyses.

* PC-OOH, hydroperoxide prepared by exposure of phosphatidylcholine liposomes to 10 µM copper sulphate for 3 hours at 37 °C in PBS, pH 7.4.

Table 3.2.5: Comparison of the extinction coefficient for synthetic hydroperoxides using FOX2-assay and the iodometric technique

Extinction coefficient (M^{-1} cm^{-1})		
Compound	FOX2(560 nm)	Iodometric (290 nm)
H_2O_2	4.4×10^4	3.9×10^4
5 (S)-HPETE	4.6×10^4	3.8×10^4
9 (S)-HPODE	4.6×10^4	3.8×10^4
13 (S)-HPODE	4.6×10^4	3.7×10^4

Figure 3.2.5.2: Standard curve for H_2O_2 and 5 (S)-HPETE in the 5-40 μM range in assay

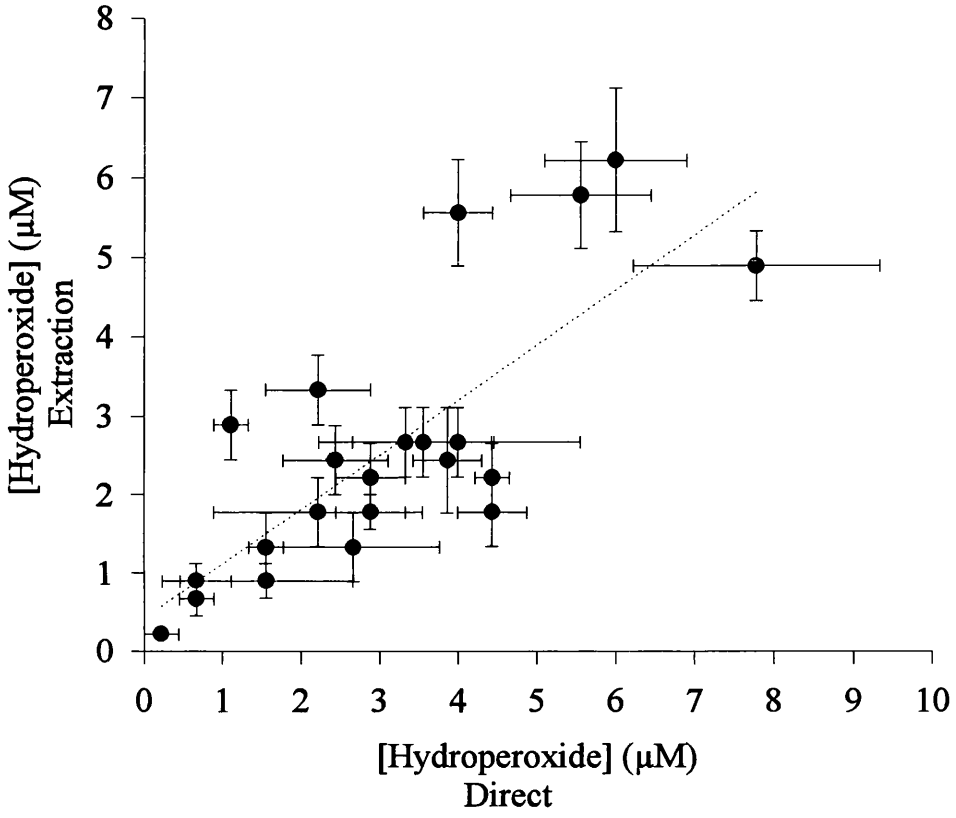


Stock solution of peroxides were diluted in PBS at a final concentration of 1 mM. Different volumes of samples were adjusted with PBS to 100 μl to give 5-40 μM peroxide in assay. Absorbance was measured following incubation of samples with FOX2 reagent for 30 minutes at room temperature.

3.2.6. Hydroperoxides in human plasma

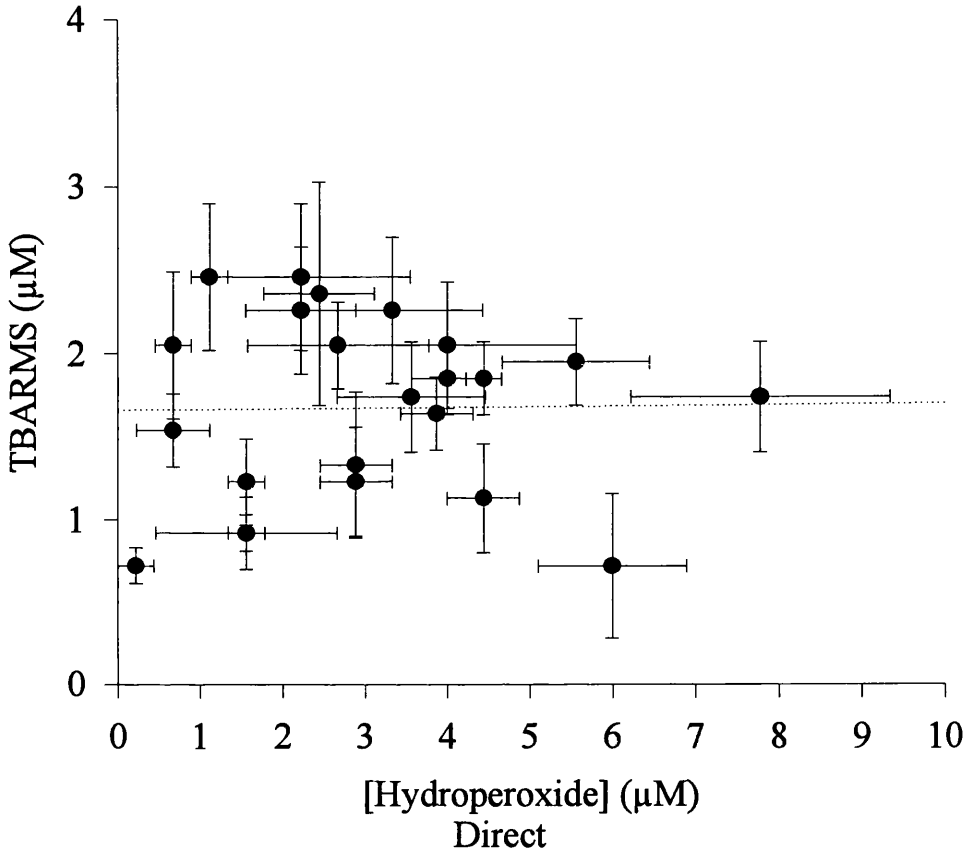
After preliminary analysis and validation of the FOX2 assay for detection of hydroperoxides in plasma using the strategy of TPP pre-treatment, hydroperoxides were measured in 23 healthy male and female volunteers in the age range 25-65. Measurement of hydroperoxides in whole plasma revealed that the levels of detectable hydroperoxide across the group ranged from 0.22 to 7.8 μM with a mean of 3.02 μM and a population standard deviation of 1.85 μM. After extraction with ethyl acetate, plasma hydroperoxide levels ranged from 0.22 to 6.22 μM with a mean value of 2.52 μM and a population standard deviation of 1.65 μM. There was a good correlation (Pearson's r = 0.78; $P<0.005$) between the measurements of whole plasma hydroperoxides in these individuals with the levels obtained after extraction of lipids (Fig 3.2.6.1). Over the entire range, approximately 80% of hydroperoxides measured in the aqueous phase were recovered after ethyl acetate extraction of total lipids. Hydroperoxide values did not correlate with the level of TBARMs (Fig 3.2.6.2), suggesting that hydroperoxides may not be the precursor for TBA-reactive materials in plasma. Furthermore, the TBA assay measures other parameters in plasma besides MDA, which reduces the specificity of the test for determination of lipid peroxidation in plasma. Alternatively since the MDA level depends on both the rate of synthesis and the rate of removal, when rates of removal vary greatly the steady state level will not reflect rates of synthesis from hydroperoxides between different individuals.

Figure 3.2.6.1: Regression of plasma hydroperoxide levels (n=23) obtained by direct and post-extraction data



For direct measurement, plasma samples (90 μl) were incubated with 10 μl of 10 mM TPP in methanol or PBS for 30 minutes at room temperature. After addition of 900 μl FOX2 reagent samples were incubated for another 30 minutes at room temperature. Absorbance of supernatant was measured at 560 nm following centrifugation at 12000g for 10 minutes. In the case of extraction, plasma lipids from the same samples were extracted with ethyl acetate (see Materials and Methods) and dried under N_2 to a final volume of 100 μl. After addition of 900 μl FOX2 reagent absorbance was measured at 560 nm after 30 minutes incubation. Data represent means ± SD of triplicate analyses.

Figure 3.2.6.2: Lack of correlation between plasma hydroperoxides and TBARMs



Direct measurement of plasma hydroperoxides was described above (Fig 3.2.6.a). The concentration of TBARMs was calculated as malondialdehyde equivalents using the extinction coefficient for the malondialdehyde-thiobarbituric acid complex of 1.56×10^5 M^{-1} cm^{-1} at 532 nm. Data represent means ± SD of triplicate analyses.

Part 3

3.3. Effect of sample handling on plasma hydroperoxide levels

There is a general agreement that it is preferable to assay plasma samples as soon as possible after collection. However, in the clinical situation immediate measurement of hydroperoxides is not always possible and storage of samples is sometimes required. In order to assess the stability of hydroperoxides in different storage conditions, the effect of temperature during short-time storage was analysed in samples from 25 healthy subjects at 4 °C and 37 °C. Also effects of long-time storage on stability of hydroperoxides in 39 healthy subjects and 169 non-insulin-dependent diabetes mellitus (NIDDM) patients were investigated after 3 months at -60 °C. Furthermore the effect of freeze and thaw cycles on the stability of hydroperoxides in plasma, authentic hydroperoxides or the combination of the two was studied. In addition, the effect of BHT as an antioxidant on the stability of peroxides at different temperatures was studied.

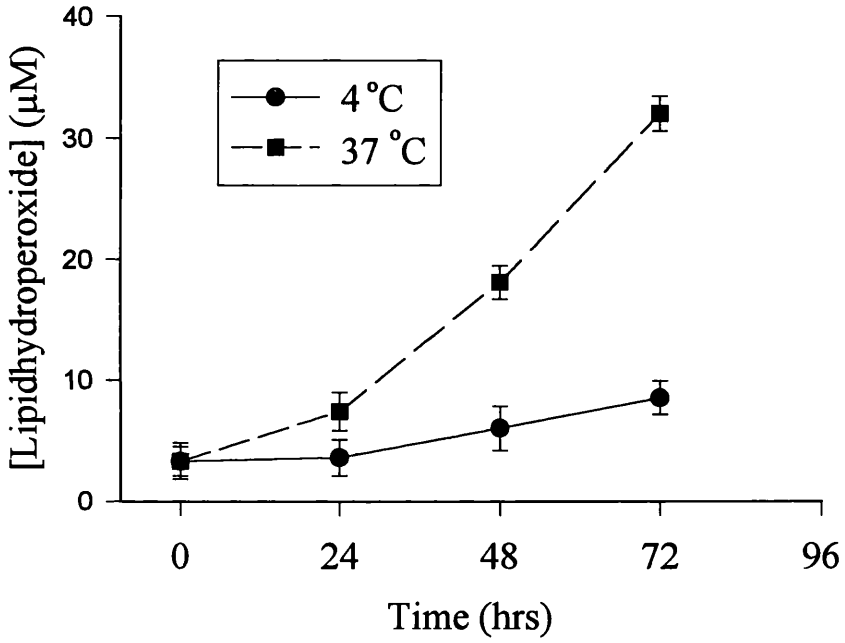
3.3.1. Temperature

To assess stability of LOOH during storage at different temperatures, plasma from 25 healthy subjects was stored at 4 °C and 37 °C and measured every 24 hours up to 3 days. As has been shown in figure 3.3.1.1., hydroperoxides were stable during storage at 4 °C for up 24 hours. After 24 hours, however, there was a rise in hydroperoxide levels (Fig 3.3.1.1.). Incubation of the same plasma samples at 37 °C accelerated hydroperoxide formation is less than 24 hours, indicating the role of the high temperature as a catalyst in the oxidation process (Fig 3.3.1.1.).

The effect of long-time storage on the stability of hydroperoxides was studied in plasma samples from healthy subjects (n=39) and NIDDM patients (n=169) that had been stored at - 60 °C for 3 months. Plasma samples from both groups were immediately analysed for hydroperoxide levels. The samples were transferred to a 1 ml microcentrifuge tube and kept for 3 months at -60 °C. Analysis of the samples after 3 months showed a big variation in hydroperoxides level, especially in NIDDM group. The control group showed loss of 5-75% with the mean level of the 40% of original signal in LOOH levels.

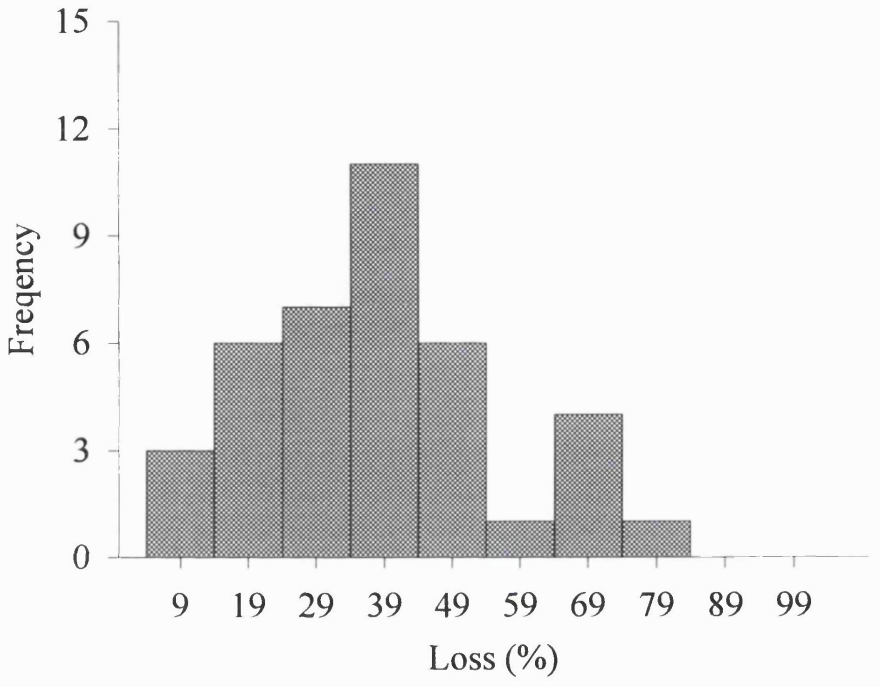
Figure 3.3.1.2. shows percentage loss of hydroperoxides with the majority of the control group samples losing around 20-30%. There was significant correlation between hydroperoxide levels in fresh samples and loss of hydroperoxides in frozen samples ($r = 0.5$; $P < 0.0005$). Loss of hydroperoxides in frozen samples had no correlation with vitamin E ($r = 0.044$; $P = 0.7$), triglyceride ($r = 0.1$; $P = 0.5$) and cholesterol levels ($r = 0.1$; $P = 0.5$) respectively. Stability of hydroperoxides in NIDDM was unpredictable. While in most of the samples ($n = 126$) hydroperoxides were lost, nevertheless there was an accumulation of hydroperoxides in some of the samples ($n = 43$). Loss of hydroperoxides was 5-90% of the original concentration with the mean level of 47%. Accumulation of hydroperoxide in the "increase" group was 0-90% with the mean level of 31%. Figure 3.3.1.3. shows percentage loss and accumulation of hydroperoxides in NIDDM patients after determination of hydroperoxides in plasma which had been frozen for 3 months. While correlation between hydroperoxides in fresh samples and those frozen samples where the loss of hydroperoxides was positive ($r = 0.54$; $P < 0.0005$), there was no correlation between loss of hydroperoxides and vitamin E ($r = -0.06$; $P > 0.05$), triglyceride ($r = 0.07$; $P > 0.05$) or total cholesterol concentration ($r = 0.1$; $P > 0.05$) respectively. Furthermore, there was no correlation between accumulation of hydroperoxide and vitamin E ($r = 0.07$; $P > 0.05$), triglyceride ($r = 0.06$; $P > 0.05$) or cholesterol ($r = -0.03$; $P > 0.05$) respectively. Lack of correlation between loss or accumulation of hydroperoxides with vitamin E or lipids may relate to the availability of transition metal ions which play an important role in the stability of plasma hydroperoxides during long-time storage.

Figure 3.3.1.1: Effect of temperature on stability of plasma hydroperoxides



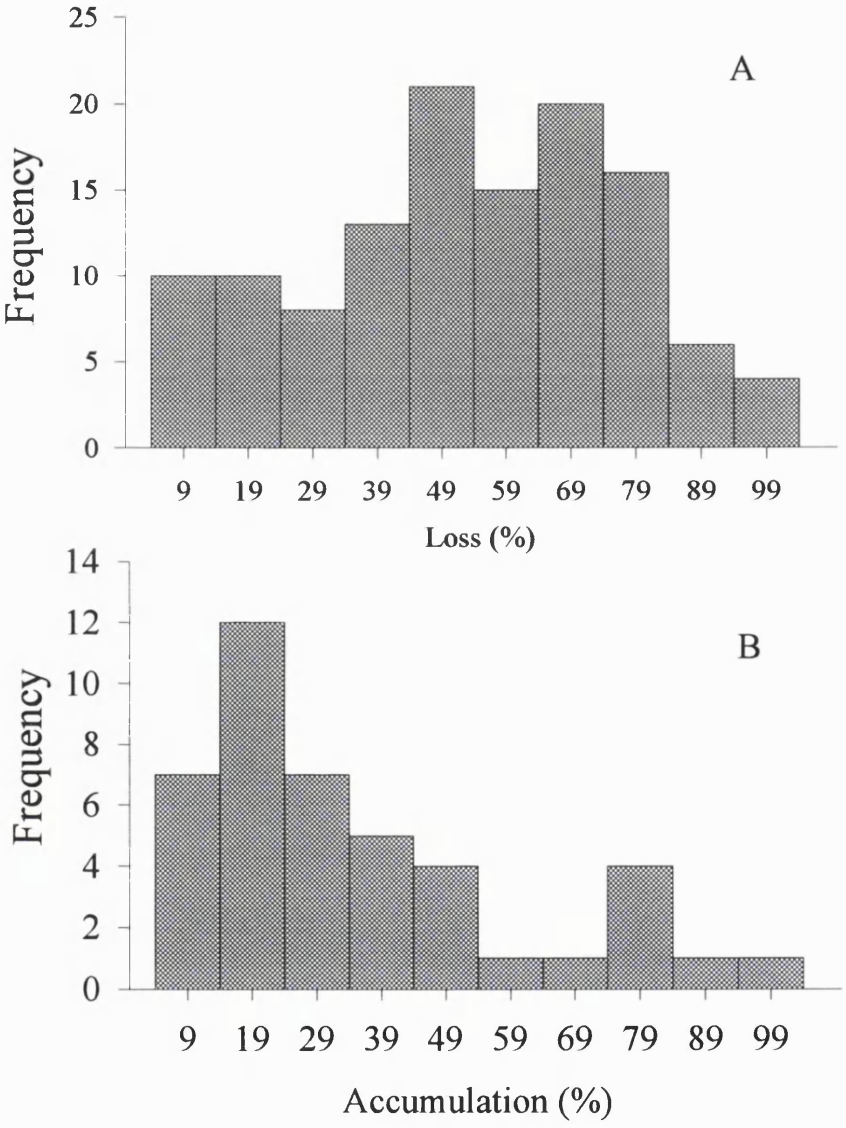
Accumulation of hydroperoxide in plasma of healthy subjects (n=25) during storage at 4 °C and 37 °C as measured as a function of time. Data represent means ± SD of triplicate analyses.

Figure 3.3.1.2: Stability of plasma hydroperoxides in healthy subjects (n=39) during storage at -60 °C



Decomposition of plasma hydroperoxides in healthy subjects (n=39) during storage at -60 °C for 3 months with high frequency around 10-50%. Hydroperoxide concentration was measured using FOX2 assay as described in Materials and Methods.

Figure 3.3.1.3: Stability of plasma hydroperoxides in NIDDM patients (n=169) during storage at -60 °C



Decomposition and accumulation of plasma hydroperoxides in NIDDM patients during storage at -60 °C for 3 months.
A: Decomposition of plasma hydroperoxides (n=126).
B: Accumulation of plasma hydroperoxides (n=43). Hydroperoxide concentration was measured using FOX2 assay as described in Materials and Methods.

3.3.2. Freeze and thaw

In order to examine the effect of freeze and thaw on the stability of hydroperoxides, samples of plasma, H_2O_2, FA-OOH (5-HPETE) and Ch-OOH were analysed alone or in combination during repeated freeze and thaw cycles at -60 °C over a period of 3 hours on the same day. Table 3.3.2., shows the significant loss in the hydroperoxide levels (H_2O_2, FA-OOH and Ch-OOH) immediately after their addition to plasma, presumably due to the effects of catalase and glutathione peroxidase. There was a further fall in hydroperoxide levels after the first cycle of "freeze and thaw" in all samples (cycle 1). Loss of hydroperoxides in plasma was notable (approximately 30%), whilst hydroperoxides in other media were less susceptible to the freeze and thaw effect. The loss of hydroperoxides prepared in water or ethanol for H_2O_2, FA-OOH and Ch-OOH was 6, 4 and 11% respectively. When plasma samples were spiked with H_2O_2, FA-OOH and Ch-OOH, loss of hydroperoxides was 24, 21 and 23% respectively. The results indicate that loss of hydroperoxides in plasma is not a simple decomposition process during freeze and thaw. However, further cycles of freeze and thaw had a very small effect on hydroperoxide levels as has been shown for the second and third cycles.

Table 3.3.2: Effect of freeze and thaw on hydroperoxide stability

[Hydroperoxide] (μM)

Cycles of freeze and thaw

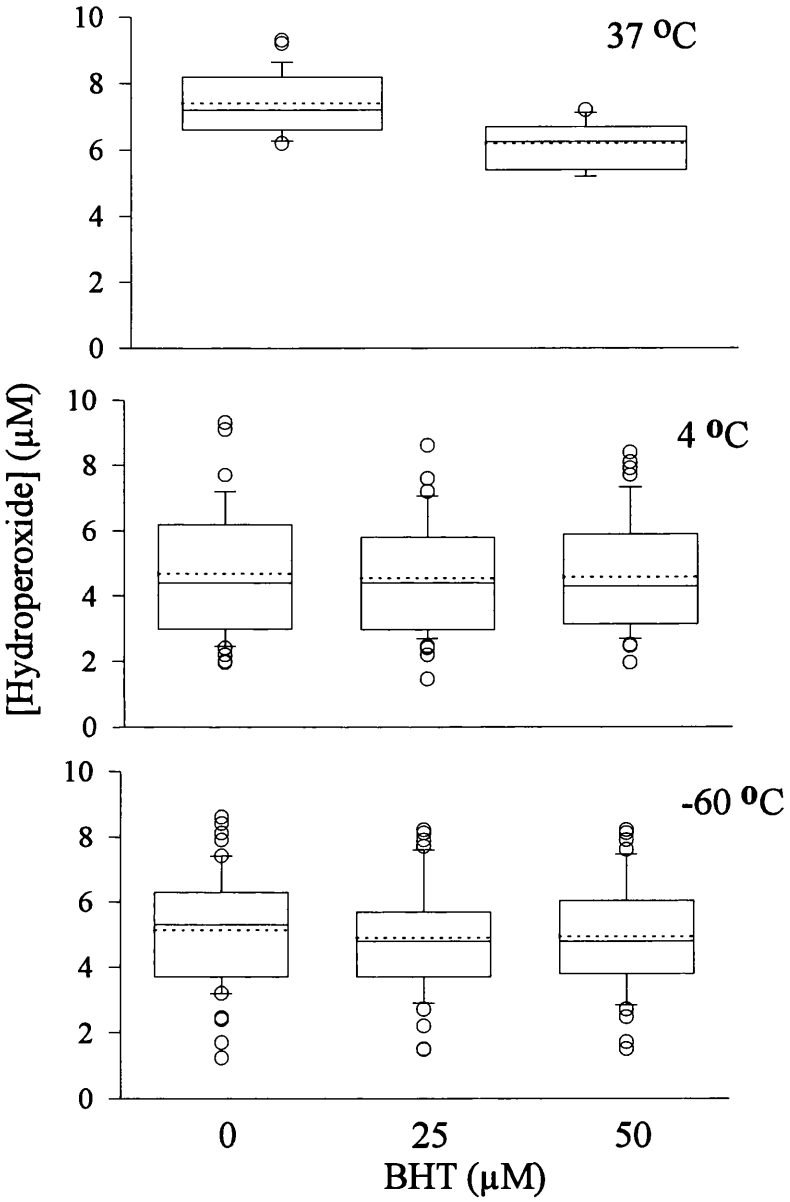
Samples	0	1	2	3
Plasma	3.9 ± 1.3	2.7 ± 0.5	2.6 ± 0.4	2.6 ± 0.5
H_2O_2	90 ± 0.8	85.6 ± 0.6	77.4 ± 0.8	76.4 ± 0.6
FA-OOH(5-HPETE)	33.3 ± 0.4	32.7 ± 0.8	33 ± 0.6	32.8 ± 0.5
Ch-OOH	49.2 ± 0.5	44 ± 0.5	44 ± 0.6	43 ± 0.4
Plasma + H_2O_2	8.1 ± 0.4	6.2 ± 0.3	6 ± 0.4	5.9 ± 0.6
Plasma + FA-OOH	9.3 ± 0.9	7.4 ± 0.7	7.2 ± 0.8	7 ± 0.9
Plasma + Ch-OOH	10.4 ± 0.8	8 ± 0.6	7.8 ± 0.6	7.4 ± 0.5

Ch-OOH was prepared by incubation of 2 mg/ml cholesterol in ethanol with 1mM AA-PH for 24 hours at 37 °C. Samples of H_2O_2, FA-OOH (10 μl of 1 and 0.3 mM respectively) and Ch-OOH (10 μl) were added to 80 μl PBS or plasma samples. Hydroperoxide concentration was measured as described in Material and Methods. Data represent means ± SD of triplicate analyses.

3.3.3. Antioxidants

The effect of butylated hydroxy toluene (BHT) as an antioxidant on the stability of plasma hydroperoxide during storage at different temperature was investigated. Plasma from 25 healthy subjects were incubated in the presence or absence of 25 μM BHT for 24 hours at 37 °C. Figure 3.3.3. shows the protective effect of BHT on oxidation rate of plasma hydroperoxides (nearly 15-20%) after 24 hours incubation at 37 °C. The effect of BHT on hydroperoxide stability was also measured in plasma samples from familial hypercholesterolaemic (FH; n=52) patients during storage at 4 °C for 24 hours or -60 °C for 3 months. There was, however, very small variation in hydroperoxide levels in samples which had been pre-treated with or without 25 and 50 μM BHT at 4 °C (4.6 ± 1.9 Vs 4.5 ± 1.7 Vs 4.6 ± 1.8; P= 0.95) and -60 °C (5.1 ± 1.8 Vs 4.9 ± 1.7 Vs 4.9 ± 1.7; P= 0.75) respectively (Fig 3.3.3.).

Figure 3.3.3: Effect of BHT on hydroperoxide levels at different temperatures



Plasma samples were incubated with or without BHT at final concentrations of 25 and 50 μM in plasma at different temperatures. The samples incubated at 37 °C were treated with 25 μM BHT for 24 hours. Storage time for samples at 4 °C and -60 °C was 24 hours and 3 months respectively. Boxes indicate the 25th and 75th percentile of values. A line inside the box marks the value of the 50th percentile. Dotted lines represent the mean value for the whole column. Bars indicate the 10th and 90th percentiles, and symbols mark all data outside the 10th and 90th percentiles. Hydroperoxide levels were measured as described in Materials and Methods.

Part 4

3.4. In vitro peroxidation of plasma lipoproteins

In order to examine the susceptibility of the major classes of plasma lipoproteins to oxidation, lipoprotein fractions from plasma of healthy subjects were oxidised under basal and stimulated conditions in vitro. Peroxidation of samples was carried out in the presence of copper ions and [2,2-Azo-bis-(2-amidinopropane) hydrochloride] (AA-PH). The commencement and the progress of oxidation of lipoproteins were analysed by measuring lipid hydroperoxides (L-OOH), conjugated diene (CD) and thiobarbituric acid reactive materials (TBARMs). During the course of peroxidation of VLDL, LDL and HDL, consumption of PUFAs was determined by GC using flame ionisation detection (FID). Vitamin E levels were analysed during incubation of lipoproteins with AA-PH and Cu^{++} at different intervals with HPLC using a fluorimetric detector.

3.4.1. Exposure to AA-PH

Isolated lipoprotein fractions were dialysed against PBS for 24 hours at 4 °C in the dark (see 2.3.2.). This step is necessary to remove any remaining EDTA from lipoprotein fractions. The EDTA-free lipoproteins were diluted in PBS to a final protein concentration of 200 μg/ml and incubated in the presence or absence of AA-PH at 37 °C for 24 hours. AA-PH is the water soluble free radical generator which undergoes spontaneous thermal decomposition to form two carbon-centred radicals and N_2 gas. These carbon-centred radicals can react with oxygen to yield peroxy radicals which are capable of abstracting hydrogen from membrane lipids and thereby initiate the peroxidation process (Halliwell and Gutteridge, 1991). The advantages of using AA-PH are water solubility and suitable rate of decomposition at physiological temperature.
In the presence of AAPH, accumulation of hydroperoxides and conjugated dienes in all 3 fractions (VLDL, LDL and HDL) was approximately linear over time and lowest in HDL (Fig 3.4.1., and 3.4.2.). When incubated in buffer, LDL and VLDL showed less of hydroperoxide and conjugated diene accumulation in the absence of AAPH than in its presence; HDL appeared to be immune to oxidation. Incubation of PBS with AA-PH

(1mM) showed that 5-10% of generated peroxides in samples was due to AA-PH alone. LDL and VLDL accumulated approximately twice as much lipid hydroperoxide as HDL in both basal and stimulated oxidation, which was to be expected from with their higher initial proportion of arachidonic (20:4 n-6) and linoleic (18:2 n-6) acids. Accumulation of TBARMs in the lipoproteins was 5 to 10-fold lower than accumulation of hydroperoxides and conjugated dienes. However, the overall trend was similar to that observed for hydroperoxide and conjugated diene accumulation. LDL and VLDL generated considerably more TBARMs than HDL (Fig 3.4.3.).

LDL and HDL contained similar levels of total linoleic and arachidonic acids prior to incubation with AAPH, approximately 40% and 5% of total fatty acids respectively, figures similar to those that have been reported previously (Esterbauer et al. 1990). These levels were higher than in the case of VLDL (25% and 3% respectively). During oxidation the levels of 18:2 and 20:4 decreased rapidly in all lipoproteins. However consumption of both arachidonic and linoleic acids were slower with AA-PH than was the case of Cu^{++} catalysed oxidation of lipoproteins (Fig 3.4.4.). This may be related to the slower accumulation of hydroperoxides during oxidation with AA-PH (Fig 3.4.1.). All lipoproteins rapidly lost arachidonic acid whereas linoleic acid was still detectable in LDL after 24 hours.

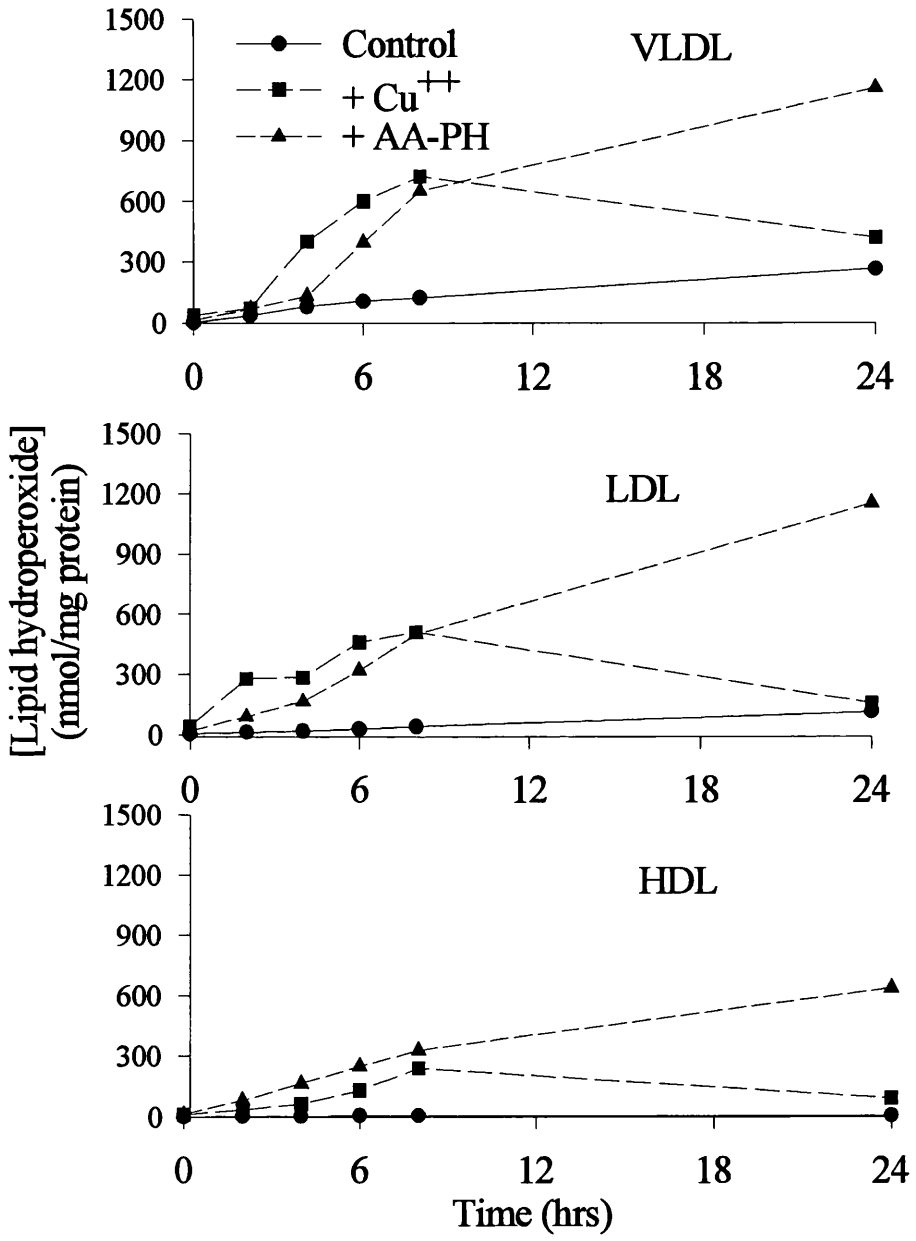
Levels of vitamin E in VLDL, LDL and HDL were 1.0, 1.1 and 1.3 μg/mg of total lipid, respectively. In all isolated lipoproteins exposed to AAPH and Cu^{++} consumption of vitamin E was rapid and had been completed prior to total loss of arachidonic acid (ie, after 2 hours). Hence variation in peroxidisability of lipoproteins is a complex process which cannot be simply explained in terms of differences in vitamin E content or fatty acid composition.

3.4.2. Metal-dependent oxidation

Peroxidation of major plasma lipoprotein classes was carried out in the presence or absence of copper ions. EDTA-free lipoproteins were diluted in PBS to a final concentration of 200 μg/ml protein and incubated with or without copper (10μM) for 24 hours at 37 °C. Lipoprotein peroxidation patterns with copper were comparable with those obtained with AA-PH, despite differences in the mechanism of peroxidation

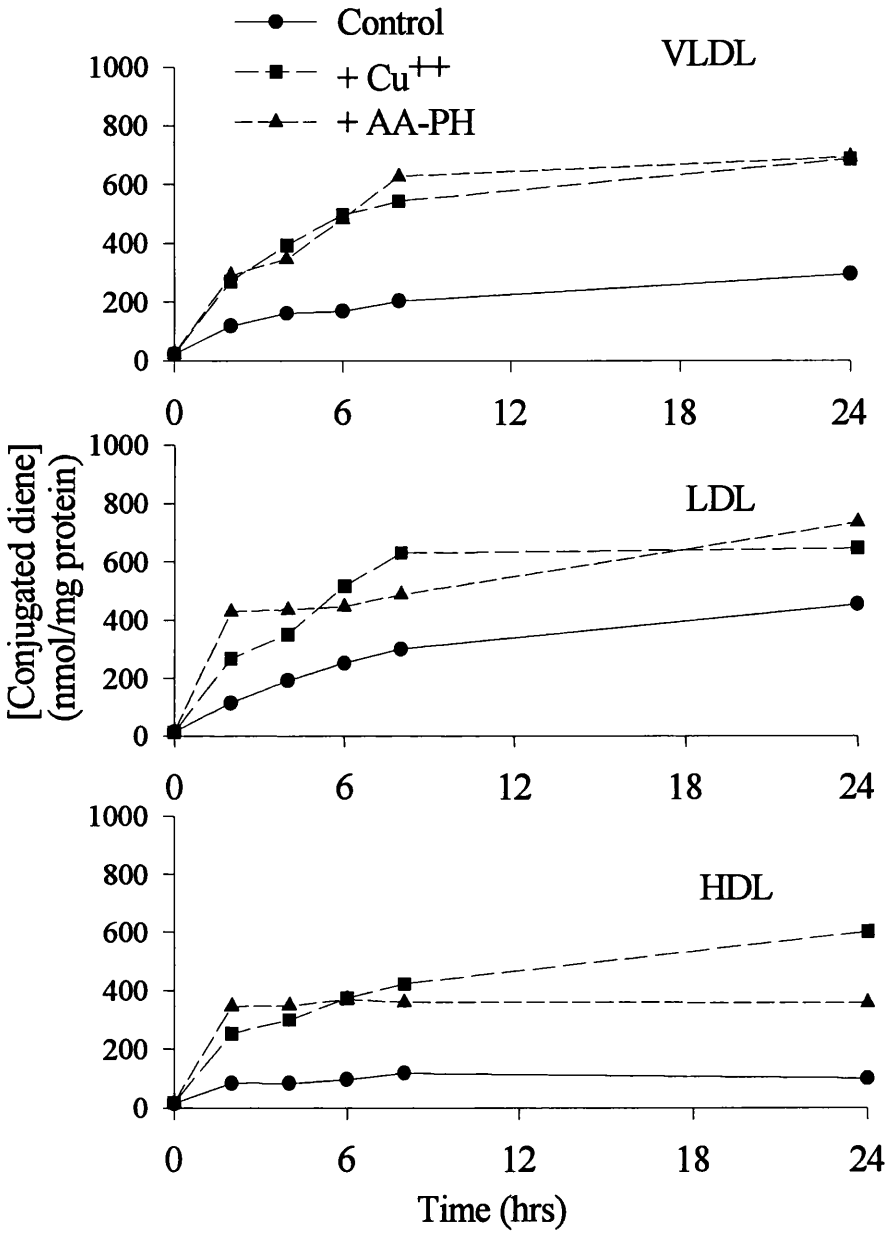
induced by this pro-oxidant. Accumulation of L-OOH, CD and TBARMs in HDL was much less than in VLDL and LDL. Maximum accumulation of hydroperoxides in Cu^{++} oxidised fractions was observed after 8 hours and, unlike AA-PH treated fractions, there was significant decomposition of hydroperoxides after 24 hours (Fig 3.4.1.). At this time point more than 70% of PUFAs were consumed in Cu^{++} oxidised samples (Fig 3.4.4.). Accumulation of TBARMs in all fractions (VLDL, LDL and HDL) was significantly higher than in AA-PH oxidised samples (Fig 3.4.3.), while there was no notable difference in conjugated diene formation between the two conditions (Fig 3.4.2.). Depletion of vitamin E was similar to AA-PH exposed samples and was rapid prior to consumption of PUFAs.

Figure 3.4.1: Accumulation of hydroperoxide in lipoproteins incubated in vitro



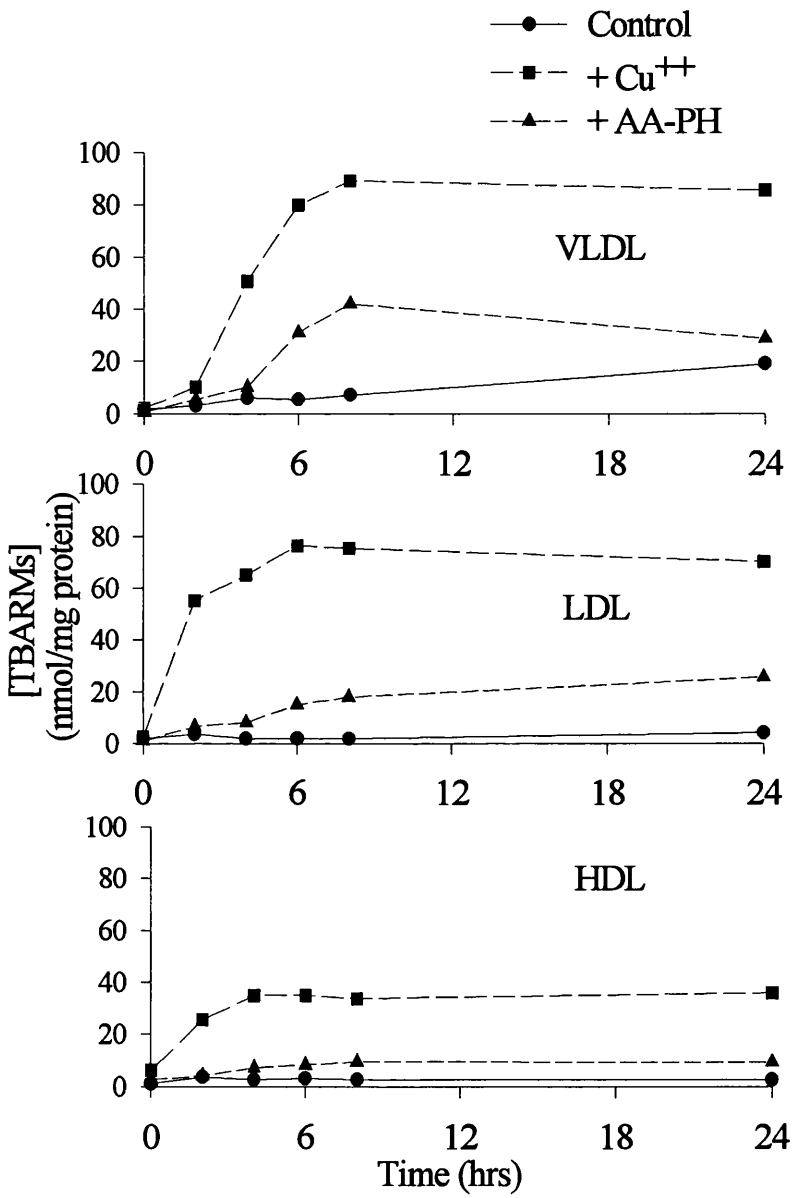
Purified lipoproteins at a final concentration of 200 µg/ml in PBS were incubated in the absence or presence of 10 µM Cu^{++} and 1 mM AA-PH at 37 °C for 24 hours. Hydroperoxide content were measured at different intervals. Data represent means ± SD of triplicate analyses. Error bars lie within the mean data points.

Figure 3.4.2: Accumulation of conjugated diene in lipoproteins incubated in vitro



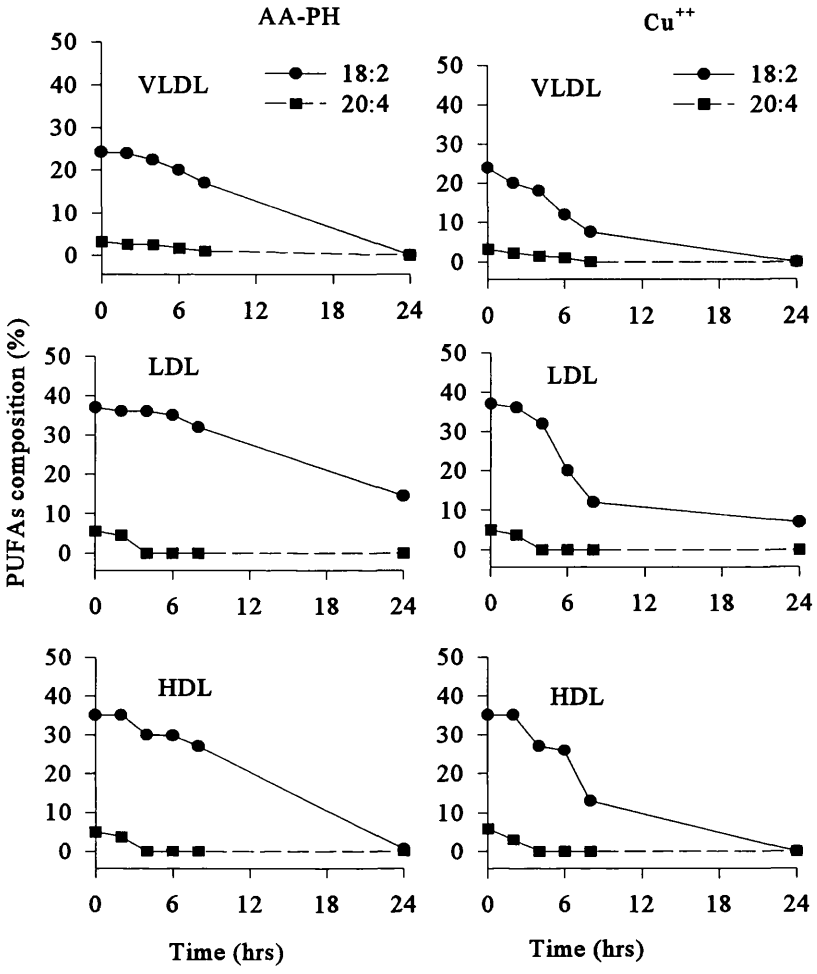
Purified lipoproteins at final a concentration of 200 μg/ml in PBS were incubated in the absence or presence of 10 μM Cu^{++} and 1 mM AA-PH at 37 °C for 24 hours. Conjugated dienes were measured at different intervals using an molar absorption extinction coefficient of 2.8 $\times 10^4$ M^{-1} cm^{-1} as described in Materials and Methods. Data represent means ± SD of triplicate analyses. Error bars lie within the mean data points.

Figure 3.4.3: Appearance of TBARMs in lipoproteins incubated in vitro



Purified lipoproteins at a final concentration of 200 µg/ml in PBS were incubated in the absence or presence of 10 µM Cu^{++} and 1 mM AA-PH at 37 °C for 24 hours. TBARMs were measured as a function of time. Error bars lie within the mean data points.

Figure 3.4.4: Consumption of PUFAs during peroxidation of lipoproteins



Arachidonic (20:4) and linoleic (18:2) acids were measured as a percentage of the total fatty acid content of the lipoproteins during the oxidation process.

Part 5

3.5. Distribution of plasma hydroperoxides in major density lipoprotein classes

In order to examine location and distribution of hydroperoxides in plasma, hydroperoxides were measured using FOX2 assay in native plasma, and after fractionation into major lipoprotein classes. Hydroperoxides were found to accumulate predominantly in LDL. Plasma was also incubated in the presence or absence of AA-PH at 37 °C. The majority of hydroperoxides generated in plasma were recovered in the LDL fraction. Furthermore, when isolated lipoproteins were subject to oxidation initiated with AA-PH and copper, VLDL and LDL showed the greatest propensity for hydroperoxide accumulation, whereas HDL seemed relatively resistant (see 3.4.1.).

3.5.1. Healthy subjects

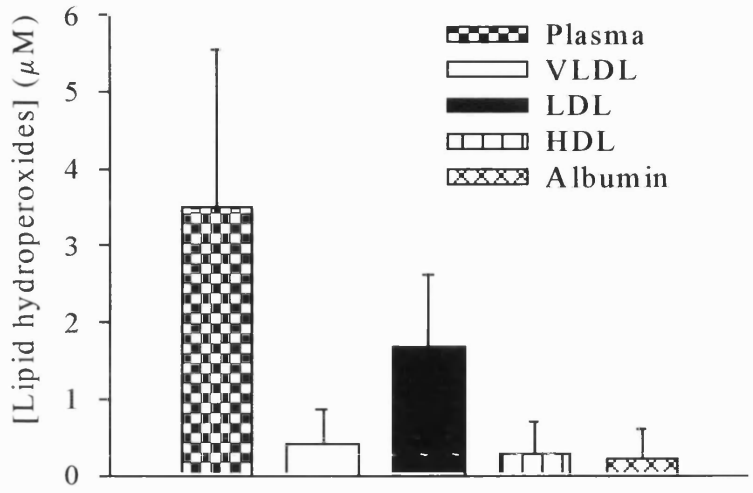
Plasma from healthy subjects (n= 4) was analysed for total hydroperoxide content. Each plasma sample was fractionated into its major lipoprotein classes (VLDL, LDL and HDL) and analysed for hydroperoxides. As shown in Figure 3.5.1. and Table 3.5.1., LDL contained more than 65% of the total hydroperoxide present in native plasma. VLDL contained 17% of plasma hydroperoxide. In contrast, HDL contained only 11% of total plasma lipid hydroperoxides. Also 8% of lipid hydroperoxides were found in the remaining fraction. This fraction contains albumin, other plasma proteins as well as "very high density lipoproteins" and lipid-poor apoprotein complexes (Beaumont et al. 1970). When hydroperoxide content in the major lipoprotein fractions was expressed on a per protein or per cholesterol basis, then VLDL, LDL and HDL were found to contain approximately 0.75, 2.5 and 0.75 mol/mg protein and 2.8, 2.0 and 1.2 nmol/mg cholesterol respectively. In repeated experiments on plasma from two of the same subjects the same distribution pattern of plasma hydroperoxide in major lipoproteins was observed.

3.5.2. Oxidised plasma

Location of hydroperoxides in plasma and the major lipoprotein classes, VLDL, LDL and HDL was analysed under basal and stimulated conditions. Whole pooled plasma from five healthy subjects (distinct from those used in Table 3.5.1.) was incubated in the presence or absence of 10 mM AA-PH at 37°C for 6 hours. The hydroperoxide content of the plasma was measured, prior to separation of the major lipoprotein classes and detection of the hydroperoxide to individual lipoprotein fractions. In plasma oxidised under basal as well as stimulated conditions the majority of hydroperoxide was found in the LDL fraction (Fig 3.5.2.). In the absence of AA-PH, but following incubation in PBS, LDL contained 32% of the hydroperoxides present in plasma. VLDL contained 8% of the plasma hydroperoxides, HDL 16%, and the remaining fraction was located in albumin and other plasma proteins. The recovery of plasma hydroperoxides from lipoproteins was 68% in basal conditions.

Incubation of plasma with AA-PH showed a similar pattern to plasma incubated only in buffer. The majority of plasma hydroperoxides were found in the LDL fraction (Fig 3.5.2.). The distribution of hydroperoxides recovered in lipoprotein fractions as a percentage of total plasma hydroperoxides was 2%, 118%, 6% and 2% for VLDL, LDL, HDL and remaining fraction respectively. The level of hydroperoxide in the LDL fraction was higher than in whole plasma (Fig 3.5.2.). This was apparently due to the oxidant effect of AA-PH. It is possible that the oxidation process continues in the lipoprotein fractions during dialysis. These results indicate that LDL is the major carrier of plasma hydroperoxides in native and peroxidised plasma.

Figure 3.5.1: Contribution of plasma lipoproteins to total plasma hydroperoxide levels



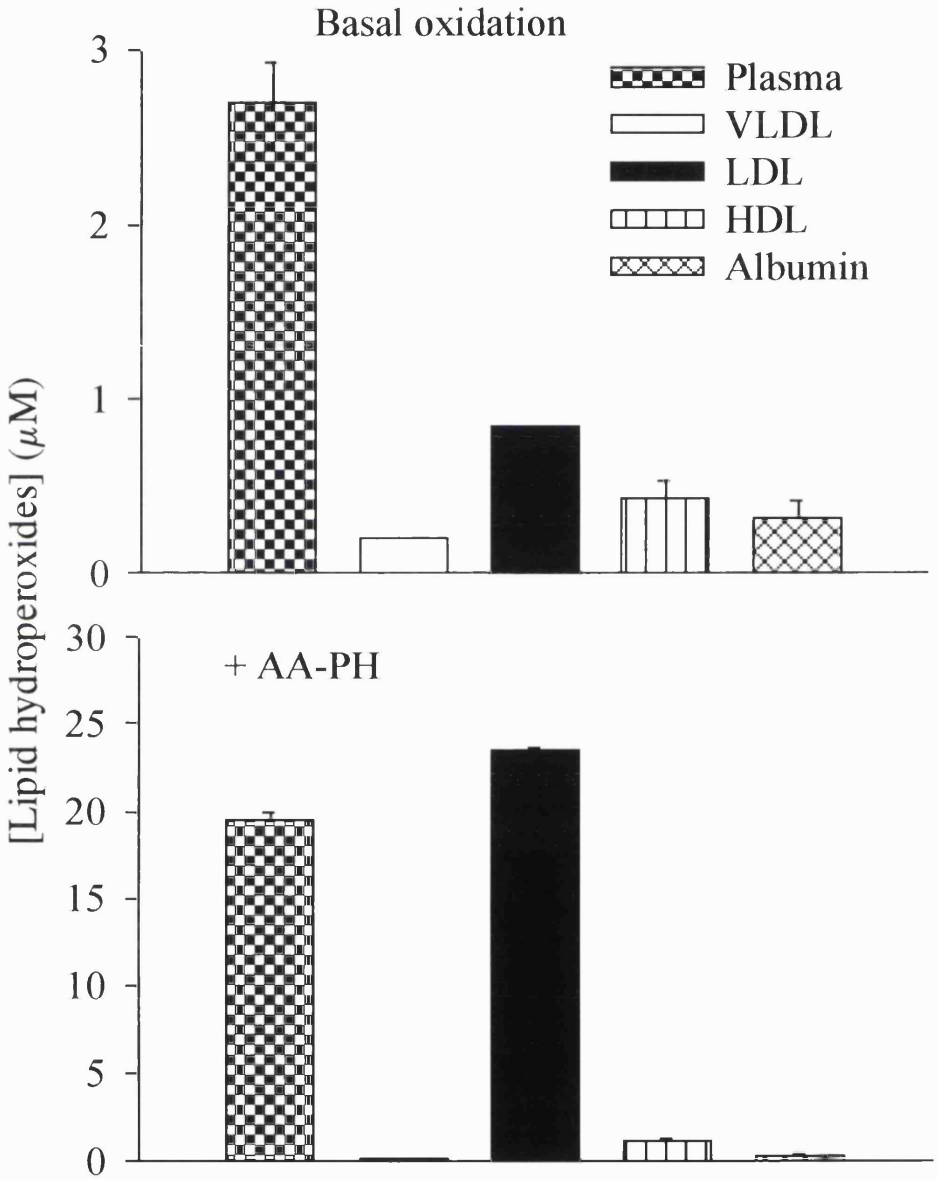
Plasma from healthy individuals (n= 4) were analysed for total hydroperoxide content. Subsequently, each plasma sample was fractionated into its major lipoprotein classes (VLDL, LDL and HDL) and hydroperoxides analysed in each fraction. Albumin refers to that fraction with $\delta > 1.21$ consisting of protein and very high density lipoprotein.

Table 3.5.1: Contribution of plasma lipoproteins to total plasma hydroperoxide level

	Hydroperoxide (μM)					
Subject	**Whole Plasma**	**VLDL**	**LDL**	**HDL**	**Protein & VHDL**	**Recovery (%)**
1	4.75	0.22	2.08	0.88	0.80	84
2	1.00	0.00	0.69	0.00	0.00	69
3	2.72	0.44	1.20	0.00	0.00	60
4	5.55	1.00	2.78	0.24	0.00	73
Summary Data	3.50 ± 2.05	0.42 ± 0.43	1.69 ± 0.93	0.28 ± 0.42	0.20 ± 0.40	72 ± 10
Location (%)	***100***	***17***	***67***	***11***	***8***	

Hydroperoxide concentrations (μM) for whole plasma and lipoprotein fractions. Percentage contributions of plasma lipoproteins to total plasma hydroperoxide level were calculated from levels of hydroperoxides present in fractions generated by ultracentrifugation. The loss of approximately 30% was presumably due to decomposition of hydroperoxides during preparation of lipoprotein fractions. Summary data means ± S.D.

Figure 3.5.2: Peroxidisability of plasma lipoproteins.



Whole pooled plasma was incubated at 37°C in the presence or absence of AAPH (10mM) for 6 hours prior to measurement of hydroperoxide content. Lipoproteins were then isolated (as described in Methods and Materials) and analysed for hydroperoxide content. Data represent means ± SD of triplicate analyses.

Part 6

3.6. Indices of lipid peroxidation in health and disease

It has been proposed diabetes mellitus is associated with increased lipid peroxidation, which may contribute to vascular and other complications of the syndrome (Baynes, 1991; Wolff, 1993). To test this hypothesis, measurements of lipid peroxidation and other biochemical parameters were analysed in a group of 60 healthy subjects and 110 well characterised individuals with non-insulin-dependent diabetes mellitus (NIDDM). Furthermore the relation between plasma hydroperoxides and other risk factors in diabetes, and the presence or absence of complications of diabetes, were investigated.

3.6.1. Clinical characteristics

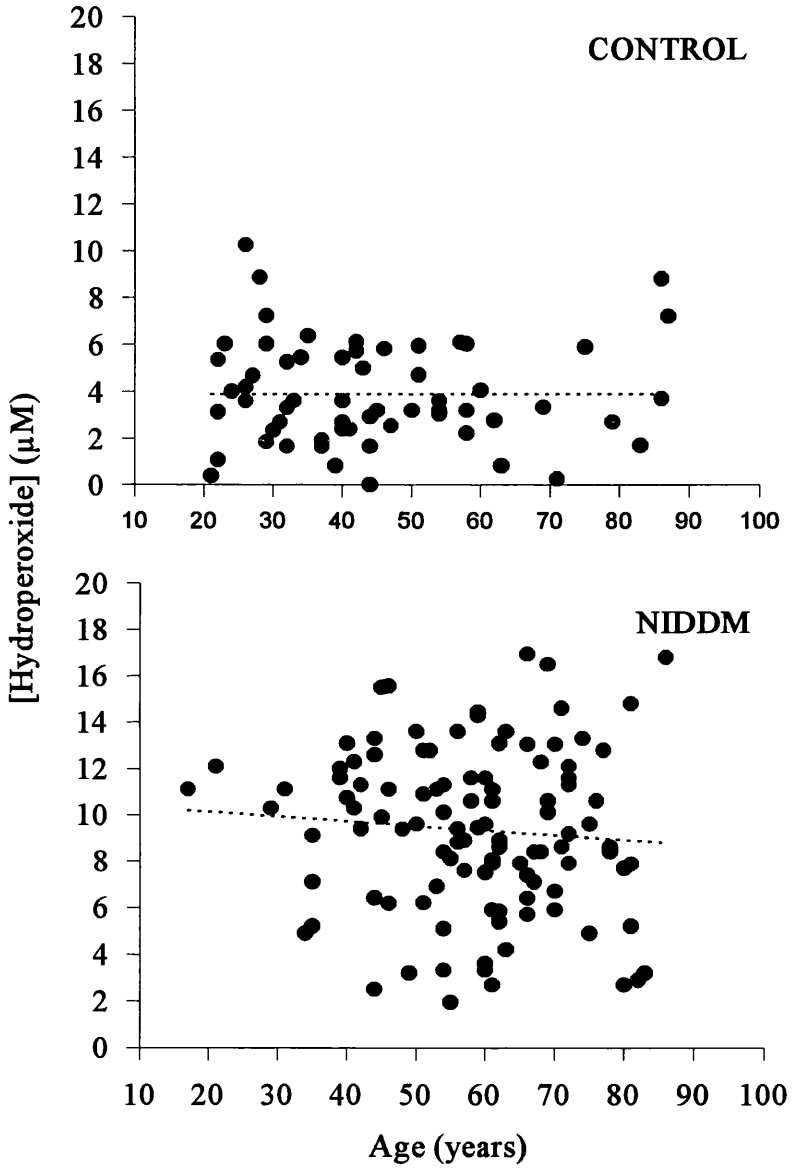
Table 3.6.1 shows some clinical characteristics for patients and control subjects as well as the differences in levels of plasma lipid hydroperoxides (LOOHs), thiobarbituric acid reactive materials (TBARMs) and vitamin E (α-tocopherol). The expected differences in fasting glucose, HbA1, triglyceride and cholesterol levels agree with those of other studies (Pickup and Williams, 1991). The mean age of the diabetic patients was significantly higher than that of the control subjects (58.3 ± 14 v 44.8 ± 18; $P < 0.0005$) respectively. However, this was the best available control group (see 2.3.1.). There was no difference in the level of vitamin E and TBARMs between two groups. Hydroperoxides did not correlate with age (Fig 3.6.1.) in either the control ($r = -0.002$; $p = 0.98$) or the patient groups ($r = -0.08$; $p = 0.39$). In fact, there was a slight negative correlation, with a very shallow slope between age and hydroperoxide levels in the patient group (Fig 3.6.1.).

Table 3.6.1: Clinical characteristics of control subjects and NIDDM patients

Variables	Control	NIDDM	P
Age (yrs)	44.8 ± 18	58.3 ± 14	<0.0005
Sex (F/M)	34/26	61/59	-
Diabetes duration (yrs)	-	11.8 ± 8.4	-
Total cholesterol (mM)	5.28 ± 0.9	6.08 ± 1.3	<0.0005
Triglycerides (mM)	1.09 ± 0.6	2.8 ± 1.7	<0.0005
Fasting Glucose (mM)	4.9 ± 0.4	11.4 ± 5	<0.0005
HbA1 (%)	(5-8)	10.8 ± 2.3	-
Hydroperoxides (μM)	3.9 ± 2.2	9.4 ± 3.5	<0.0005
TBARM (μM)	1.4 ± 0.8	1.5 ± 0.7	NS
Vitamin E (μM)	20.6 ± 8.6	19.8 ± 7.4	NS

* NS= not significant. Numbers in parentheses are normal range. Measurement of clinical chemistry data carried out at University College London Hospitals' Diabetic Service.

Figure 3.6.1: Correlation between age and hydroperoxide levels in plasma



Lack of any positive association between age and hydroperoxide levels in control ($r = -0.002$; $p = 0.98$) or patient groups ($r = -0.08$; $p = 0.39$).

3.6.2. Hydroperoxides

Hydroperoxide levels in plasma were measured using FOX2 assay. For 60 control subjects the hydroperoxide concentration varied from 0 (ie below level of detection, < 0.1 μM) to 10.27 μM with the mean level of 3.89 ± 2.2 μM (mean ± SD). The hydroperoxide values for 110 non-insulin-dependent diabetes mellitus patients were significantly higher than those of control subjects and were found to range from 1.94 to 16.94 μM with the mean level of 9.4 ± 3.5 μM (Fig 3.6.2.). Hydroperoxide levels for male and female subjects in the control group were 3.5 ± 1.6 and 4.1 ± 2.2 μM respectively. The corresponding hydroperoxide levels for male and female NIDDM patients were 9.1 ± 3.4 and 9.7 ± 3.6 μM respectively. The mean level of hydroperoxide in the control group in this study was not different (separate-variance *t* test, P > 0.05) from the level (3.02 ± 1.85 μM; n=23) determined in the previous study with a different set of control subjects (see 3.2.6.). There was no correlation between hydroperoxides and total triglycerides in the patient group (r =0.13; p =0.21), while in the control group some correlation was observed (r = 0.29; p = 0.028). Hydroperoxide levels did not correlate with total cholesterol levels in controls (r = 0.03; p = 0.82) or patients (r = -0.02; p = 0.83). There was no correlation between hydroperoxide and glucose levels in the control group (r = 0.31; p = 0.059), while a positive association between hydroperoxide and glucose levels was found in the patient group (r = 0.2; p = 0.037).

3.6.3. Thiobarbituric acid reactive materials (TBARMs)

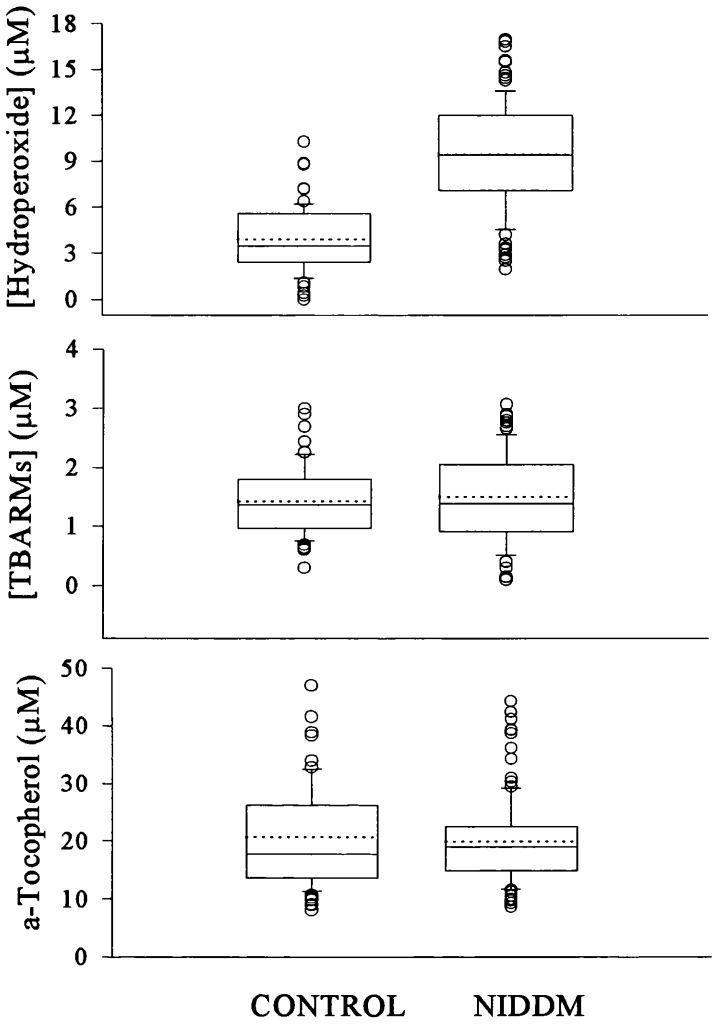
Thiobarbituric acid reactive materials were determined in both control and patient groups with the TBA-assay. As has been indicated in figure 3.6.2. there was no difference in TBARMs level between the two groups. The concentration of TBARMs in the control subjects varied from 0.3 to 3 μM with the mean level of 1.42 ± 0.6 μM. This value varied from 0.1 to 3.07 μM with the mean level of 1.49 ± 0.72 μM in the patient group. As expected, MDA level (and that of other aldehydes which react to form chromophores with TBA) was significantly lower than hydroperoxide levels in both the control and patient groups. Figure 3.6.3. shows lack of correlation between TBARMs

and L-OOH levels in control (r = -0.12; p = 0.36) and patient groups (r = 0.029; p = 0.76), which is in agreement with previous data (see 3.2.6.).

3.6.4. Vitamin E status (α-tocopherol)

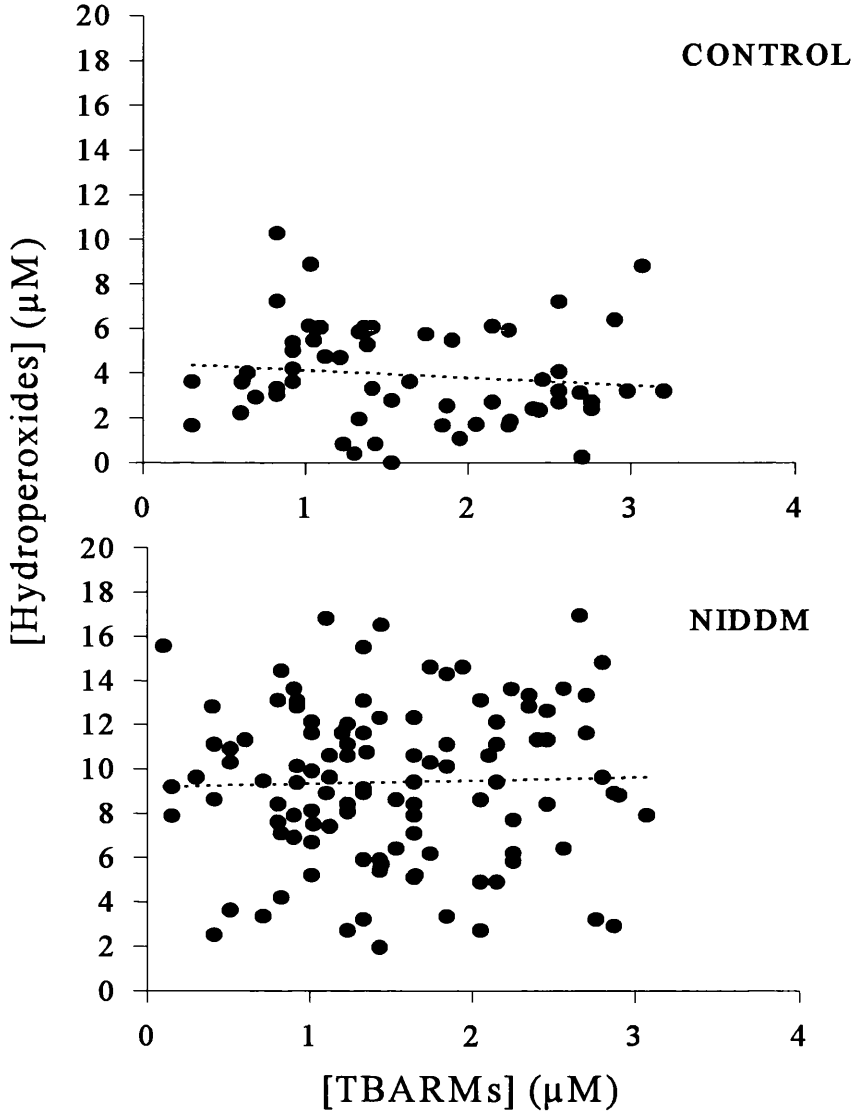
Vitamin E status as an α-tocopherol concentration was analysed with HPLC using fluorometric detector. There was, however, no significant difference in vitamin E concentration between control and patient groups (Fig 3.6.2.). The concentration of vitamin E for control subjects varied from 8 to 47 μM with the mean level of 20.6 ± 8.9 μM; that for patients varied from 8.63 to 44.27 μM with the mean level of 19.8 ± 7.5 μM. Vitamin E levels did not correlate with hydroperoxide levels (Fig 3.6.4.) in control (r = 0.074; p = 0.57) or patient groups (r = 0.031; p = 0.74). There was no correlation between triglyceride and vitamin E in control (r = 0.16; p = 0.23) or patient groups (r = 0.16; p = 0.14). Vitamin E did not correlate with total cholesterol in the control group (r = -0.043; p = 0.75), while the correlation in patient groups was significant (r = 0.312; p = 0.002).

Figure 3.6.2: Hydroperoxide, TBARMs and vitamin E in control subjects and NIDDM patients



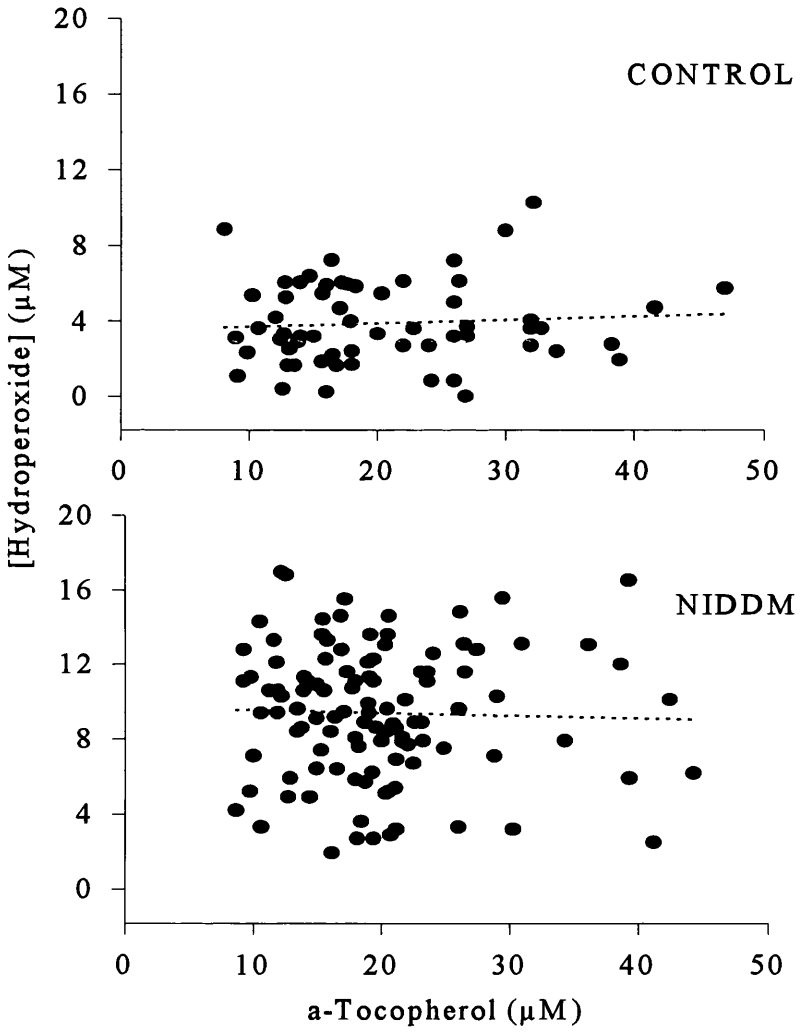
Levels of hydroperoxide are elevated of the in NIDDM group ($p < 0.0005$). There is no significant difference in TBARMs and vitamin E levels in either group ($P > 0.05$). Boxes indicate the 25th and 75th percentile of column. A line inside the box marks the value of the 50th percentile. Dotted lines represent the mean value for the whole column. Bars indicate the 10th and 90th percentiles, and symbols mark all data outside the 10th and 90th percentiles. Data represent the means of triplicate analyses for TBARMs and hydroperoxide. The data points given for vitamin E are based on single measurements.

Figure 3.6.3: Correlation between hydroperoxide and TBARMs levels in control subjects and NIDDM patients



Lack of correlation between TBARMs and L-OOH levels in control ($r = -0.12$; $p = 0.36$) and patient groups ($r = 0.029$; $p = 0.76$). Data represent the means of triplicate analyses for each point.

Figure 3.6.4: Correlation between hydroperoxide and vitamin E levels in controls and patients



Lack of correlation between vitamin E and hydroperoxide levels in both control ($r = 0.074$; $p = 0.57$) and patient groups ($r = 0.031$; $p = 0.74$). Data represent the mean of triplicate analyses for hydroperoxide. The data points for vitamin E are based on single measurements.

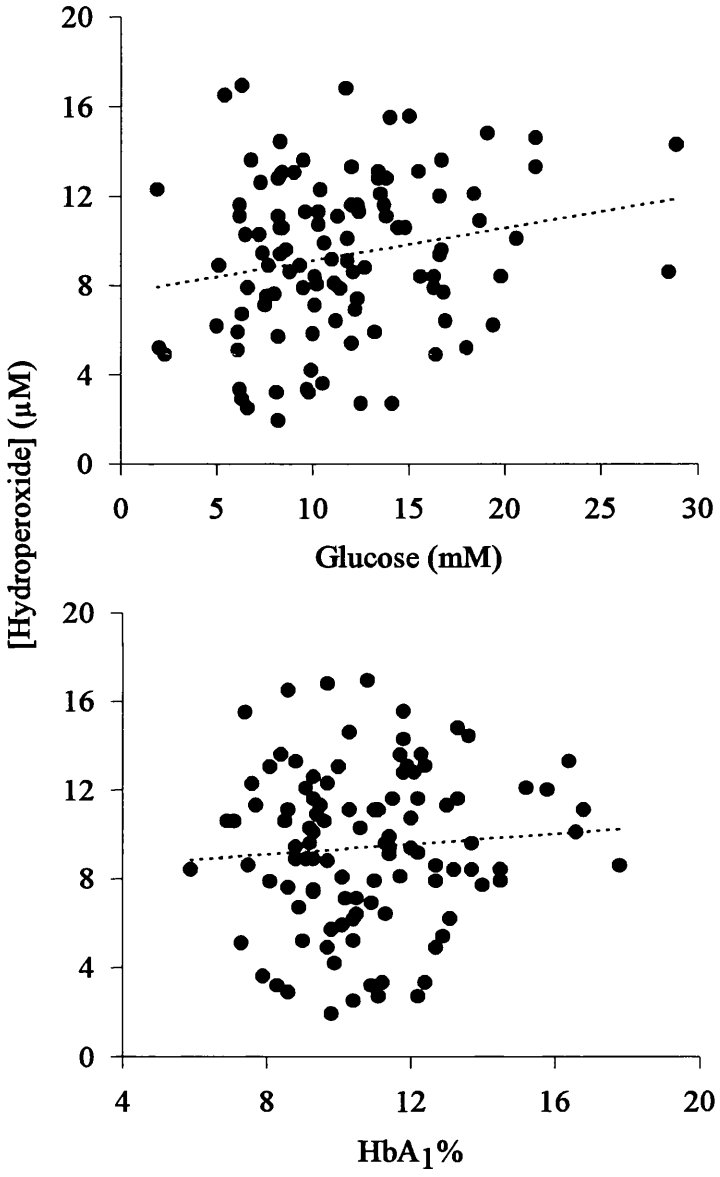
3.6.5. Relation between lipid hydroperoxides and other clinical characteristics in diabetes

Since diabetes has been associated with abnormalities in lipid metabolism and glucose homeostasis, the relationships between plasma hydroperoxides and these parameters were studied. Clinical characteristics of NIDDM patients are shown in Table 3.6.5.1. Hydroperoxides did not correlate with age ($r = -0.08$; $p = 0.39$), total cholesterol ($r = -0.02$; $p = 0.83$), triglyceride ($r = 0.13$; $p = 0.21$) or vitamin E ($r = 0.031$; $p = 0.74$). This suggests that hydroperoxide levels are not a simple function of total lipid levels. Hydroperoxides correlated with fasting glucose levels, but not with HbA_1 (Fig 3.6.5.1.). This indicates that hyperglycaemia may itself be a cause of the increased hydroperoxide levels (Wolff, 1993). There was also no correlation between vitamin E and triglyceride levels, although correlation between vitamin E and cholesterol was strongly positive (Fig 3.6.5.2.). There was significant negative correlation between HDL cholesterol and triglyceride ($r = -0.40$; $p = 0.0008$), while HDL did not correlate with cholesterol ($r = -0.11$; $p = 0.35$).

Table 3.6.5.1: Clinical characteristics of NIDDM patients

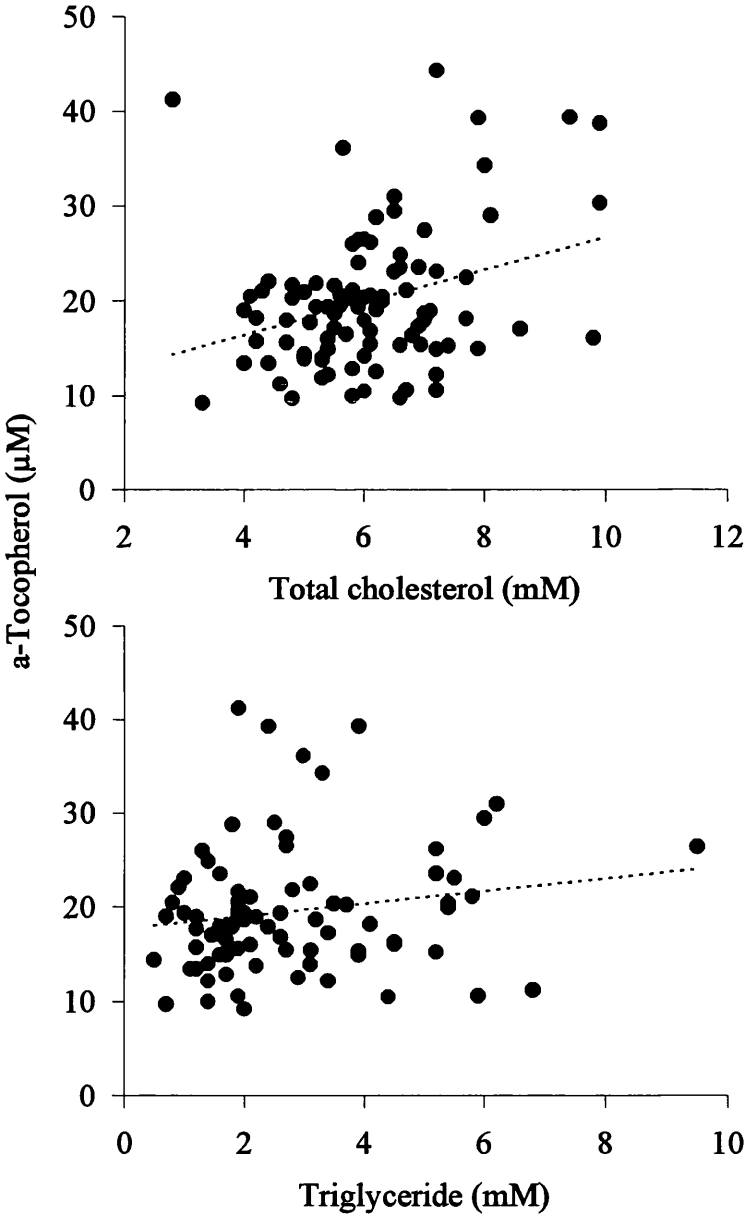
Variables	Number	Range	Mean ± SD
Sex (F/M)	110	61/59	-
Age (years)	110	17.0 - 86.0	58.3 ± 14.0
Duration (yrs)	110	0.0 - 44.0	12.0 ± 8.3
Total cholesterol(mM)	94	2.8 - 9.9	6.1 ± 1.3
Triglycerides (mM)	82	0.5 - 9.5	2.8 ± 1.7
HDL cholesterol(mM)	79	0.5 - 3.5	1.3 ± 0.6
Glucose (mM)	106	1.9 - 28.9	11.4 ± 5.0
HbA_1 (%)	106	5.9 - 17.8	10.8 ± 2.3
Hydroperoxides (μM)	110	2.7 - 16.8	9.5 ± 3.3
TBARMs (μM)	110	0.1 - 3.0	1.49 ± 0.7
Vitamin E (μM)	110	8.6 - 44.3	19.8 ± 7.5

Figure 3.6.5.1: Scatter plot with trend of fasting plasma glucose, HbA_1 and plasma hydroperoxide levels in NIDDM patients



Positive correlation between hydroperoxide and fasting glucose levels ($r = 0.2$; $p = 0.037$). Lack of correlation between hydroperoxide and HbA_1 ($r = 0.075$; $p = 0.44$).

Figure 3.6.5.2: Scatter plot with trend of cholesterol, triglyceride and vitamin E levels in NIDDM patients



Lack of correlation between vitamin E and triglyceride ($r = 0.16$; $p = 0.14$), while correlation between vitamin E and cholesterol is significant ($r = 0.312$; $p = 0.002$).

3.6.6. Comparison of hydroperoxides in NIDDM patients with or without complications

The main complications of diabetes were defined as retinopathy, neuropathy and/or nephropathy and/or macroangiopathy. From the clinical results the patients were divided into two groups, those with and those without complications of NIDDM. Some clinical characteristics of the two groups of NIDDM patients are shown in Table 3.6.6.1.

As shown in figure 3.6.6.1., there was no difference in hydroperoxide level between those with (9.4 ± 3.5 μM) and without complications (9.2 ± 3.6 μM). Total cholesterol was significantly higher in the presence of complications (Fig 3.6.6.1.). The mean age of patients with complications was significantly higher than in those without (62 ± 14 Vs 54 ± 13; $P< 0.0005$) respectively. Smoking status was also different in the two groups. However, there was no difference in other measures of lipid peroxidation or biochemical markers in the two groups (Table 3.6.6.1.).

The data from the groups with and without complications was analysed to take account of smoking status. There was no significant difference in clinical data and other biomarkers of lipid peroxidation between smokers and non-smokers in the absence of complications (Table 3.6.6.2). The levels of total cholesterol, HDL cholesterol and TBARMs were approximately similar in both groups in the presence of complications (Table 3.6.6.2). However, triglyceride levels of in smokers were higher than those of non-smokers (3.4 ± 2.4 Vs 2.5 ± 1.3) respectively. Vitamin E levels in smokers were lower than those non-smokers (18.5 ± 6.7 Vs 21.6 ± 9.4). The levels of both glucose and HbA_1 in smokers were higher than those of non-smokers (12.1 ± 6.1 Vs 11 ± 4.4 and 11.7 ± 2.4 Vs 10.1 ± 2 respectively). Hydroperoxide levels remained unchanged in both smokers and non-smokers in the presence (9.6 ± 3.4 Vs 9.3 ± 3.5) and absence (9.3 ± 3.2 Vs 9.1 ± 3.6) of complications (Table 3.6.6.2).

Table 3.6.6.1: Clinical characteristics of NIDDM patients with and without complications

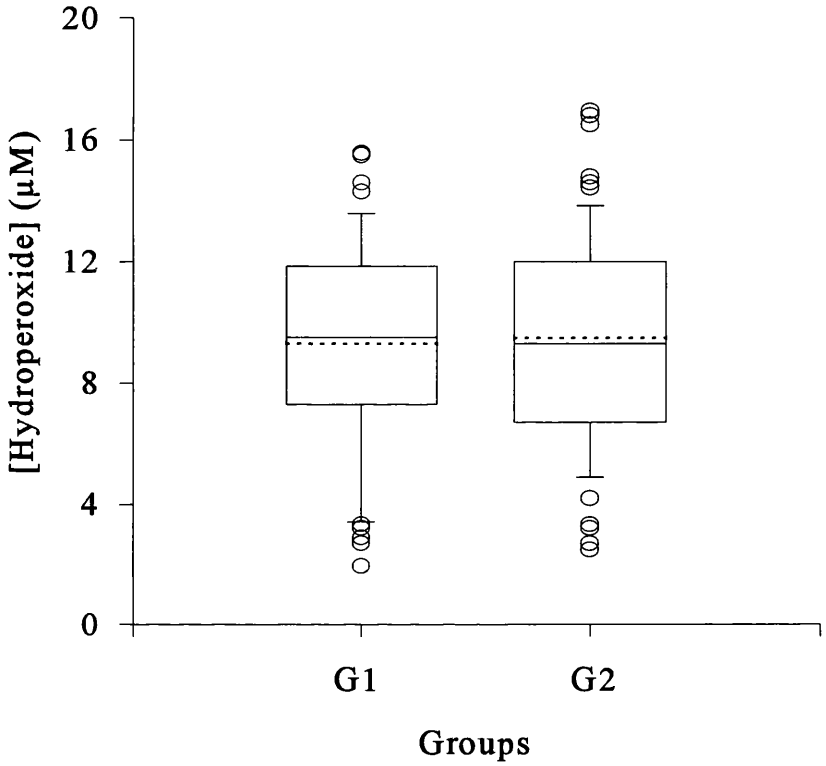
Variables	No complications	Complications	P
Number	48	62	-
Smoking status	11	20	
Age (yrs)	54 ±13	62 ±14	(p = 0.001)
Total Cholesterol (mM)	5.6 ±1.2	6.3 ±1.3	(p = 0.014)
HDL cholesterol (mM)	1.4 ±0.6	1.3 ±0.6	NS
Triglycerides (mM)	2.6 ±1.7	2.9 ±1.7	NS
Glucose (mM)	11.2 ± 5.2	11.5 ± 5	NS
HbA_1 (%)	10.8 ± 2.4	10.8 ± 2.2	NS
Hydroperoxides (μM)	9.2 ±3.6	9.4 ±3.5	NS
TBARMs (μM)	1.5 ±0.7	1.47 ± 0.7	NS
Vitamin E (μM)	19.6 ± 5.5	20.2 ± 8.7	NS

NS= not significant

Table 3.6.6.2: Clinical characteristics of NIDDM patients with and without complications in the presence or absence of smoking status

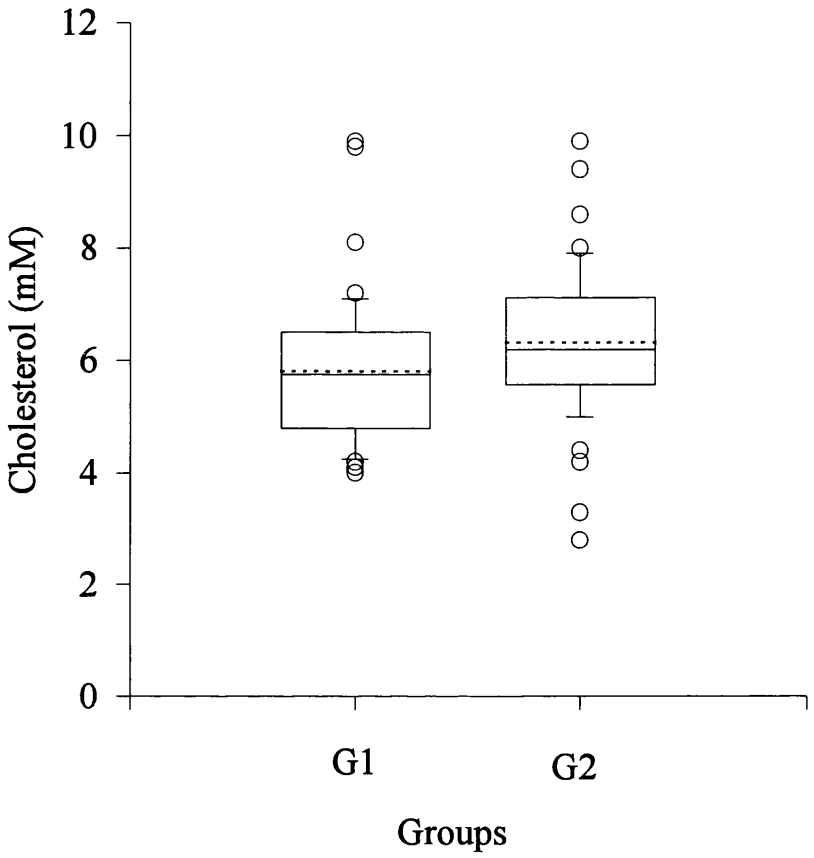
Variables	No complications		Complications	
	Non-smoker	**Smoker**	**Non-smoker**	**Smoker**
Number	**37**	**11**	**42**	**20**
Age (yrs)	**52 ±13**	**56 ±16**	**60 ±12**	**64 ±14**
Total Cholesterol (mM)	**5.6 ±1.2**	**5.7 ±1.7**	**6.2 ± 1.4**	**6.5 ±1.2**
HDL cholesterol (mM)	**1.6 ±0.6**	**1.1 ±0.6**	**1.3 ±0.4**	**1.3 ±0.9**
Triglycerides (mM)	**2.5 ±1.6**	**2.7 ±1.8**	**2.5 ±1.3**	**3.4 ±2.4**
Glucose (mM)	**11.1 ± 5.4**	**11.3 ± 4.7**	**11 ± 4.4**	**12.1 ± 6.1**
HbA_1 (%)	**10.7 ± 2.4**	**11 ± 2.4**	**10.1 ± 2**	**11.7 ± 2.4**
Hydroperoxides (μM)	**9.1 ± 3.6**	**9.3 ±3.2**	**9.3 ± 3.5**	**9.6 ± 3.4**
TBARMs (μM)	**1.5 ±0.8**	**1.5 ±0.6**	**1.3 ± 0.7**	**1.7 ±0.7**
Vitamin E (μM)	**19.8 ± 5.6**	**19.3 ± 6.1**	**21.6 ± 9.4**	**18.5 ± 6.7**

Figure 3.6.6.1: Plasma hydroperoxide levels in individuals with and without complications in NIDDM



Hydroperoxide levels in NIDDM patients without complications (G1) and with complications (G2). Boxes indicate the 25th and 75th percentile of value. A line inside the box marks the value of the 50th percentile. Dotted lines represent the mean value for whole column. Bars indicate the 10th and 90th percentiles, and symbols mark all data outside the 10th and 90th percentiles.

Figure 3.6.6.2: Total plasma cholesterol distribution in groups with and without complications



Total cholesterol concentration in NIDDM patients without complications (G1) and with complications (G2). Boxes indicate the 25th and 75th percentile of value. A line inside the box marks the value of the 50th percentile. Dotted lines represent the mean value for whole column. Bars indicate the 10th and 90th percentiles, and symbols mark all data outside the 10th and 90th percentiles.

Chapter IV Discussion

It has been proposed that peroxidation of lipids plays an important role in the aetiology of degenerative diseases such as atherosclerosis (Witztum, 1994; Bruckdorfer, 1990), diabetes (Baynes, 1991; Wolff, 1993) and ageing (Pal Yu, 1993). The major initial reaction products of lipid peroxidation are hydroperoxides, and these are known to affect the prostaglandin cascade and vascular endothelium. Furthermore lipid hydroperoxides decompose enzymatically and non-enzymatically to give secondary products such as hydroxy-alkanals, MDA and other compounds which are very reactive and cytotoxic.

The assay of hydroperoxides offers a direct measurement of a lipid peroxidation product and seems more reliable than measuring the secondary breakdown products or metabolites to prove that there is occurrence of lipid peroxidation in biological samples (Miyazawa, 1989). However, it must be emphasised that analysis of lipid peroxidation products in plasma is far more complex than in simple systems such as pure fatty acids. In particular, the pharmacokinetics of removal of the lipid hydroperoxides will be just as important as the rate of formation in determining plasma steady state levels. Both the rate of formation, and the factors that affect removal in vivo remain essentially unknown. As a result it is not known whether elevated levels of plasma lipid hydroperoxide are due to increased formation or decreased clearance. It is not known if some diabetics have grossly elevated rates of lipid hydroperoxide production, compensated by increased clearance resulting in normal plasma levels. The varied degree of unsaturation of PUFAs will generate a mixture of lipid hydroperoxides. Decomposition of each of them will produce a variety of aldehydes and other products. Moreover the presence and absence of transition metal ions, antioxidants, source of diet and pathophysiological condition of subjects are major factors which may influence the formation of the peroxidation products and their measurement (Cheeseman, 1993).

4.1. Detection of hydroperoxides in biological systems

Determination of hydroperoxides in biological samples such as plasma have been reported by several research groups (see 1.7.8.1 & 1.7.8.2.). The techniques can be classified into two main groups. Those measuring total hydroperoxides normally using spectrophotometric and fluorimetric methods, or individually different classes of lipid in plasma using HPLC coupled with UV or fluorimetric detection.

Individual classes

It is generally agreed that free fatty hydroperoxides are present in the lower nM range in plasma due to rapid degradation by hydroperoxide metabolising enzymes such as glutathione peroxidase (Terao et al. 1988). Furthermore free fatty acids (FFAs) bound to albumin in plasma are protected from oxidation possibly due to albumin bound bilirubin (Frei et al. 1988). On the other hand, opinions are divided regarding plasma cholesterol-ester and phospholipid hydroperoxides. It has been stated that plasma from healthy donors has approximately 3nM of cholesterol ester hydroperoxides (Yamamoto et al. 1987), whereas values ranging from 0.0 to 0.92 μM have been reported by Coghlan et al (1991). The phospholipid hydroperoxides concentration in plasma of healthy donors (n=43) has been reported as ranging from 0.01-0.5 μM whereas the values for "unhealthy" subjects (n=144) were between 0.5-9 μM (Miyazawa, 1989) . However, (Frei et al. 1988) reported that phospholipid hydroperoxide content in plasma was not greater than 0.03 μM. In contrast, (Holey and Slater, 1991) could not detect phospholipid hydroperoxides in plasma from healthy volunteers using a similar HPLC/CL system to that of Miyazawa. Such discrepancies in levels of plasma hydroperoxide found by different laboratories have not been adequately explained. Some of the disagreement may arise from inadequate preparation of standards, differential reactivity of various classes of hydroperoxide in the chemiluminescence cocktails or gross artefact (Zhang et al. 1995); variable recoveries of lipid hydroperoxides using the many lipid extraction techniques available prior to HPLC-chemiluminescence analysis may be a source of error (Miyazawa, 1989); while oxidative side-reactions and presence of unrecognised quenchers of chemiluminescence may

contribute to the under estimation of hydroperoxides using HPLC-chemiluminescence technique (Zamburlini et al. 1995). The focus on CE and PL classes may also lead to underestimation since the plasma TG fraction, which contains a large proportion of peroxidisable fatty acid is not separately scrutinised for hydroperoxide content but may possibly contain hydroperoxides which are poorly reactive in the chemiluminescence assays. Nevertheless, because of their great sensitivity and specificity HPLC techniques may provide more information about the individual hydroperoxides when this is required.

Total hydroperoxides

Techniques that measure total hydroperoxides, rather than individual fractions using HPLC-chemiluminescence, suggest higher and consistent values for lipid hydroperoxides. Thus, total hydroperoxides in pooled plasma samples have been estimated to be 4.0 ± 1.7 μM by a complex but exact iodometric technique (Cramer et al. 1991) and 2.1-4.6 μM (n = 5; mean = 3.1μM) by an automated iodometric technique (Thomas et al. 1989). The iodometric assays require simple apparatus, but often need strict anoxia to perform well and are severely limited by high and mobile baselines. Need for extraction of lipids is another limitation of iodometric methods in assessment of hydroperoxides in plasma. Using a cyclooxygenase activation assay Marshall et al. (1985) suggested that plasma hydroperoxides in healthy individuals were approximately 500 nM. However, it is not clear which classes of hydroperoxide activate cyclooxygenase and the same authors recorded higher levels using their exact iodometric technique.

4.1.1. Measurement of hydroperoxides in plasma using FOX2

Interfering factors

In response to need for a simple and sensitive test for measurement of total hydroperoxides in plasma a new method was developed based on the oxidation of ferrous to ferric ions in the presence of hydroperoxides under acidic conditions. Ferric ions generated by the hydroperoxides in the assay are complexed by the ferric ion indicator, xylenol orange, generating a blue-purple complex with an absorption maximum

of 550-600 nm (FOX2; see 2.4.1.). The method had previously been used in our laboratory for measurement hydroperoxide in low density lipoprotein (Jiang et al. 1992). However application of the method for direct measurement of hydroperoxides in whole plasma was problematic. In order to implement the FOX2 assay for direct measurement of hydroperoxides in plasma, the effect of possible interfering factors were studied.

Since plasma contains various iron proteins, it was possible that ferric ions released from the plasma protein "transferrin" in the acidic environment of the FOX2 reagent would react directly with xylenol orange. Ferric ions are bound tightly to transferrin at physiological pH, however ferric ions are released from this protein in acidic conditions (Aisen and Liskowsky, 1980). This was confirmed by using commercial transferrin in the FOX2 assay (see 3.1.1.). Comparison of hydroperoxide values for plasma and for extractable plasma lipids using ethyl acetate, showed that only a small proportion of the FOX2-generated signal was due to the authentic hydroperoxides. Hence, a high proportion of the signal generated by direct addition of the plasma to the FOX2 assay was attributable to ferric ions released from plasma.

The presence of ascorbate in plasma was the another factor which might alter the FOX2-generated signal during direct measurement of hydroperoxides in plasma. Vitamin C has been known as an important antioxidant both within cells and in the plasma (Stocker et al. 1991). However, in the presence of transition metal ions and oxygen it is oxidised and produces H_2O_2 and hydroxyl radicals. Since the FOX2 reagent contains ferrous ions, it was thought that part of the FOX2 generated signal could be due to production of H_2O_2 as a result of oxidation of ascorbate by oxygen and the ferrous ions of FOX2 reagent. However, I have shown that there was no interference between ascorbate in the concentration range of 10-100 μM (normal range in plasma) and hydroperoxide recovery in plasma (see Fig 3.1.3.2.).

Reduction of hydroperoxides in the FOX2-assay

When I confirmed that a proportion of FOX2-generated signal was due to ferric ions, it became necessary to distinguish between the signal from hydroperoxides and that from ferric ions. For this, plasma samples were treated with various hydroperoxide reducing agents.

TPP, a specific reductant for hydroperoxides was used to confirm presence of hydroperoxides in plasma. TPP reduces hydroperoxides to the corresponding alcohols while becoming oxidised to triphenylphosphine oxide. TPP was found to be effective in reducing a variety of authentic hydroperoxides, in aqueous and non-aqueous environments (see Fig 3.2.1.), but had no effect on H_2O_2. TPP can thus be used in aqueous and non-aqueous environments to discriminate between H_2O_2 and non-H_2O_2 hydroperoxides. In addition, TPP does not alter the interaction between the FOX2 reagent and ferric ions in the plasma, nor does it interact with the FOX2 reagent itself.

Since TPP had no effect on the H_2O_2 reaction with FOX2, it was possible that some of the remaining signal in plasma after pre-treatment with TPP besides ferric ions would be due to H_2O_2. Hence, plasma samples were treated with glutathione peroxidase. This enzyme in the presence of reduced glutathione (GSH) reduces H_2O_2 but also reduces lipid hydroperoxides. However, when FA-OOH, oxidised LDL, and plasma were pre-treated with G/PX and TPP, hydroperoxides in oxidised linoleic acid were significantly reduced by both TPP and G/PX, while in oxidised LDL and plasma samples G/PX was less effective than TPP in destroying hydroperoxides (see 3.2.2.). It has been shown that plasma G/PX can reduce PC-OOH but not CE-OOH (Yamamoto et al. 1993). Since LDL and plasma contains various free and esterified peroxides, it is possible that G/PX is not as effective as TPP in reducing different classes of peroxides.

When it was found that TPP was effective in reducing a variety of authentic hydroperoxides, samples of plasma were pre-treated with TPP together with catalase to detect the presence of any H_2O_2 in plasma (Catalase selectively breaks down H_2O_2 to H_2O and O_2, while TPP has no effect on H_2O_2). It was found that pre-exposure of plasma samples to catalase had no effect on FOX2 generated signal. The result indicated that hydroperoxide in plasma samples was not H_2O_2, and that any H_2O_2 present was below the detection limit of the spectrophotometric measurement.

Hydroperoxides in plasma

In plasma samples from 23 individuals, using the strategy of pre-treatment with and without TPP, it was found that hydroperoxide levels ranged from 0.22 to 7.8 μM with a mean of 3.02 μM and a population standard deviation of 1.85 μM. The corresponding

hydroperoxide levels after partitioning with ethyl acetate varied from 0.22 to 6.22 μM with a mean of 2.52 μM and a population standard deviation of 1.85 μM. Inter assay coefficient of variation was <5%. These results, were in reasonable agreement with earlier studies (Cramer et al. 1991; Thomas et al. 1989; Marshall et al. 1985). Thus, the FOX2 reagent can be used to determine hydroperoxide levels in whole plasma by a strategy of measuring signal development in the FOX2-assay in the presence and absence of TPP pre-treatment of the sample. This method can be corroborated by extraction of lipid with ethyl acetate prior to addition of FOX2 reagent.

4.2. Hydroperoxides and storage conditions

Because of the instability of the hydroperoxides, it is advisable to assay plasma samples immediately after collection. However, in the clinical situation the immediate analysis of samples is often not possible and storage of samples is sometimes inevitable. A tendency to increased lipid peroxidation has been reported during long-term storage of samples (Gutteridge et al. 1985). They reported increased TBARMs measured fluorimetrically and spectrophotometrically along with increased levels of free copper using a phenanthroline copper assay, in serum samples which had been kept at 4 °C. This increase was slower during storage at -20 °C or -70 °C. Using a fluorimetric assay increased apparent TBARMs in plasma samples during storage at 4 °C and -20 °C were found, and this was prevented by the use of EDTA or reduced glutathione (Hendriks and Assmann, 1988). Measuring MDA in EDTA treated plasma samples kept at different temperature Young and Trimble (1991) reported the accumulation of MDA at 20 °C, while samples were stable at 4 °C up to 10 days and for 3 weeks at -20 °C. They also reported that the most suitable condition for long term storage is -70 °C. Loss of exogenously added 15(s)-HPETE in plasma samples during storage at different temperature has also been reported (Holley and Slater, 1991). They showed rapid loss of hydroperoxides in plasma samples which had been kept at 0 °C or 27 °C, while samples were stable at -70 °C up to 2 weeks and 3 months in liquid nitrogen in the presence of BHT and Desferal.

We have found that, using the FOX2 assay hydroperoxide levels in healthy subjects (n = 25) were stable for up to 24 hours at 4 °C. However, hydroperoxide levels rose

after 24 hours. When the same samples were stored at 37 °C, accumulation of hydroperoxides was accelerated, and the rise in hydroperoxide levels could be detected before 24 hours. After 3 days this increase had gone up to nearly 10 fold of the original levels after 3 days (see 3.3.1.1).

Storage of plasma samples over a long term led to large variations in hydroperoxide levels when compared with those found in the fresh samples. There was approximately 40% loss of hydroperoxide in plasma samples of control group (n = 39) with most samples losing around 20-30% (see 3.3.1.2) after 3 months storage at - 60 °C .

Plasma samples of NIDDM patients (n = 169) showed considerable variation after 3 months storage at - 60 °C. While, there was approximately 47% loss of hydroperoxide in most of the samples (n = 126), increased hydroperoxide levels were seen in some of the samples (n = 43) with the mean level of 31% (see 3.3.1.3). Loss of hydroperoxide correlated significantly with the amount of hydroperoxides in fresh samples in both control and NIDDM patients (see 3.3.1). There was, however no correlation between loss or accumulation of hydroperoxides with lipids or vitamin E content of the samples in control or in NIDDM groups (see 3.3.1). Clearly there is simultaneous breakdown of hydroperoxides and generation of fresh hydroperoxides in stored samples.

Addition of the BHT (25 μM) decreased the rate of hydroperoxides generation in plasma samples (n = 25) stored at 37 °C for 24 hours, but only by 15-20% (see 3.3.3.). However, BHT at concentration of 25 and 50 μM had no effect on hydroperoxide levels in plasma samples from familial hypercholesterolemic patients (n = 52), which had been kept at 4 °C and -60 °C for 24 hours and 3 months respectively (see 3.3.3.).

The reason for this discrepancy in the stability of hydroperoxides is unclear. The availability of the metal ions, antioxidants other than vitamin E, diet and drug medication may have a role in the stability of hydroperoxides during storage. The different techniques and sample handling procedures could possibly be the source of this discrepancy. Furthermore, because of the complexity of plasma, effect of different unknown factors on the stability of hydroperoxides could not be excluded. These factors could alter accumulation or decomposition of hydroperoxides. The levels which have been measured by different techniques during storage in various conditions, indicate that it is advisable that measurement of hydroperoxides be performed on fresh samples and that stored samples be used only for confirmatory studies.

4.3. Plasma lipoproteins and susceptibility to in vitro peroxidation

The oxidation of plasma lipoproteins is a complex process during which the chemical and functional properties of both lipid and protein moieties are altered (Esterbauer et al. 1990). The commencement and progression of lipid peroxidation can be determined by measuring lipid hydroperoxides, conjugated dienes, TBARMs and fluorescent proteins or lipids. Other possibilities include measurement of consumption of PUFAs, antioxidants and also fragmentation of the apolipoprotein moiety (Gebicki et al. 1991). In order to compare the susceptibility of lipoprotein fractions (VLDL, LDL and HDL) to oxidation, purified lipoproteins from healthy subjects were incubated in the presence and absence of 10 μM copper and 1mM AA-PH at 37 °C for 24 hours. The results showed that LDL and VLDL were the fractions most susceptible to oxidation as indicated by accumulation of hydroperoxides, conjugated dienes and MDA (as measured with the TBA assay). HDL appeared to be resistant to oxidation in all cases (see 3.4.1.). Comparison of the oxidation process in copper stimulated samples, indicated that all of three methods (hydroperoxides, conjugated dienes, TBARMs) gave similar patterns for lag phase and propagation phase in copper-stimulated lipoprotein. However, the decomposition phase was different. During the first 8 hours hydroperoxide levels reached a maximum and then decomposition of hydroperoxides started and continued for a next 24 hours. The apparently steady levels of the conjugated dienes after the propagation phase which continued until 24 hours was not due to continued formation of conjugated dienes but due to accumulation of lipid degradation products absorbing around 230 nm. This agrees with the general mechanism of lipid peroxidation during which conjugated dienes are intermediate products, which can then decompose to a variety of products, many of which have a strong absorption at 230 nm (Esterbauer et al. 1990). The formation of TBARMs was rapid and the levels then remained more or less unchanged up to 24 hours (see figures 3.4.1.; 3.4.2.; 3.4.3.). The data was similar to that which has been reported by Esterbauer et al.(1989).

The time-profile of oxidation of lipoproteins differed markedly in the presence of AA-PH in comparison with copper. For hydroperoxides, the propagation phase was slower with AA-PH than in copper stimulated samples. In contrast for VLDL and LDL accumulation of hydroperoxide continued for the next 24 hours (see Fig 3.4.1.). This

difference between copper and AA-PH oxidised samples can be attributed to the mechanism of action of these pro-oxidants. AA-PH, itself is a free radical generator with suitable rate of decomposition at physiological pH (Dooley et al. 1990). Hence, in the presence of AA-PH the continuous level of production of peroxides during the incubation time could be related to a steady-state production of carbon-centred radicals and subsequently production of peroxyl radical in the reaction medium. Accumulation of conjugated dienes and TBARMs was more or less similar to that found with copper stimulated samples but at much lower rates (see figures 3.4.1.; 3.4.2.; 3.4.3.). Propensity of the lipoproteins to peroxidation appeared to be unrelated to vitamin E levels or the PUFAs composition of the different lipoprotein fractions (see 3.4.4.).

The reason for the greater propensity of LDL to peroxidation is not entirely clear. Triglyceride content of LDL (Regnstrom et al. 1992), the presence of pre-existing lipid hydroperoxides (Thomas et al. 1994; O'Leary et al. 1992), the ratio of 18:1 to 18:2 (Parthasarathy et al. 1990; Abbey et al. 1993; Kleinveld et al 1993), ubiquinol-10 content (Stocker et al. 1991; Tribble et al 1994), the ratio of vitamin E to cholesterol (Frei and Gaziano, 1993), size and density of the particles (Chait et al. 1993; deGraaf et al. 1991) have all been suggested to contribute to peroxidisability of individual LDL samples. Similar factors may also provide an explanation for gross differences in behaviour of the major lipoprotein classes. Simple mass action effects may also play a role. For example, the higher concentration of LDL than HDL and VLDL in plasma could imply that initiators of lipid peroxidation are simply more likely to interact with LDL than other lipoproteins. However, it cannot be excluded that discrete physicochemical differences or differences in molecular architecture of the lipoproteins may also contribute to differences in peroxidisability in vivo and in vitro.

4.4. Distribution of hydroperoxides in plasma

In addition to differences in levels of plasma lipid hydroperoxides as determined by various techniques, there is conflict concerning the location and distribution of lipid hydroperoxides in plasma. Since LDL is the fraction most closely associated with atherosclerosis risk, and becomes modified in a manner making it susceptible to uptake by scavenger receptors it would seem natural to consider LDL as the principal carrier

of lipid hydroperoxides. HDL, by contrast, is recognised to be a negative risk factor for atherosclerosis, so that this fraction might reasonably be considered to contain only very low levels of hydroperoxides; if hydroperoxides are causative in atherosclerosis. However, Bowry et al (1992), using HPLC-chemiluminescence technique, suggested that HDL is the major carrier of plasma lipid hydroperoxides. Perhaps the plasma hydroperoxides are a transport form on their way to metabolic disposal and HDL functions to remove hydroperoxides. Without turn over rates one can not tell which fraction has the greatest flux of hydroperoxides.

Fractionation of plasma from healthy subjects (n= 4) to its major lipoprotein classes showed that most of the hydroperoxides appear to be principally resident in LDL. Indeed, more than 65% of hydroperoxides in native plasma were present in LDL (see 3.5.1.). Furthermore, when native plasma was oxidised in vitro in the presence and absence of AA-PH at 37 °C, the great majority of the hydroperoxides which accumulate were found to be resident in the LDL fraction. HDL, by contrast, showed a small fraction of the total native plasma hydroperoxide pool (see 3.5.2.). The TBARMs value for LDL in healthy subjects using TBA-test have been reported to be 1.18 ± 0.33 μM; n=32 (Nishigaki et al. 1981), 0.84 0.25 μM; n=50, under 40 years and 1.09 0.31 μM; n=52, over 40 years (Hagihara et al. 1984). In these studies the sum of TBARMs measured in the lipoprotein fractions was higher than the amounts found in parent plasma samples. It has been suggested that in whole plasma some component such as HDL inhibits the chemical reaction of TBA with the precursor of TBARMs (Szczeklik and Gryglewski, 1980). This shows the limitation of the TBA-test. Nevertheless, the TBARMs value for HDL fraction was much lower than for the LDL fraction. Using an iodometric assay, the total LDL hydroperoxide content has been reported as 5.4 ± 0.3 nmol/mg protein (El-Saadani et al. 1989). The mean hydroperoxide value in LDL fraction in ten healthy subjects using chemiluminescence modified by a 'photon counting' luminescence technique has been reported as approximately 0.95 nmol/mg protein (Zamburlini et al. 1995). Using HPLC-chemiluminescence technique Stocker et al (1991) suggested that the LDL fraction from healthy subjects was free from cholesterol ester hydroperoxides, phospholipid hydroperoxides and triglyceride hydroperoxides. In contrast, I found that the total LDL hydroperoxide

content using FOX2 assay in apparently healthy individuals was approximately 2.5 nmol hydroperoxide/mg LDL protein or 2 nmol hydroperoxide/mg cholesterol.

Using the FOX2 assay as a measure of total lipid hydroperoxide content, the data suggest that LDL is the major carrier of plasma lipid hydroperoxides. This conclusion, is supported by physicochemical considerations of the peroxidisability of various lipoprotein classes, by other considerations of the mechanism of lipoprotein peroxidation as well as by various estimates of plasma and lipoprotein peroxidation. HPLC-chemiluminescence techniques may underestimate the concentration of hydroperoxides in biological samples. However, they should be more sensitive and provide more information on individual hydroperoxides. Since, there are many factors in biological systems which can affect the amounts of product formed, It is reasonable to use more than one measurement technique. This may help to increase our understanding of the mechanisms of lipid peroxidation as they relate to atherosclerosis.

4.5. Diabetes and lipid peroxidation

It has been shown that diabetes is associated with significant changes in lipid structure and metabolism, especially in patients with vascular complications (Sato et al. 1979). These structural changes cause oxidation of lipids in plasma lipoproteins and also in cell membranes which in turn is associated with the development of vascular disease in diabetes (Lyons, 1991). Hyperglycaemia itself is the independent factor for increased oxidation via various mechanism which participate in the complication of vascular diseases and further damage to the tissues (Tesfamariam, 1994). It has been reported that glycosylation of LDL in diabetes leads to increased susceptibility to oxidation (Lyons, 1991; Hunt et al. 1990). Although increased peroxidation of lipids in plasma lipoprotein and other tissue has been reported by different research groups in diabetes, the relative importance of enzymatic versus non-enzymatic sources of lipid peroxidation in diabetes is unknown (Godin and Wohaieb 1987; Lyons 1991). Increased enzymatic peroxidation may result from vascular inflammation, which leads to increased levels of prostaglandins and lipooxygenase products. Alternatively, non-enzymatic formation of hydroperoxides can occur as a result of reaction of PUFAs with hydrogen peroxide, superoxide radicals and transition metal ions in the circulation or extravascular space or

at the surface of phagocytic and endothelial cells (Baynes, 1991). Hence, the distinction between enzymatic and non-enzymatic peroxidation of lipids in vivo is difficult. Enzymatically generated lipid hydroperoxides may undergo reactions with metal ions and cause initiation of autoxidation reaction. Alternatively enzymatic synthesis of prostaglandins may be stimulated by lipid hydroperoxides derived from non-enzymatic pathways. Some lipid peroxidation products may be formed by both pathways. In general it is reasonable to consider the possible involvement of both enzymatic and non-enzymatic pathways of lipid peroxidation in diabetes. However the origin of the hydroperoxides in plasma is not understood. Various proposals for the oxidation of plasma lipids include (a) oxidative reactions of glucose, (b) decompartmentalised transition metals, (c) dietary hydroperoxides and (d) enzymatically-generated hydroperoxides.

4.5.1. Elevated levels of hydroperoxides in NIDDM

A number of studies have compared plasma lipid peroxidation in control individuals and those with NIDDM using the thiobarbituric acid assay and have reported higher values of TBARM in patients (Kaji et al. 1985; Uzel et al. 1987; Griesmacher et al. 1995). However, in the present work measurement of lipid peroxidation products in a group of 60 healthy subjects and 110 NIDDM patients showed no difference in TBARMs levels (1.4 ± 0.8 Vs 1.5 ± 0.7 μM respectively. However, using the FOX2 assay hydroperoxide levels in NIDDM patients were significantly higher than in control groups (9.4 ± 3.5 vs. 3.9 ± 2.2 μM respectively; $p < 0.0005$). In this study no correlation was found between TBARMs and hydroperoxide levels within the groups (see 3.6.3). Lack of correlation between hydroperoxides and TBARMs and also lack of distinct difference in TBARMs levels between patients and control groups indicate that plasma TBA-assay is not a suitable marker of lipid peroxidation in vivo. This is because of presence of many substances in plasma that react in the TBA assay to give coloured compounds, including bile pigments, amino acids and carbohydrates (Gutteridge, 1986; Gutteridge and Halliwell, 1990). Lipid content of samples also affect the TBA reactivity in the assay (Chirico et al. 1993). Furthermore, the type and concentration of acid added with the

TBA also influences the rate of peroxide decomposition and hence the amount of TBARMs formed in the reaction mixture (Gutteridge, 1982).

A slight trend to lower vitamin E levels in the NIDDM group (19.8 ± 7.4 vs. 20.6 ± 8.6 μM in control group) failed to achieve significance at the 95% confidence level. Similar data have been reported previously by Griesmacher et al (1995). Hyperglycaemia (Tesfamariam, 1994; Lyons, 1991) disturbances of antioxidants and micronutrient status (Yoshida et al. 1995; Sinclair et al. 1991; Stankova et al. 1984; Strain, 1991) and alteration in trace element levels (Walter et al. 1991) are possibly the important factors for the proposed increased levels of lipid peroxidation in diabetes.

However, the conflicting reports indicate that formation of hydroperoxide is more complex in the in vivo system than in vitro. The role of antioxidative defence mechanism is controversial (Selvam and Anuradha, 1988; Kaji et al. 1985). Different reports have shown increased (Asayama et al. 1993; Krempf et al. 1991) and normal (Griesmacher et al. 1995; Tasi et al. 1994) level of vitamin E in plasma of diabetic patients as compared with control subjects. The reason for this variability in plasma vitamin E level in diabetic patients may result from an altered plasma lipid transport capacity (Vandewoude et al. 1987) or related to the associated hyperlipidemia, specially to levels of non-HDL cholesterol (Blanchard et al. 1992). Changes in trace elements content of the plasma have been suggested as a cause of increased lipid peroxidation in diabetes. However, there is no strong evidence for altered metabolism of trace elements in plasma of diabetic patients. Both increased [1.37 ± 0.29 vs. 1.05 ± 0.27 μg/ml] (Schlienger et al. 1988) and normal copper value [male, 133 ± 5.2 vs. 132 ± 5.6 ; female 152 ± 5.9 vs. 145 ± 5.1 μg/100 ml] (pidduck et al. 1970) have been reported in diabetic patients as compared with control subjects respectively. The normal value for copper in plasma is 13-23 μM, o.8-1.4 μg/ml (Williams and Marks, 1994) and copper levels seem not to change significantly in diabetes. Furthermore increased lipid peroxidation has been shown in diabetic patients with or without complications (Ceriello et al. 1993; Gallou et al. 1993; Velazquez et al. 1991). On the other hand increased levels of copper in diabetic patients with complications and normal level in patients without complication has been reported (Walter et al. 1991).

4.5.2. Hydroperoxides and metabolic control

Increased lipid peroxidation has been associated with poor metabolic control in diabetes (Ceriello et al. 1991). But the relationship between lipid peroxidation and the metabolic control of diabetes is conflicting (Niskanen et al. 1995). The increased lipid peroxidation probably reflects a part of a continuous cycle of oxidative stress in diabetes (Baynes, 1991). I have found no correlation between vitamin E and hydroperoxide levels in plasma. It has been reported that plasma vitamin E levels depend mainly on the lipoprotein levels in diabetic patients (Vandewoode et al. 1987).

The lack of correlation between plasma lipids and hydroperoxides was consistent with other findings (Niskanen et al. 1995; Griesmacher et al. 1995), which indicate that formation of hydroperoxides depends not only on the availability of lipids, as has been suggested by Mooradian (1991), but also other factors are involved. The positive correlation between hydroperoxide and glucose levels particularly in the NIDDM patient groups ($r = 0.2$; $p < 0.05$) suggests that hyperglycaemia is associated with increased lipid peroxidation. This agrees with in vitro data which indicates glycosylated plasma lipoproteins are more susceptible to oxidation (Bowie et al. 1993; Hunt et al. 1990). Hydroperoxide, however did not correlate with age in control or a patient groups, which may be related to changes in diet between cohorts, compensating for increased age and time of diabetic status.

4.5.3. Hydroperoxides and complications of diabetes

Elevated levels of peroxides have been shown in diabetic patients. However, it is a matter of speculation whether elevated levels of peroxide take place in patient whether with or without complication or only occur in patients with complication. Analysis of unspecified diabetic patients (n=110) and control subjects (n=331) has shown that elevated levels of peroxides (as measured with TBA method) were present only in those patient with complication (Sato et al. 1979). Measurement of conjugated dienes in diabetic patients (n=62) and control subjects (n=36) has shown that only patients with complications had elevated levels of conjugated dienes (Jennings et al. 1987). In another similar study Kaji et al (1985) have shown increased levels of peroxides (as measured

with TBA method) in diabetic patients (n=60) as compared with a control group (n=71) but the study did not specify whether or not complication were present. On the other hand (Velazquez et al. 1991) have shown increased peroxide levels (as measured by TBA method) in non-diabetic (n=20) and diabetic (n=18) patients with macrovascular disease as compared with control (n=28) subjects.

Using FOX2 assay it was observed that hydroperoxide levels in NIDDM patients were significantly higher than in the control groups (9.4 ± 3.5 vs. 3.9 ± 2.2 μM respectively); but were similar in those with (n= 62; 9.5 ± 3.5) and without complications (n= 48; 9.3 ± 3.6). Similarly there was no difference in TBARMs and vitamin E levels between two groups (see Table 3.6.6.1.). However the total cholesterol level in patients with complications was higher than those without complications. Furthermore the mean age of patients in the presence of complications was significantly higher than those without complications (see Table 3.6.6.1.). This may well be because the older patients had suffered from NIDDM for longer periods and had developed more biochemical changes and complications of the disease.

After taking account of smoking status the hydroperoxide levels and other biomarkers of lipid peroxidation in the presence and absence of complications were analysed. The data was similar in non-smokers and smokers in these patients without complications, except for HDL-cholesterol which was lower in smokers (see Table 3.6.6.2). Leonard et al (1995) have reported similar plasma levels of TBARMs in smokers and non-smokers in diabetic patients without macrovascular disease. They suggested that any increased free radical activity due to smoking is adequately scavenged in diabetic patients who are free of macrovascular disease. I found that in the patients with complications the levels of triglyceride, glucose and HbA_1 in smokers were higher than those non-smokers. However, hydroperoxide levels were not different between the two groups (see Table 3.6.6.2). A decreased vitamin E level was observed in smokers as compared with non-smokers (18.5 ± 6.7 Vs 21.6 ± 9.4).

The increased level of hydroperoxides in groups with or without complication (see 3.6.6.1.) suggests that increased lipid peroxidation could be a cause of complications rather than the consequences of complications in diabetes. However, in view of the poorly understood pathogenesis of NIDDM further studies that include assessment of specific markers of free radical activity and other factors linked with

complications of the disease are required. This would be a valuable way to increase our understanding of this disease.

The data indicate that NIDDM is associated with oxidative stress as was shown by increased plasma lipid hydroperoxides in comparison with control subjects. This phenomenon appears to be unrelated to the abnormalities in overall lipid metabolism, suggesting that hydroperoxide levels are not a simple function of total lipids. This is despite the fact that the majority (>70%) of hydroperoxides in pooled plasma were found to reside in the LDL fraction (see 3.5.1.), which is elevated in NIDDM. The absence of any correlation between LOOHs and other risk factors for complications of diabetes could be due to the fact that hydroperoxides generation take place via primary reactions in the circulation or alternatively, the peroxides might first appear in membrane components of parenchymal or circulating cells (erythrocytes, leukocytes) and then be transferred or exchanged into LDL (Thomas et al. 1994). The increased level of hydroperoxides in groups with or without complications (Fig 3.6.6.1.) may suggest that oxidative stress is found even in the early stages in the pathology of the disease and may be the cause of complications rather than the consequences of complications.

4.6. Concluding remarks

With the increasing acceptance of the role of free radicals as important biochemical intermediates in the pathogenesis of diseases, more attention has been focused on developing analytical methods for studying free radicals and their reactions in biological systems. Most of analytical methods are based on the measurement of secondary products. Hydroperoxides are the major initial products of the reaction of free radicals with lipids in biological systems. Hence, using simple and sensitive methods for determination of hydroperoxides will assist the better understanding of role of free radicals in pathology of those diseases which are related to oxidative stress. The study presented here proposes a new simple and sensitive method (FOX2) as an alternative to other methods and it may be useful in this respect.

I modified and improved the previously used FOX assay for measurement of hydroperoxides in plasma. The FOX2 assay was developed for direct determination of hydroperoxides in plasma by applying a specific hydroperoxide reductant "TPP". The

resulting method obviates the need for extraction procedures which are a major disadvantage of alternative spectrophotometric methods. Location and distribution of hydroperoxides in plasma was measured after fractionating of plasma into its major lipoprotein classes. Furthermore, using this method a significant increase in levels of hydroperoxides was observed in plasma of NIDDM patients in comparison with control subjects.

However, this study had limitations. The control group was younger than the patients. The duration of the disease since diagnosis was subject to uncertainty in the NIDDM patients. Although, vitamin E levels in these patients and control subjects was analysed, I could not investigate effect of other important factor such as ascorbic acid and antioxidant enzymes in relation to hydroperoxides because of the limitation of time. These limitations require cautious interpretation of the data. Hence, it is premature to point out the involvement of one or two factor as a cause of increased lipid peroxidation in type two diabetes, where pathogenesis of disease is still unclear. Moreover, in the absence of pharmacokinetic data it is not clear whether the accumulation of hydroperoxides in diabetic patients is due to overproduction or failure in clearance from plasma. Nor do we know where the hydroperoxides are formed, whether in cells or in the circulation.

Further research regarding of link between diabetes and oxidative stress is required to answer most of the questions in the future. The following considerations may assist better understanding of relation between lipid peroxidation and related disease:

The complexity of lipid peroxidation in biologic systems specially in disease, leads to formation of many different products may be affected by many parameters. Hence, it is desirable to measure more than one product. Using much more specific methods such as HPLC and GC-MS is likely to give more precise information in this respect. Measurement of prostaglandin-F_2 like compound as a specific marker of lipid peroxidation is becoming feasible and more interesting.

Assessment of antioxidants, micronutrient status and determination of the effect of dietary supplements should be carried out in the future. For instance loading with water soluble and fat soluble phenolic antioxidants such as paracetamol and vitamin E might

bring down hydroperoxide levels and the rate of fall could give an indication of turnover time.

Although in practise simultaneous determination of all of these parameter is difficult, yet a combination of specific methods for measurement of specific markers of the phenomenon of lipid peroxidation will help the better understanding of role of lipid peroxidation in cell injury and human diseases.

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