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Peroxidation of Human Low-Density Lipoprotein (LDL) by Charged and Uncharged Water-Soluble Peroxyl Radicals

by

Leanne Bedard, B.Sc. (Hons.)

A thesis submitted to
the Faculty of Graduate Studies and Research
in partial fulfillment of
the requirements for the degree of

Master of Science

Department of Chemistry

Carleton University
Ottawa, Ontario
August, 1999

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Chair

Department

Thesis Supervisor

Carleton University
December 1999

Abstract

The peroxidation of human low-density lipoprotein (LDL) by charged and uncharged water-soluble peroxy radicals was investigated. Kinetic studies at physiological pH and temperature using a superoxide thermal source (SOTS-1) showed that superoxide ($O_2^{\bullet-}$) was much less efficient at oxidizing LDL than water-soluble alkylperoxy radicals. It was also found that superoxide was unable to oxidize LDL particles that had been depleted of α -tocopherol, whereas water-soluble alkylperoxy radicals were able to do so. Although $O_2^{\bullet-}$ was unable to oxidize vitamin E-depleted LDL, $O_2^{\bullet-}$ was capable of oxidizing mixed micelles (in the absence of vitamin E) and this oxidation was found to be pH-dependent. It was concluded that superoxide probably initiates LDL oxidation via its conjugate acid and may act as an antioxidant by regenerating α -tocopherol from the α -tocopheroxy radical, terminating the radical chains that fuel peroxidation.

Since LDL particles have a negative zeta potential and superoxide is a negatively charged radical, it is possible that LDL peroxidation depends on the electrostatic charge of the initiating radical. Azo initiators having different electrostatic charges were examined in relation to their ability to oxidize LDL. In matched experiments (for the rates of α -tocopherol consumption), the charged and neutral alkylperoxy radicals oxidized LDL with same efficiencies. Therefore, the initiation of LDL peroxidation by water-soluble alkylperoxy radicals and superoxide is probably independent of the electrostatic charge of the initiating radical.

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Table of Contents

Acceptance.....	ii
Abstract.....	iii
Acknowledgments.....	iv
Table of Contents.....	v
List of Figures.....	viii
List of Symbols.....	xi

Chapter 1. Introduction.....	1
1.1. Free Radicals and Disease.....	1
1.2. Superoxide Thermal Source (SOTS).....	4
1.3. Lipid Peroxidation.....	8
1.4. Structure of LDL, Oxidized LDL and Atherogenesis.....	11
1.5. Tocopherol-Mediated Peroxidation (TMP).....	17
1.6. Controlling LDL Peroxidation with Plasma Antioxidants.....	24

Chapter 2. Can Superoxide Oxidize Low-Density Lipoprotein?.....	30
2.1. The Reaction of SOTS-1 with LDL and the Effect of SOD and CAT.	30
2.1.1. Introduction.....	30
2.1.2. Results.....	31
2.2. LDL Oxidation by an Authentic Superoxide Source (AA/XO).....	35
2.2.1. Introduction.....	35
2.2.2. Results.....	35
2.3. Comparison Between ROO [•] -Initiated and O ₂ ^{•-} -Initiated LDL Oxidation.....	38
2.3.1. Introduction.....	38
2.3.2. Results.....	39
2.4. Can O ₂ ^{•-} Oxidize LDL in the Absence of Vitamin E?.....	41

2.4.1.	Introduction.....	41
2.4.2.	Results.....	42
2.5.	Oxidation of PLPC/Cholate Mixed Micelles by $O_2^{\bullet-}$	42
2.5.1.	Introduction.....	42
2.5.2.	Results.....	44
2.6.	The pH Dependence of Lipid Peroxidation by $O_2^{\bullet-}$	49
2.6.1.	Introduction.....	49
2.6.2.	Results.....	49
2.6.2.1.	The pH Dependence of LDL Oxidation by $O_2^{\bullet-}$	49
2.6.2.2.	The pH Dependence of the Oxidation of PLPC/Cholate Mixed Micelles by $O_2^{\bullet-}$	54
2.7.	Discussion.....	54
APPENDIX 2A. Tables of Data From Experiments Described in Chapter		
2.		60

Chapter 3. The Effect of Electrostatic Charge on Alkylperoxyl

Radicals Used to Initiate the Oxidation of LDL.	71
3.1.	Introduction.....71
3.2.	Hydrolysis of ABAP.....71
3.2.1.	Introduction.....71
3.2.2.	Results.....74
3.3.	Rate of Decomposition of the Azo Initiators by 1H NMR.....75
3.3.1.	Introduction.....75
3.3.2.	Results.....78
3.3.2.1.	Thermal Decomposition of ABAP.....78
3.3.2.2.	Thermal Decomposition of ABCBS.....78
3.3.2.3.	Thermal Decomposition of ABDBA.....81
3.3.2.4.	Thermal Decomposition of ABMHP.....81
3.4.	Concentration Dependence of LDL Oxidation.....84

3.4.1.	Introduction.....	84
3.4.2.	Results.....	85
3.4.2.1.	TMP Range of ABAP.....	85
3.4.2.2.	TMP Range of SOTS-1.....	87
3.4.2.3.	TMP Range of ABCBS.....	89
3.4.2.4.	TMP Range of ABDBA.....	92
3.4.2.5.	TMP Range of ABMHP.....	94
3.5.	Effect of Electrostatic Charge in Experiments Matched for Total Radical Flux.....	95
3.5.1.	Results.....	95
3.6.	Does ABMHP Dissolve in LDL Particles?.....	99
3.6.1.	Introduction.....	99
3.6.2.	Results.....	100
3.7.	LDL Peroxidation by ABMHP.....	102
3.7.1.	Introduction.....	102
3.7.2.	Results.....	102
3.8.	Effect of Electrostatic Charge in Experiments Matched for the Rate of Initiation.....	104
3.8.1.	Introduction.....	104
3.8.2.	Results.....	104
3.9.	Summary and Conclusions.....	105
APPENDIX 3A. Tables of Data From Experiments Described in Chapter 3.....		110

Chapter 4. Experimental Procedures.....	121
--	------------

Chapter 5. References and Notes.....	133
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List of Figures

	Page
Chapter 1.	
1.1. Mechanism of the thermal decomposition of SOTS-1 under neutral pH conditions.	6
1.2. Mechanism of lipid peroxidation.	9
1.3. Post-column HPLC chemiluminescence detection of lipid hydroperoxides by isoluminol/microperoxidase.	12
1.4. Schematic diagram of an LDL particle.	14
1.5. Oxidative modification of LDL and atherogenesis.	16
1.6. α -Tocopherol-inhibited peroxidation of esters of polyunsaturated fatty acids in homogeneous solution.	18
1.7. Tocopherol-mediated peroxidation.	20
1.8. ABAP-induced peroxidation of LDL.	23
1.9. Radical export and inhibition of tocopherol-mediated peroxidation by ascorbate.	26
1.10. Inhibition tocopherol-mediated peroxidation by ubiquinol-10.	28
Chapter 2.	
2.1. The reaction pathways of catalase.	32
2.2. Reaction of SOTS-derived $O_2^{\bullet -}$ with LDL and inhibition by superoxide and catalase.	34
2.3. Superoxide formation from the acetaldehyde/xanthine oxidase system.	36
2.4. Reaction of LDL with superoxide generated by acetaldehyde/xanthine oxidase and inhibition by superoxide dismutase and catalase.	37

2.5.	Reaction of LDL with SOTS-1-derived $O_2^{\bullet-}$ and ABAP-derived peroxy radicals.	40
2.6.	Reaction of vitamin E-depleted LDL with SOTS-1 and ABAP.	43
2.7.	Structure of phosphatidylcholine (PLPC).	45
2.8.	Size distribution of PLPC/cholate mixed micelles.	47
2.9.	Reaction of SOTS-1-derived $O_2^{\bullet-}$ with PLPC/cholate mixed micelles and inhibition by superoxide dismutase and catalase.	48
2.10.	Size distribution of LDL at pH 7.4.	51
2.11.	Size distribution of LDL at pH 9.0.	52
2.12.	Size distribution of LDL at pH 6.0.	53
2.13.	The pH dependence of the reaction of SOTS-1-derived $O_2^{\bullet-}$ with PLPC/cholate mixed micelles	55

Chapter 3.

3.1.	Structures of azo initiators with different electrostatic charges.	72
3.2.	Stacked NMR plot showing the thermal decomposition of ABAP.	76
3.3.	Stacked NMR plot showing the base-catalysed hydrolysis of ABAP.	77
3.4.	Decomposition of ABAP.	79
3.5.	Decomposition of ABCBS.	80
3.6.	Decomposition of ABDBA.	82
3.7.	Decomposition of ABMHP.	83
3.8.	Concentration dependence of the oxidation of LDL by ABAP.	86
3.9.	Concentration dependence of the oxidation of LDL by SOTS-1.	88
3.10.	Concentration dependence of the oxidation of LDL by ABCBS.	91
3.11.	Concentration dependence of the oxidation of LDL by ABDBA.	93

3.12. Concentration dependence of the oxidation of LDL by ABMHP.	96
3.13. Effect of electrostatic charge of the oxidation of LDL in experiments matched for total radical flux.	98
3.14. LDL peroxidation by a lipid-soluble azo initiator.	101
3.15. Reaction of LDL with ABMHP.	103
3.16. Effect of electrostatic charge on the oxidation of LDL in experiments matched for the rate of initiation.	106

Chapter 4.

4.1. Schematic diagram of the HPLC set-up for the separation of lipid oxidation products.	125
4.2. Calibration curve for cholesteryl benzoate.	127
4.3. Calibration curve for <i>tert</i> -butyl hydroperoxide.	129
4.4. Calibration curve for free cholesterol.	130
4.5. Calibration curve for α -tocopherol.	132

List of Symbols

AA	Acetaldehyde
ABAP	2,2'-Azobis(2-amidinopropane) hydrochloride
ABCBS	3,3'-Azobis(3-cyano-1-butanesulfonic acid) disodium salt
ABDBA	2,2'-Azobis(<i>N,N</i> -dimethylene- isobutyramidine) dihydrochloride
ABMHP	2,2'-Azobis[2-methyl- <i>N</i> -(2- hydroxyethyl)propionamide]
α -Toc $^{\bullet}$	α -Tocoperoxyl radical
α -TocH	α -Tocopherol
χ	Apparent chain length
CAT	Catalase
CEOOH	Cholesteryl ester hydroperoxide
Ch18:2	Cholesteryl linoleate
Ch18:2OOH	Cholesteryl linoleate hydroperoxide
Ch20:4	Cholesteryl arachidonate
CL-HPLC	HPLC with chemiluminescence detection
CoQH ₂	Ubiquinol-10
DEF	Deferoxamine mesylate
EC-HPLC	HPLC with electrochemical detection
FIVE	Familial isolated vitamin E deficiency
HOO $^{\bullet}$	Hydroperoxyl radical
HPLC	High performance liquid chromatography
<i>k</i>	Rate constant
LDL	Low-density lipoprotein

LO [•]	Lipid alkoxyl radical
LOO [•]	Lipid peroxy radical
LOOH	Lipid hydroperoxide
ν	Kinetic chain length
NRPs	Non-radical products
O ₂ ^{•-}	Superoxide
[•] OH	Hydroxyl radical
PBS	Phosphate-buffered saline
PC	Phosphatidylcholine
PCS	Photon correlation spectroscopy
PCOOH	Phosphatidylcholine hydroperoxide
PLPC	1-Palmitoyl-2-Linoleoyl- <i>sn</i> -Glycero-3- Phosphocholine
PUFA	Polyunsaturated fatty acid
R_g	Rate of radical generation
R_i	Rate of initiation
R_p	Rate of chain propagation
ROO [•]	Alkylperoxy radical
⁺ ROO [•]	Positively charged alkylperoxy radical
⁻ ROO [•]	Negatively charged alkylperoxy radical
ROS	Reactive oxygen species
SOD	Superoxide dismutase
SOTS-1	Superoxide Thermal Source
$t_{1/2}$	Half-life
tBuOOH	<i>tert</i> -butyl hydroperoxide
TG	Triacylglycerol
TMP	Tocopherol-mediated peroxidation
X	Xanthine
XO	Xanthine oxidase

1. Introduction

1.1. Free Radicals and Disease

A variety of reactive oxygen species (*ROS*) are produced *in vivo* and are generally believed to initiate or, at least, to contribute to most of the major degenerative diseases including, in particular, atherosclerosis and cancer. The principal *ROS* are the hydroxyl radical ($\text{HO}^\bullet$), the superoxide radical anion ($\text{O}_2^{\bullet-}$), and its conjugate acid, the hydroperoxyl radical ($\text{HOO}^\bullet$), hydrogen peroxide (H_2O_2), lipid peroxy and alkoxy radicals ($\text{LOO}^\bullet$ and $\text{LO}^\bullet$). Some or all of these *ROS* can damage biological membranes, enzymes and DNA. In quantitative terms superoxide is the most abundant *ROS* because during respiration ca. 1 - 4 % of the oxygen metabolized in the mitochondrial electron transport chain escapes complete 4-electron reduction to water after accepting a single electron. Superoxide, despite its name, is not a particularly reactive radical. That is, it is unable to abstract a hydrogen atom from, or add to, most biological target molecules at a significant rate. However, it is known to deactivate a number of dehydratase enzymes containing iron-sulfur (4-Fe/4-S) clusters and there are reports that it, or its conjugate acid can initiate lipid peroxidation and damage DNA. The corresponding hydroperoxyl radical produced by protonation of $\text{O}_2^{\bullet-}$ is known to have a reactivity comparable to that of lipid peroxy radicals ($\text{LOO}^\bullet$) which are responsible for membrane degradation via lipid peroxidation.

However, peroxy radicals are relatively unreactive oxyl radicals compared with alkoxy and hydroxyl radicals.

Despite the low reactivities of superoxide and hydroperoxyl radicals in typical radical reactions the ubiquitous presence of superoxide dismutase (SOD) enzymes in the cells of all aerobic organisms implies that these radicals are toxic. That is, although superoxide undergoes fairly rapid dismutation to oxygen and hydrogen peroxide at physiological pH and temperature ($k \sim 10^5 \text{ M}^{-1} \text{ s}^{-1}$), evolution has produced enzymes which catalyze this reaction.

The formation of hydrogen peroxide from superoxide dismutation is a mixed blessing because this compound serves, in the presence of transition metal ions, as a source of the hydroxyl radical (via Fenton chemistry):

The hydroxyl radical is the most aggressive of all *ROS*. It reacts at, or close to, the encounter-(diffusion)-controlled rate with virtually all bioorganic molecules. This potentially damaging chemistry is minimized *in vivo* by sequestration of nearly all soluble iron and copper into storage and transport proteins and by the catalytic decomposition of H_2O_2 to oxygen and water by the enzyme catalase (CAT) and by glutathione peroxidase (GP_x)

Thus, the aerobic organism has a host of protective mechanisms against the damaging effects of *ROS*. The fact that these mechanisms are so comprehensive implies that *ROS* pose a serious threat to living organisms. Obviously no protective system will ever be 100 % effective. Therefore, although the protective mechanisms outlined above (and others) will reduce damage due to *ROS*, they will not totally eliminate it.

To understand this “residual” *ROS* damage is the goal of many research workers. The problems are complicated by uncertainty as to which *ROS* is responsible for some particular observed bio-damage.

The best way to cope with the particular problem of “which *ROS* does what?” is to generate one specific *ROS* at a controlled rate under physiological conditions and see what it does with various biological targets. The *ROS* chemistry described above starts with superoxide. Recently, a new and novel tool has become available to study the effect of $O_2^{\bullet -}$ on biological targets, such as low-density lipoproteins.

1.2. Superoxide Thermal Source (SOTS)

The usual method for generating $O_2^{\bullet -}$ for *in vitro* studies of its effect on biological targets such as DNA and cells in culture involves the enzyme xanthine oxidase (XO). Xanthine oxidase contains a molybdenum center and two 2Fe-2S clusters.¹ *In vivo*, this enzyme is involved in purine and pyrimidine metabolism. Under aerobic conditions, purines are oxidized to xanthine (X) which is then further oxidized to uric acid.² In this last step, $O_2^{\bullet -}$ is generated. A variety of aromatic heterocycles and simple aldehydes are also substrates for xanthine oxidase and can serve in place of xanthine to generate superoxide.

Although the aerobic XO/X couple has been widely used to generate $O_2^{\bullet -}$, it has several disadvantages. *In vitro* xanthine oxidase systems generate a burst of superoxide over a relatively short period of time, which does not mimic the situation *in vivo*. *In vivo*, $O_2^{\bullet -}$ is produced slowly and more or less steadily over the entire lifetime of the cell. Another problem with *in vitro* xanthine oxidase systems is that this enzyme is often contaminated with iron. Under such

conditions, $\cdot\text{OH}$ will be generated by Fenton chemistry and any biological damage due to $\text{O}_2^{\cdot-}$ itself becomes difficult to assess. Xanthine oxidase is also deactivated by $\text{O}_2^{\cdot-}$ produced during the enzyme's turnover.

Superoxide Thermal Source (SOTS-1) is the first source that generates $\text{O}_2^{\cdot-}$ at a precisely known rate which more closely mimics the situation *in vivo*.³ Unlike the enzymatic sources described above, SOTS-1 is a metal-free source that provides a slow and steady flux of superoxide under physiological conditions (pH 7, 37°C).

Figure 1.1. shows the mechanism of the decomposition of SOTS-1 in aqueous solution at physiological pH where the carboxylic acid group is deprotonated. Within the radical cage, the alkoxyl radicals disproportionate to form the aldehyde and alcohol products. However, those alkoxyl radicals which escape the cage undergo a fast 1,2 hydrogen atom shift in water to form the electron-rich carbon-centered radical, $\cdot\text{O}_2\text{CC}_6\text{H}_4\text{C}^*\text{HOH}$.⁴ This radical reacts with O_2 to form a peroxy radical which decomposes to form $\text{O}_2^{\cdot-}$.

For my undergraduate thesis, I proved that the chemistry shown in Figure 1.1. actually occurred. Two probes were used to determine the rate of decomposition and the efficiency of $\text{O}_2^{\cdot-}$ generation from SOTS-1. These probes were tetranitromethane and cytochrome c. The reduction of tetranitromethane by $\text{O}_2^{\cdot-}$ produces the nitroform anion, $\text{C}(\text{NO}_2)_3^-$, which has a strong absorption band with a maximum at 350 nm ($\epsilon_{350} = 15\,000\text{ M}^{-1}\text{ cm}^{-1}$).⁵

Figure 1.1. Mechanism of the thermal decomposition of SOTS-1 under neutral pH conditions.³

The rate constant for this reaction is $1.9 \times 10^9 \text{ M}^{-1} \text{ s}^{-1}$.⁶ Superoxide was also detected using ferricytochrome c. The reduction of ferricytochrome c by $\text{O}_2^{\bullet-}$ produces ferrocycytochrome c ($k = 2.6 \times 10^5 \text{ M}^{-1} \text{ s}^{-1}$), which has a strong absorption band with a maximum at 550 nm.⁷

Both forms of cytochrome c absorb at 550 nm, however, they have different extinction coefficients.⁷ The oxidized form of cytochrome c has an absorptivity coefficient of $8\,000 \text{ M}^{-1} \text{ cm}^{-1}$, whereas the reduced form, it has a value of $28\,000 \text{ M}^{-1} \text{ cm}^{-1}$. Therefore, to estimate the yield of $\text{O}_2^{\bullet-}$ by absorption spectroscopy at 550 nm, an extinction coefficient of $20\,000 \text{ M}^{-1} \text{ cm}^{-1}$ has to be used.

Using these probes, two methods were employed to measure the efficiency of $\text{O}_2^{\bullet-}$ generation from SOTS-1. First, measurement of the total change in absorbance at the monitoring wavelength after essentially complete decomposition of SOTS-1 (4 half-lives, ca. 6 hours at 37°C). Second, measurement of the initial rate of growth of the absorbance (10 min experiments) and a comparison of this rate with the rate of SOTS-1 decomposition. Results from both methods with both probes indicate that SOTS-1 generates $\text{O}_2^{\bullet-}$ with a mean molar efficiency of 0.40. The rate constant and half-life for SOTS-1 decomposition were determined by three independent methods: i) from the

complete (6 hour) first-order grow-in of the absorbance using cytochrome c, ii) from the complete (6 hour) first-order grow-in of the absorbance using tetranitromethane, iii) by ^1H NMR. All three methods gave the same half-life, 4 750 s in water at 37°C and pH 7.6, corresponding to a rate constant of $k = 1.46 \times 10^{-4} \text{ s}^{-1}$.³ The ^1H NMR spectra of decomposed SOTS-1 revealed that the alcohol and aldehyde are the only organic products of SOTS-1 decomposition and they are formed in a 40:60 ratio which is fully consistent with the mean molar efficiency of superoxide formation of 0.40.

1.3. Lipid Peroxidation

Lipid peroxidation has been broadly defined as the oxidative degradation of lipids. Polyunsaturated fatty acids (PUFA) are fatty acids containing two or more carbon-carbon double bonds and are the lipids most susceptible to peroxidation. Oxidative deterioration, which leads to rancidity, has been recognized as a problem in the storage of fats and oils since antiquity.⁸ However, the relevance of oxidative damage in biological systems (including ourselves!) was not recognized until much later.

The major constituents of biomembranes (cell and organelle membranes) are lipids, that are susceptible to oxidation. Lipid peroxidation is a chain reaction, the mechanism of which is shown in Figure 1.2. The products of lipid peroxidation are lipid hydroperoxides which can affect the proper functioning of biomembranes by reducing membrane fluidity. Membrane lipids are not the only lipids that contain polyunsaturated fatty acid moieties. The lipoproteins in blood

Figure 1.2. Mechanism of lipid peroxidation.⁹

plasma also contain PUFA moieties that can be oxidized. The implications of oxidative damage to lipoproteins, in particular, low-density lipoprotein will be discussed later.

Lipid peroxidation is initiated by the attack of a species that has sufficient reactivity (e.g. $\text{ROO}^\bullet$, $\text{LOO}^\bullet$, $^\bullet\text{OH}$) to abstract a hydrogen atom from the methylene group of a methylene interrupted diene, producing a carbon-centered radical. The resonance-stabilized pentadienyl radical reacts with O_2 to give a peroxy radical ($\text{LOO}^\bullet$) adjacent to a conjugated diene. This peroxy radical can abstract a hydrogen atom from another PUFA moiety to give a lipid hydroperoxide (LOOH) and a new pentadienyl radical which continues the chain.

Lipid hydroperoxides in biological samples formed as shown in Figure 1.2. can be quantified by their absorbance at 234 nm. Lipid peroxidation has also been assessed by oxygen consumption and the measurement of secondary reaction products (e.g. malonaldehyde).¹⁰ However, the measurement of LOOH by high pressure liquid chromatography coupled with postcolumn chemiluminescence detection is a much more sensitive method which allows measurement of peroxides at picomole levels.¹¹ The HPLC separation removes antioxidants present in blood plasma that could react with oxygen radicals and interfere with the detection of lipid hydroperoxides. This chromatography also allows the determination of which lipid classes become oxidized.¹¹ This method is well suited for the detection of the early stages of oxidation.¹⁰

The method is based on the detection of chemiluminescence emitted by isoluminol in the presence of hydroperoxides and microperoxidase.¹¹ Microperoxidase, a proteolytic fragment of cytochrome c, catalyzes the decomposition of the lipid hydroperoxide (LOOH) to produce a lipid alkoxyl radical (LO^{*}) which oxidizes isoluminol and leads to the emission of light (Figure 1.3.).

1.4. Structure of LDL, Oxidized LDL and Atherogenesis

Human blood plasma contains several lipoproteins. As their name implies, lipoproteins are protein-lipid packages that function in the transport of cholesterol (and other sterols), triglycerides and lipid-soluble vitamins from sites of absorption to sites of utilization and storage.¹² Five major classes of lipoproteins have been defined: i) chylomicrons, ii) very low density lipoproteins (VLDL), iii) intermediate density lipoproteins (IDL), iv) low density lipoproteins (LDL), v) high density lipoproteins (HDL).¹² Most of the research has concentrated on LDL and HDL because quantitative abnormalities in these lipoproteins are symptomatic of various diseases, including atherosclerosis. Low density lipoproteins are sometimes known as "*bad cholesterol*". LDL transports cholesterol from the liver to cholesterol-requiring cells. Oxidative modification of LDL leads to the accumulation of cholesterol in certain cells and hence to the development of atherosclerosis (to be discussed later). Contrarily, high density lipoproteins have been nicknamed "*good cholesterol*" because they are involved in "reverse cholesterol transport".¹² HDL can deliver cholesterol to cells but it is also an

Figure 1.3. Post-column HPLC chemiluminescence detection of lipid hydroperoxides by isoluminol/microperoxidase.¹¹ $\text{Q}^{\bullet-}$ = semiquinone radical.

effective acceptor of cell membrane cholesterol. Thus HDL prevents the excessive accumulation of cholesterol in cells.

The core of an LDL particle consists of neutral non-polar lipids, mostly cholesteryl esters (40% cholesteryl linoleate, Ch18:2).¹³ LDL is surrounded by a coat of polar lipids, mostly phosphatidylcholine (PC). The apoprotein, Apo B₁₀₀ (MW ~ 550 kDa) intercalates the lipoprotein and stabilizes its surface. Apo B₁₀₀ occupies ~20% of the particle's total volume. Apo-B₁₀₀ is produced in the liver and regulates lipoprotein metabolism.

The plasma lipoproteins are classified by their densities. Cholesteryl esters and triglycerides have a specific gravity less than 0.9 g/mL. The apoproteins have a density greater than 1.29 g/mL. Different lipid to protein compositions give rise to lipoproteins with intermediate densities. LDL has a density ranging between 1.063 and 1.019 g/mL and consists of ~ 75% lipid and ~25% protein.¹²

There are several antioxidants in blood plasma. In the aqueous phase, both ascorbate and urate are efficient at protecting LDL from peroxidation. LDL also contains some lipid-soluble antioxidants such as carotenoids (0.4 molecules per particle) and the quinolic antioxidant ubiquinol-10 (0.5 to 1.2 molecules per particle). However, α -tocopherol is by far the most abundant lipid-soluble antioxidant in LDL with 6-12 molecules per particle.¹³

Dimensions:

diameter = 21 nm

mass = 2.5×10^6 g/molvolume = 4×10^{-12} m³Apoprotein B₁₀₀Antioxidants: α -TocH, 6-12 molecules γ -TocH, 0.5CoQH₂, 0.5-1.2

carotenoids, 0.4

LH groups: $\Sigma = 1450$

720 in Ch18:2

180 in Ch20:4

150 in TG

400 in PC

Figure 1.4. Schematic diagram of an LDL particle. PUFA = polyunsaturated fatty acids, PC = phosphatidylcholine, Ch18:2 = cholesteryl linoleate, Ch20:4 = cholesteryl arachidonate, TG = triacylglycerol, α -TocH = α -tocopherol, γ -TocH = γ -tocopherol, CoQH₂ = ubiquinol-10.¹³

Oxidative modification of human LDL is implicated in the initiation of atherosclerosis. Atherosclerosis is a disease of the arteries which will prove fatal to roughly 2/3 of all Canadians. The development of atherosclerosis eventually leads to heart disease and stroke. Endothelial cells exposed to LDL express adhesion molecules that bind monocyte-macrophages.¹² These scavenger cells can oxidize LDL. Oxidized LDL can no longer interact properly with the LDL receptor on the surface of cholesterol-requiring cells, leading to the accumulation of cholesterol in the bloodstream. However, the receptor on the surface of macrophages have a high affinity for the oxidatively modified LDL.¹⁴ Oxidized LDL is internalized at a high rate by macrophages which proliferate and become foam cells.¹² These lipid-laden foam cells deposit themselves in the subendothelial region of the artery and can be observed in fatty streaks which are the first visible sign of atherosclerosis. Fatty streaks will eventually develop into arterial plaques which can continue to grow until they completely block the artery, leading to a heart attack or stroke.

There has been a great deal of interest in LDL oxidation and its inhibition by antioxidants. A number of animal and human studies have indicated that antioxidants can attenuate atherosclerosis. α -Tocopherol is the most abundant lipid soluble antioxidant in LDL and accordingly, most research has concentrated on both the *in vivo* and *in vitro* effects of having more or less of this vitamin.

Figure 1.5. Oxidative modification of LDL and atherogenesis.¹⁴

1.5. Tocopherol-Mediated Peroxidation

Vitamin E (α -Tocopherol, α -TocH) is the major lipid-soluble, free radical-trapping antioxidant in LDL. When compared to other forms of vitamin E (e.g. β -TocH, γ -TocH, δ -TocH), α -tocopherol is chemically and biologically the most active. α -Tocopherol is also present in a much higher concentration than other antioxidants associated with plasma lipoproteins. The large amount of vitamin E present in LDL lead to the expectation that lipid peroxidation would be strongly inhibited in LDL. However, relatively recent studies of the kinetics of LDL peroxidation showed that α -TocH can act as a *prooxidant* under certain experimental conditions.¹⁵

The conventional picture shows α -tocopherol as an antioxidant, suppressing lipid peroxidation by trapping two peroxy radicals per molecule. The reactions involved in α -tocopherol-inhibited lipid peroxidation in homogeneous solution are shown in Figure 1.6. α -Tocopherol can prevent lipid peroxidation by trapping the initiating peroxy radicals ($\text{ROO}^\bullet$) or it can break a peroxidation chain by reacting with lipid peroxy radicals $\text{LOO}^\bullet$. Both scenarios yield the relatively inert α -tocoperoxyl radical ($\alpha\text{-Toc}^\bullet$) which can terminate a second peroxidation chain by trapping a second peroxy radical. For α -TocH inhibited lipid peroxidation, the overall kinetic rate law is

$$R_p = d[\text{LOOH}]/dt = R_g (k_p/2k_{inh}) [\text{LH}]/[\alpha\text{-TocH}] \quad (1.13.)$$

Initiation *Autoxidation Chain* *Inhibition by α -Tocopherol*

Figure 1.6. α -Tocopherol-inhibited peroxidation of esters of polyunsaturated fatty acids (PUFA = LH) in homogeneous solution.¹⁶

R_g is the rate of generation of the radicals ($ROO^\bullet$) which initiate peroxidation chains by hydrogen atom abstraction from LH (reaction 1.15.). Propagation of the chain occurs by i) the very fast (near encounter controlled) reaction of $L^\bullet$ with O_2 , to form the lipid peroxy radical, $LOO^\bullet$, ii) hydrogen atom abstraction from another LH molecule by $LOO^\bullet$ forming another $L^\bullet$. Inhibition of the first radical chain occurs by the reaction of $LOO^\bullet$ with α -TocH, forming the relatively inert α -tocoperoxy radical (α -Toc $^\bullet$) which can terminate a second radical chain via reaction 1.19.

Bowry *et al.* were surprised to find that α -TocH behaves differently in aqueous LDL dispersions.^{13,15} Their studies showed that α -tocopherol acts as a *prooxidant* in LDL under conditions of low radical flux. Only one α -tocoperoxy radical can exist in an LDL particle for a significant period of time. This is because LDL particles are very small and if they contained two α -tocoperoxy radicals, these two radicals would react with one another to give non-radical products within about 1 ms. A single α -Toc $^\bullet$ may persist within an LDL particle for a much longer time (estimated to be about 17 min!) because it is unable to diffuse out of the LDL particle in which it was born. The α -Toc $^\bullet$ is therefore forced to propagate a three step radical chain by reacting with lipids within the LDL particle in a process which has been christened Tocopherol-Mediated Peroxidation (TMP). The pathway for TMP is shown in Figure 1.7.

Figure 1.7. Tocopherol-mediated peroxidation (TMP). NRPs = non-radical products.¹³

Initiation of TMP occurs with the formation of $\alpha\text{-Toc}^\bullet$ inside the LDL particle. Propagation of the radical chain carried by $\alpha\text{-Toc}^\bullet$ occurs by i) hydrogen atom abstraction from LH (rate-limiting step) to form $\text{L}^\bullet$, ii) addition of O_2 to $\text{L}^\bullet$ (near encounter controlled), iii) trapping (i.e. nominal "inhibition") of $\text{LOO}^\bullet$ by its reaction with $\alpha\text{-TocH}$, which of course regenerates $\alpha\text{-Toc}^\bullet$. Termination of this radical chain can only occur when $\alpha\text{-Toc}^\bullet$ captures a second $\text{ROO}^\bullet$ from the aqueous phase.

In the experiments done by Bowry and Stocker, native LDL was reacted with a water-soluble azo-initiator, 2,2'-azobis(2-amidinopropane hydrochloride) (ABAP). Thermolabile azo compounds were introduced as a means to oxidize lipids by alkylperoxyl radicals (generated at a steady rate R_g) in a controlled and chemically defined manner.

$$R_g (37^\circ\text{C}) = 1.1 \times 10^{-6} [\text{ABAP}] \text{ s}^{-1}$$

The amounts of cholesteryl ester hydroperoxide (CEOOH), mostly cholesteryl linoleate hydroperoxides (Ch18:2OOH), $\alpha\text{-TocH}$, phosphatidylcholine hydroperoxides (PCOOH) and ubiquinol-10 (CoQH_2) were measured over 24 hours. Their results clearly indicated that the rate of peroxidation, R_p , was faster in the presence of $\alpha\text{-TocH}$. Initially, there was a lag period before R_p reached its

maximum value. This delay period was due to the small amounts of ubiquinol-10 present in freshly prepared LDL. Once CoQH₂ was consumed, the peroxidation was not inhibited by the relatively much larger amount of α -TocH in the LDL. Even more surprising was that in the presence of α -TocH, LDL peroxidation was propagated by a *radical chain* (*apparent* chain length $\chi = R_p/R_g$). As many as 100 lipid hydroperoxide molecules were formed per initiating ROO[•] radical. Another characteristic of TMP was that the rate of peroxidation (R_p) was independent of the rate of radical generation (R_g) over a rather large concentration range of the initiator (0.5 to 55 mM ABAP). As the last few percent of α -TocH was consumed, the peroxidation rate fell before accelerating again to reach a *slower* rate in the uninhibited phase (Figure 1.8.).

To prove the existence of TMP in LDL, Bowry *et al.* developed a kinetic model known as the *modified emulsion polymerization model*, consistent with experimental findings. This model was developed using the following principles i) no transfer of radicals between particles, ii) a one-radical-per-particle limit, iii) α -TocH retains its “normal” high radical scavenging activity in LDL. Accordingly (premise iii), LOO[•] is much more likely to react with α -TocH than with LH. Therefore the majority of LOOH must be formed by the reaction of LOO[•] with α -TocH ($k_{inh} \sim 10^6 \text{ M}^{-1} \text{ s}^{-1}$) (see Figure 1.7.). The following expression holds true for the rate of peroxidation

$$R_p = -d[\text{LH}]/dt \approx d[\text{LOOH}]/dt = [\alpha\text{-Toc}^*] [\text{LH}] k_{TMP} \quad (1.21.)$$

Figure 1.8. ABAP-induced peroxidation of LDL. Purified LDL ($2.1 \mu\text{M}$) was incubated at 37°C with 1.0 mM ABAP.¹³

The prooxidant activity of α -Toch is due the *phase-transfer* activity of α -Toch (i.e. its ability to transfer the “radical character” of $\text{ROO}^\bullet$ from the aqueous phase into the particle). It was suggested that LDL particles are not uniformly reactive towards initiating radicals. According to premise iii), the likelihood of a non-peroxidizing particle reacting with $\text{ROO}^\bullet$ is dominated by the amount of α -Toch it contains. Assuming that i) all initiation occurs via the reaction of α -Toch with $\text{ROO}^\bullet$ and ii) all termination via the reaction of $\alpha\text{-Toc}^\bullet$ and $\text{ROO}^\bullet$, then, at steady state:

Rate of initiation = Rate of termination

$$k_i [\alpha\text{-Toch}] [\text{ROO}^\bullet] = 2 k_{ter} [\alpha\text{-Toc}^\bullet] [\text{ROO}^\bullet] \quad (1.22.)$$

and therefore

$$[\alpha\text{-Toc}^\bullet] = (k_i/2k_{ter}) [\alpha\text{-Toch}] \quad (1.23.)$$

$$R_p^{inh} = k_{TMP} [\text{LH}] [\alpha\text{-Toch}] (k_i/2k_{ter}) \quad (1.24.)$$

Equation 1.24. is consistent with the “sigmoid” shapes of LOOH (CEOOH) yield versus time plots.

1.6. Controlling LDL Peroxidation with Plasma Antioxidants

The question remains whether TMP is important *in vivo* where there are a number of other lipid-soluble and water-soluble antioxidants which can affect the LDL peroxidation rates.

Urate is commonly thought to act as an antioxidant in blood plasma. Previous studies have shown that urate is a scavenger of water-soluble peroxy radicals

(but not lipid-soluble peroxy radicals). Bowry and Stocker showed that urate had a *prooxidant* effect on LDL oxidation initiated by water-soluble peroxy radicals (ABAP). Addition of urate at its blood plasma concentration (~ 0.3 mM) increased the rate of lipid hydroperoxide formation in LDL by $\sim 50\%$ while inhibiting α -tocopherol consumption by $\sim 90\%$. Urate cannot reduce α -Toc $^{\bullet}$ to α -TocH in LDL, therefore it spares α -TocH and prolongs TMP by intercepting ROO $^{\bullet}$ in the aqueous phase.

In contrast to urate, ascorbate (the reduced form of vitamin C) retards LDL peroxidation efficiently. Ascorbate is the only endogenous water-soluble antioxidant in plasma that can completely protect all plasma lipids from aqueous ROO $^{\bullet}$ -mediated oxidation.¹⁷ Unlike urate, ascorbate can reduce the chain carrying α -Toc $^{\bullet}$ in an LDL particle and can also intercept initiating ROO $^{\bullet}$ in the aqueous phase. Only small amounts of ascorbate (micromolar) are needed to prevent TMP. It is postulated that ascorbate attenuates LDL peroxidation by radical export, a mechanism by which "radical character" is exported from LDL into the aqueous phase.

Ascorbate ($\text{AsCH}^{\bullet}$, $\text{ZH}_{(\text{aq})}$) reacts with α -Toc $^{\bullet}$ to form the "harmless" semidehydroascorbic acid radical ($\text{Asc}^{\bullet-}$, $\text{Z}^{\bullet}_{(\text{aq})}$) in the aqueous phase and at the same time, regenerates α -TocH in the particle. Ascorbate is recycled by the termination reaction of two $\text{Asc}^{\bullet-}$ radicals, leading to the formation of dehydroascorbic acid (Asc). $\text{Asc}^{\bullet-}$ can also react with O_2 , forming $\text{O}_2^{\bullet-}$.

AscH₂ Asc^{•-}

Asc

Figure 1.9. Radical export and inhibition of tocopherol-mediated peroxidation by ascorbate.¹⁶

Superoxide can attenuate the initiation of other radical chains by reducing peroxy radicals in the aqueous phase or by trapping the chain carrying $\alpha\text{-Toc}^\bullet$ in LDL via reaction 1.28.

In their studies of tocopherol-mediated peroxidation, Bowry and Stocker showed that ubiquinol-10 (CoQH_2 , the reduced form of coenzyme Q_{10}) is much more efficient at inhibiting LDL oxidation than lycopene, β -carotene or α -tocopherol.¹³ The LOOH vs time plots showed a lag period before R_p reaches its maximal value in the presence of CoQH_2 . After the consumption of CoQH_2 , there was a significant increase in the rate of peroxidation despite the presence of carotenoids and α -tocopherol. Ubiquinol-10 most likely prevents LDL peroxidation by radical export. Ubiquinol-10 also reduces $\alpha\text{-Toc}^\bullet$, however, radical export occurs indirectly because the ubisemiquinone radical ($\text{CoQ}^{\bullet-}$) remains attached to the LDL particle by its very long hydrocarbon tail. It has been suggested that radical export occurs via the reaction of $\text{CoQ}^{\bullet-}$ with O_2 leading to the formation of $\text{O}_2^{\bullet-}(\text{aq})$, which can prevent the initiation of other radical chains, as described above. This may explain why small amounts of CoQH_2 (as little as one molecule per three LDL particles) significantly reduces the rate of LDL peroxidation.

Figure 1.10. Inhibition of tocopherol-mediated peroxidation by ubiquinol-10.

The mechanism by which LDL peroxidation is initiated *in vivo* is unknown. The most likely suspect is $O_2^{\bullet-}$ because it is the major free radical formed *in vivo*. As mentioned earlier, superoxide is not a particularly reactive radical. It is unable to initiate TMP by hydrogen-atom abstraction from α -TocH for thermodynamic reasons. However, its conjugate acid, $HOO^\bullet$, may be able to do so, in which case, $O_2^{\bullet-}$ would be a *prooxidant*.

On the other hand, $O_2^{\bullet-}$ may act as an *antioxidant* by regenerating α -TocH from α -Toc $^\bullet$.

My project was to determine the ability of SOTS-1-derived $O_2^{\bullet-}$ to initiate the peroxidation of LDL. The electrostatic charge of the superoxide radical anion may also play a role in its ability to oxidize LDL and therefore I also investigated the effect of the electrostatic charge on alkylperoxyl radicals on LDL oxidation.

2. Can Superoxide Oxidize Low-Density Lipoprotein?

2.1. The Reaction of SOTS-1 with LDL and the Effect of SOD and CAT

2.1.1. Introduction

As mentioned in the Introduction, superoxide dismutase (SOD) provides an *in vivo* defense against $O_2^{\bullet-}$ toxicity. The SOD used in the experiments described below contains a copper-zinc prosthetic group and catalyzes the disproportionation of superoxide radicals with a rate constant of $2.3 \times 10^9 \text{ M}^{-1} \text{ s}^{-1}$.¹⁸ These enzymes are remarkably active and are present in many cells at high concentrations.

By competition for superoxide, SOD should be able to inhibit the oxidation of LDL by $O_2^{\bullet-}$.

The enzyme catalase (CAT) provides an *in vivo* defense against H_2O_2 toxicity. As described in the Introduction, H_2O_2 serves as a source of the hydroxyl radical (the most aggressive *ROS*) in the presence of transition metals (via Fenton chemistry). Catalase contains an iron center and catalyzes the decomposition of H_2O_2 ($k \sim 10^7 \text{ M}^{-1} \text{ s}^{-1}$) to water and oxygen.

The decomposition of H_2O_2 by catalase proceeds via two steps. In the first step, the native catalase (ferricatalase) is oxidized by H_2O_2 to an intermediate called Compound I.¹⁹ In the second step, Compound I is reduced back to the native catalase with release of O_2 . However, catalase mediates other redox reactions which leads to the formation of other intermediates, namely Compound II and Compound III (Figure 2.1.).

In the presence of a one-electron donor (AH), Compound I is converted to Compound II. At low concentrations of H_2O_2 (nanomolar), Compound II slowly reverts back to the native state in a one step process. At higher concentrations of H_2O_2 , Compound II reverts back to the native enzyme via the intermediacy of Compound III. Compound III is catalytically inactive and its formation from the reaction of Compound II with H_2O_2 is irreversible.

2.1.2. Results

Freshly prepared LDL ($0.4\ \mu\text{M}$) from a normal donor was incubated at pH 7.4 and 37°C with SOTS-1 ($0.57\ \text{mM}$) and in the presence of SOD (25 units) and CAT (4560 units) for 3 hours. In this time $180\ \mu\text{M}$ of superoxide would have been formed and the initial rate of formation of superoxide was $33\ \text{nM/s}$. After 14 s, 50% of the LDL particles could, in principle, have contained 1 radical. Before analysis of the samples, the coat and core lipids of LDL were separated by extraction into a methanol/hexane mixture. The lipid hydroperoxides of the core

Figure 2.1. The reaction pathways of catalase.¹⁹

non-polar lipids were analysed by reverse phase HPLC with postcolumn chemiluminescence detection.

SOTS-1 was able to oxidize LDL and this oxidation was retarded by the addition of SOD (Figure 2.2.). By varying the amounts of SOD, LDL oxidation was also found to show SOD inhibition in a dose-dependent manner. Catalase also had a protective effect on LDL oxidation by SOTS-1 (Figure 2.2.).

LDL was also incubated with decomposed SOTS-1 to confirm that the observed oxidation was actually due to $O_2^{\bullet-}$. The decomposition products of SOTS-1 did not initiate LDL peroxidation (Table 2A.1.).

LDL oxidation by SOTS-1 was also studied in the presence of a metal chelator. Only small amounts of metal ions are needed to generate the very aggressive hydroxyl radical (via Fenton chemistry) and initiate LDL peroxidation. The addition of a metal chelator (100 μ M deferoxamine mesylate, DEF) decreased the amount of lipid hydroperoxides formed but did not completely inhibit oxidation (Table 2A.1.).

To further confirm the inhibitory effects of the enzymes, LDL was incubated with both the native and denatured forms of SOD and CAT. No oxidation was observed with SOD alone or in its denatured form. However, both the native and denatured forms of CAT were able to oxidize LDL. This oxidation was attributed

Figure 2.2. Reaction of SOTS-1-derived $O_2^{\cdot -}$ (0.57 mM SOTS-1) with LDL (0.4 μ M) and inhibition by SOD (25 units) and CAT (4 560 units), incubated for 3 hours in 50 mM phosphate-buffered saline (pH 7.4) at 37°C.

to the presence of exogenous metal ions associated with catalase because it was strongly inhibited with 100 μM DEF (Table 2A.1.).

2.2. LDL Oxidation by an Authentic Superoxide Source (AA/XO)

2.2.1. Introduction

The aerobic acetaldehyde/xanthine oxidase (AA/XO) system was used as an authentic $\text{O}_2^{\bullet-}$ source. Acetaldehyde is commonly used as a substitute for xanthine for *in vitro* studies because it is more soluble in water.

Under aerobic conditions, acetaldehyde is oxidized to acetic acid, with the concomitant generation of H_2O_2 and of $\text{O}_2^{\bullet-}$ (Figure 2.3.).

2.2.2. Results

The concentrations of acetaldehyde/xanthine oxidase were adjusted to match the first 10 minutes of superoxide formation from the SOTS-1 system (0.57 mM) as measured with cytochrome c (100 μM , $\lambda = 550 \text{ nm}$, $\epsilon_{550} = 20\,000 \text{ M}^{-1} \text{ cm}^{-1}$). That is, the initial rate of formation in the AA/XO system was adjusted to 33 nM/s by varying the XO concentration. Freshly prepared LDL (0.3 μM) was incubated at pH 7.4 and 37°C with AA/XO (20 mM AA, 1.08×10^{-3} units XO) and in the presence of SOD (25 units) and CAT (4 560 units) for 3 hours. The $\text{O}_2^{\bullet-}$ generated by the AA/XO system was also able to oxidize LDL. This oxidation was inhibited by SOD and CAT (Figure 2.4.).

Figure 2.3. Superoxide formation from the acetaldehyde/xanthine oxidase system.

Figure 2.4. Reaction of LDL (0.3 μ M) with superoxide generated by acetaldehyde (AA, 20 mM), xanthine oxidase (XO, 1.08×10^{-3} units) and inhibition by SOD (25 units) and CAT (4 560 units), incubated for 3 hours in 50 mM phosphate-buffered saline (pH 7.4) at 37°C.

LDL was incubated separately with both acetaldehyde and xanthine oxidase to confirm that the observed oxidation was due to $O_2^{\bullet-}$. A minimal amount of lipid hydroperoxides (0.0284 nmol) was detected after the incubation of LDL with acetaldehyde (Table 2A.2.). A much larger amount of lipid hydroperoxides (0.689 nmol) was detected after the incubation of LDL with xanthine oxidase (Table 2A.2.). This oxidation was attributed to the contamination of xanthine oxidase with metal ions. The oxidation of LDL in the presence of xanthine oxidase was attenuated by the addition of the metal chelator DEF (100 μ M) (Table 2A.2.).

2.3. Comparison Between $ROO^{\bullet}$ -Initiated and $O_2^{\bullet-}$ -Initiated LDL Oxidation

2.3.1. Introduction

The role of $O_2^{\bullet-}$ in the oxidation of LDL was investigated. Superoxide is the simplest peroxy radical and is relatively unreactive towards most biological target molecules. Unlike most alkylperoxy radicals ($ROO^{\bullet}$), superoxide is unable to initiate TMP by hydrogen-atom abstraction from α -TocH. However, its conjugate acid, $HOO^{\bullet}$, may be able to do so, in which case, $O_2^{\bullet-}$ would be *prooxidant*.

On the other hand, $O_2^{\bullet-}$ may act as an *antioxidant* by regenerating α -TocH from $\alpha\text{-Toc}^{\bullet}$ in LDL.

The kinetics of the reaction of LDL with $O_2^{\bullet -}$ and with water-soluble alkylperoxyl radicals ($ROO^{\bullet}$) were studied.

2.3.2. Results

Freshly prepared LDL (3.7 μ M) was incubated at pH 7.4 and 37°C with SOTS-1 (200 μ M) and ABAP (10 mM) for 80 minutes (1 half-life for SOTS-1). The amounts of SOTS-1 and ABAP were matched in terms of their total radical fluxes. These were set at 45 μ M $O_2^{\bullet -}$ and 45 μ M $ROO^{\bullet}$ for the 80 minute experiments and the initial rates of formation of superoxide and $ROO^{\bullet}$ were both 11 nM/s. In these matched experiments, $O_2^{\bullet -}$ was much *less* efficient at oxidizing LDL than $ROO^{\bullet}$ (Figure 2.5.). In fact, the rate of LDL peroxidation by $ROO^{\bullet}$ was ca. 9 times faster than by $O_2^{\bullet -}$. After 80 minutes, nearly all of the α -TocH in the LDL was consumed by the reaction with ABAP but only a small amount of the α -TocH was consumed by the reaction with SOTS-1 (0.11 nmol α -TocH, 24%). Treatment of LDL with $ROO^{\bullet}$ resulted in the formation of lipid hydroperoxides (LOOH) at a rate R_p which was greater than the rate at which $ROO^{\bullet}$ were being generated (R_g), leading to an apparent chain length ($\chi = R_p/R_g$) of 4.0 (Table 2A.3.). In contrast, LDL peroxidation by SOTS-1-derived $O_2^{\bullet -}$ was not propagated by a radical chain ($\chi = 0.6$).

The radical chain lengths were also calculated according to the rate of consumption of α -tocopherol (Table 2A.3.). Two alkylperoxyl radicals are

Figure 2.5. Reaction of LDL (3.7 μ M) with SOTS-1-derived $O_2^{\bullet-}$ (200 μ M SOTS-1, ●) and ABAP-derived $ROO^{\bullet}$ (10 mM ABAP, ■), incubated for 80 minutes in 50 mM phosphate-buffered saline (pH 7.4) at 37°C. Cholesteryl linoleate hydroperoxides were measured by reverse phase HPLC with post-column chemiluminescence detection. α -Tocopherol was measured by reverse phase HPLC with electrochemical detection.

needed to destroy one molecule of α -tocopherol (see Figure 1.7.). Assuming that all $\text{ROO}^\bullet$ react to destroy α -ToCH in the LDL and that all initiation occurs via the reaction of $\text{ROO}^\bullet$ with α -ToCH, the rate of initiation, R_i , is 2 (rate of α -ToCH consumption). The radical chain length can then be expressed as $\chi' = R_p/R_i$. LDL peroxidation by $\text{ROO}^\bullet$ and $\text{O}_2^{\bullet-}$ were both propagated by a radical chain with chain lengths χ' of 7.6 and 3.5 respectively.

These results suggest either that $\text{O}_2^{\bullet-}$ is much less efficient at initiating the peroxidation of LDL than the peroxy radicals from ABAP and/or that $\text{O}_2^{\bullet-}$ inhibits LDL peroxidation by regenerating α -ToCH from $\alpha\text{-Toc}^\bullet$.

2.4. Can $\text{O}_2^{\bullet-}$ Oxidize LDL in the Absence of Vitamin E?

2.4.1. Introduction

Neuzil *et al.* have shown that plasma lipids are resistant to oxidation in the absence of vitamin E.²⁰ In their study, vitamin E-depleted LDL was isolated from a *FIVE* (familial isolated vitamin E deficiency) patient. *FIVE* is a rare syndrome caused by a defect in the hepatic α -ToCH-binding protein which results in very low circulating levels of α -ToCH with essentially none in the LDL. It was found that vitamin E-depleted LDL from the *FIVE* patient was resistant to oxidation initiated by water-soluble alkylperoxyl radicals (from ABAP) and other oxidants. It was also discovered that supplementation of the patient's diet with large doses of α -ToCH "pushed" α -ToCH into the LDL which now became oxidizable.

These findings are consistent with the TMP model of lipid peroxidation. The TMP model predicts that α -Toch acts both as a phase-transfer and lipid peroxidation chain-transfer agent.²⁰ α -Toch is the most reactive molecule on the surface of LDL and it transfers the radical character of radicals in the aqueous phase into the particle. In the absence of α -Toch, there is no efficient transfer of radicals into LDL.

2.4.2. Results

Having no access to a FIVE patient, vitamin E-depleted LDL was “chemically” prepared. Freshly isolated LDL from a normal donor was incubated at pH 7.4 and 37°C with ABAP (100 mM) for 12 minutes to destroy the α -Toch. HPLC with electrochemical detection of the sample revealed that more than 95% of the α -tocopherol had been consumed. The vitamin E-depleted LDL (0.3 μ M) was incubated alone (LDL control) and with ABAP (10 mM) and SOTS-1 (230 μ M) for 80 minutes. The amounts of ABAP and SOTS-1 were matched in terms of their total radical fluxes (45 μ M $O_2^{\bullet -}$ and $ROO^{\bullet}$). The initial rates of formation of superoxide and $ROO^{\bullet}$ were both 11 nM/s. $O_2^{\bullet -}$ was unable to oxidize LDL in the absence of α -Toch, whereas $ROO^{\bullet}$ was able to do so (Figure 2.6.). LDL peroxidation initiated by $ROO^{\bullet}$ in the absence of α -Toch was not propagated by a radical chain ($\chi = 1.5$).

2.5. Oxidation of PLPC/Cholate Mixed Micelles by $O_2^{\bullet -}$

2.5.1. Introduction

Figure 2.6. Reaction of vitamin E-depleted LDL with SOTS-1 (230 μ M, ●) and ABAP (10 mM, ■, initial $\chi = 1.5$), LDL control (○), incubated for 80 minutes in 50 mM phosphate-buffered saline (pH 7.4) at 37°C.

The results with vitamin E-depleted LDL showed that $O_2^{\bullet -}$ was unable to oxidize LDL in the absence of vitamin E. Lipid peroxidation by $O_2^{\bullet -}$ in the absence of vitamin E was studied further using PLPC (1-Palmitoyl-2-Linoleoyl-*sn*-Glycero-3-Phosphocholine) in mixed micelles with sodium cholate. The coat of an LDL particle consists mostly of phosphatidylcholine (85%). PLPC is amphipathic, i.e. it contains both a polar head group that is hydrophilic and two hydrocarbon chains which are hydrophobic (Figure 2.7.).

In aqueous solution, the hydrophobic regions of the phospholipids aggregate and form liposomes which are bags of aqueous solution bound by a lipid bilayer. The phospholipids can also be solubilized into aqueous solution via the intermediacy of detergents. Detergents associate with the phospholipid molecules and screen the hydrophobic portions from water, forming micelles. The shape and size of the micelles depends on the chemical nature of both the detergent and the lipid and their concentrations.²¹ Detergents can form micelles in the absence of any other lipids providing their concentration is high enough. Micelles which contain other components in addition to the detergents are known as “mixed micelles”.

2.5.2. Results

PLPC/cholate mixed micelles (0.76 mM PLPC, 0.97 mM sodium cholate) were prepared in phosphate-buffered saline (50 mM, pH 7.4) and passed through a polycarbonate membrane (pore diameter = 100 nm) in a hand-held extrusion apparatus. The size of the PLPC/cholate mixed micelles was measured by

Figure 2.7. Structure of phosphatidylcholine (PLPC).

photon correlation spectroscopy (PCS, laser light scattering). PCS is the analysis of the time dependence of intensity fluctuations in scattered laser light due to the motion of particles in solution.²¹ The diameter of the mixed micelles was determined to be 68.5 ± 16.0 nm, approximately three times larger than an LDL particle (diameter ~ 20 to 25 nm). The size distribution of the PLPC/cholate mixed micelles is shown in Figure 2.8. (Table 2A.4.).

The PLPC/cholate mixed micelles were incubated at pH 7.4 and 37°C with SOTS-1 (0.47 mM) and in the presence of SOD (8 units) and CAT (3 890 units) for 80 minutes. The PLPC/cholate mixed micelles were oxidized by SOTS-1 and this oxidation was attenuated by the addition of SOD and CAT (Figure 2.9.).

The oxidation of PLPC/cholate mixed micelles was also studied in the presence of the metal chelator DEF (100 μ M). As in the LDL studies, it was found that the addition of this metal chelator decreased the amount of lipid hydroperoxides formed (Table 2A.5.).

I also attempted to reproduce tocopherol-mediated peroxidation using PLPC/cholate mixed micelles enriched with α -tocopherol. PLPC/cholate mixed micelles (0.76 mM PLPC, 0.97 mM sodium cholate) enriched with α -tocopherol (180 μ M) were incubated at pH 7.4 and 37°C with SOTS-1 (0.52 mM) for 80 minutes. It was found that under the experimental conditions necessary for TMP to occur (i.e. low radical flux), α -tocopherol acted as an *antioxidant* (Table 2A.6.).

Figure 2.8. Size distribution of PLPC/cholate mixed micelles measured by PCS in 50 mM phosphate-buffered saline (pH 7.4) at 23°C. Mean diameter = 68.5 ± 16.0 nm.

Figure 2.9. Reaction of SOTS-1-derived $O_2^{\bullet-}$ (0.47 mM SOTS-1) with PLPC/cholate mixed micelles (0.76 mM PLPC, 0.97 mM sodium cholate) and inhibition by SOD (8 units) and CAT (3 890 units), incubated for 80 minutes in 50 mM phosphate-buffered saline (pH 7.4) at 37°C.

Unlike LDL, micelles are “dynamic” structures that can fuse or exchange components, including α -TocH and α -Toc $^{\bullet}$. The exchange of α -TocH and α -Toc $^{\bullet}$ between particles is not consistent with the model of TMP and would lead to the termination of the radical chain.

2.6. The pH Dependence of Lipid Peroxidation by $O_2^{\bullet-}$

2.6.1. Introduction

Superoxide is not sufficiently reactive (for thermodynamics reasons) to abstract a hydrogen atom from LH or α -TocH, and therefore it cannot initiate lipid peroxidation. This raises the question as to how superoxide initiates lipid peroxidation in LDL and in PLPC/cholate mixed micelles. It appears likely that it is the conjugate acid of $O_2^{\bullet-}$, $HOO^{\bullet}$, that actually initiates lipid peroxidation.

The *Henderson-Hasselbach* equation relates the pH of a solution to the pK_a .

$$pH = pK_a + \log_{10} [O_2^{\bullet-}]/[HOO^{\bullet}] \quad (2.6.)$$

At physiological pH, the ratio of $HOO^{\bullet}/O_2^{\bullet-}$ is ca. 1/100 so there may be just sufficient $HOO^{\bullet}$ to initiate lipid peroxidation.

2.6.2. Results

2.6.2.1. The pH Dependence of LDL Oxidation by $O_2^{\bullet-}$

LDL was isolated from human blood plasma in 50 mM phosphate-buffered saline at pH 7.4. The pH of the LDL solution was adjusted to pH 6.0, 8.0, 9.0 and 10.0 by overwhelming the buffer capacity of PBS by adding either dilute acid or base. The size of the LDL particles at pH 6.0, 7.4 and 9.0 was measured by photon correlation spectroscopy. The size distribution of LDL at pH 7.4 is shown in Figure 2.10. The diameter of the LDL particles was determined to be 19.1 ± 4.9 nm.

Figure 2.11. shows the size distribution of LDL at pH 9.0. Under alkaline conditions, the distribution was much larger and the diameter of the LDL particles decreased to 7.1 ± 5.3 nm, indicating that the particles were not stable at pH 9.0.

At acidic pH, the LDL particles were intact. The size distribution of LDL at pH 6.0 is shown in Figure 2.12. The mean diameter of the lipid particles at pH 6.0 was determined to be 20.0 ± 2.3 nm.

Due to the limited solubility of SOTS-1 at acidic pH and the instability of LDL at alkaline pH, the pH dependence of LDL oxidation by $O_2^{\bullet-}$ was impossible to study. However, the pH dependence of lipid peroxidation in PLPC/cholate mixed micelles was examined.

Figure 2.10. Size distribution of LDL in 50 mM phosphate-buffered saline at pH 7.4 measured by PCS at 23°C. Mean diameter = 19.1 ± 4.9 nm.

Figure 2.11. Size distribution of LDL in 50 mM phosphate-buffered saline at pH 9.0 measured by PCS at 23°C. Mean diameter = 7.1 ± 5.3 nm.

Figure 2.12. Size distribution of LDL in 50 mM phosphate-buffered saline at pH 6.0 measured by PCS at 23°C. Mean diameter = 20.0 ± 2.3 nm.

2.6.2.2. The pH Dependence of the Oxidation of PLPC/Cholate Mixed Micelles by $O_2^{\bullet-}$

PLPC/cholate mixed micelles (0.72 mM PLPC, 0.97 mM sodium cholate) were incubated at pH 6.0, 7.0, 7.8, 9.0 and 10.0 and 37°C with SOTS-1 (0.46 mM) for 80 minutes. Figure 2.13. shows that the oxidation of PLPC/cholate mixed micelles by SOTS-1-derived $O_2^{\bullet-}$ was pH dependent. At pH 10 there is very little $HOO^{\bullet}$ ($HOO^{\bullet}/O_2^{\bullet-} = 1/160\ 000$) and essentially no lipid hydroperoxides could be detected. At physiological pH, there is ca. 1% $HOO^{\bullet}$ and lipid hydroperoxides were readily detected.

2.7. Discussion

Superoxide generated from both SOTS-1 and the aerobic acetaldehyde/xanthine oxidase system was able to oxidize LDL. As expected, this oxidation was inhibited by superoxide dismutase. As shown in Figures 2.2. and 2.4., catalase is also very efficient at inhibiting LDL oxidation by $O_2^{\bullet-}$ which I initially presumed to be due to the decomposition of H_2O_2 generated by the dismutation of $O_2^{\bullet-}$. Thus, these results with catalase suggested that $O_2^{\bullet-}$ toxicity was due mostly to the presence of H_2O_2 . However, if this really were the case it would be expected that SOD would not have a protective effect on LDL oxidation since H_2O_2 is generated by the disproportionation of $O_2^{\bullet-}$ catalyzed by SOD. The only plausible explanation for my results was that catalase also had the ability to trap $O_2^{\bullet-}$. My results are, in fact, consistent with the report that catalase is inhibited by $O_2^{\bullet-}$ and that this inhibition is due to the reaction of $O_2^{\bullet-}$ with catalase and Compound I:¹⁹

Figure 2.13. Reaction of SOTS-1-derived $O_2^{\bullet-}$ (0.46 mM SOTS-1) with PLPC/cholate mixed micelles (0.72 mM PLPC, 0.97 mM sodium cholate) incubated in sodium phosphate buffer (0.1 M, pH 6.0, 7.0 and 7.8) or sodium borate buffer (0.1 M, pH 9.0 and 10.0) for 80 minutes at 37°C.

The trapping of $\text{O}_2^{\bullet-}$ by CAT and by Compound I competes with the initiation of lipid peroxidation by the $\text{O}_2^{\bullet-}/\text{HOO}^{\bullet}$ couple and, therefore, it is to be expected that CAT will be rather efficient at inhibiting LDL oxidation by $\text{O}_2^{\bullet-}$.

Kinetic studies at physiological pH and temperature showed that $\text{O}_2^{\bullet-}$ was much less efficient at oxidizing human LDL than water-soluble alkylperoxyl radicals ($\text{ROO}^{\bullet}$). These results imply that $\text{O}_2^{\bullet-}$ may act as an antioxidant by regenerating $\alpha\text{-TocH}$ from $\alpha\text{-Toc}^{\bullet}$. To check whether or not $\text{O}_2^{\bullet-}$ is acting as an antioxidant in LDL, the reaction between the $\alpha\text{-Toc}^{\bullet}$ and $\text{O}_2^{\bullet-}$ would need to be studied by generating $\alpha\text{-Toc}^{\bullet}$ in LDL and studying its reaction with SOTS-1. I have not attempted to do this because there would be many practical problems to overcome. In particular, it would be very difficult to control the generation of $\alpha\text{-Toc}^{\bullet}$ in the LDL and to monitor the concentration of $\alpha\text{-Toc}^{\bullet}$ in the LDL during the treatment with SOTS-1.

Superoxide cannot oxidize LDL particles that have been depleted of α -tocopherol, whereas water-soluble alkylperoxyl radicals were able to do so. At first sight these results are not consistent with the findings of Neuzil *et al.* Their studies showed that LDL from a *FIVE* patient was resistant to oxidation initiated by $\text{ROO}^{\bullet}$. The length of the radical chain was calculated to further understand

the kinetics driving the reaction between the vitamin E-depleted LDL and $\text{ROO}^\bullet$. The *kinetic* chain length, ν , is defined as (LH consumption rate)/(chain initiation rate). The kinetic chain length is difficult to determine experimentally because R_i cannot be measured in the uninhibited phase of LDL peroxidation. However, the *apparent* radical chain length, $\chi = R_p/R_g = (\text{LOOH formation rate})/(\text{chain generation rate})$, can be readily measured throughout peroxidation. For the reaction between the vitamin E-depleted LDL and $\text{ROO}^\bullet$, the apparent chain length was calculated to be only 1.5, meaning that this peroxidation was not really a chain reaction.

In my experiment, the vitamin E-depleted LDL contained 0.27 nmoles of lipid hydroperoxides, which corresponds to 3% of the total amount of core lipids in LDL before incubation with the ABAP or SOTS-1. It seems possible that the lipid hydroperoxides are positioned with their hydrophilic $-\text{OOH}$ group on the outside of the LDL particle so that they are exposed to the aqueous phase. This would allow the initiating $\text{ROO}^\bullet$ radicals to abstract a hydrogen atom from the lipid hydroperoxides (LOOH) to form $\text{LOO}^\bullet$. The lipid hydroperoxides transfer the radical character of $\text{ROO}^\bullet$ into the LDL particle. Very surprisingly, it would appear at first sight that the $\text{LOO}^\bullet$ radicals generated in this way do not carry a radical chain very well since the apparent chain length χ was only 1.5. However, this is unreasonable as hydrogen atom abstraction by peroxy radicals from polyunsaturated fatty acids (reaction 1.15.) is very much faster ($k \sim 100 \text{ M}^{-1} \text{ s}^{-1}$) than the abstraction by α -tocoperoxyl radicals ($k_{\text{TMP}} \sim 0.1 \text{ M}^{-1} \text{ s}^{-1}$). We conclude

that initiation of peroxidation, the aqueous peroxy radical reaction with lipid hydroperoxides in LDL, is very much less efficient than via their reaction with α -tocopherol. This conclusion is consistent with the (gas phase) thermodynamic driving forces for these two reactions viz. roughly thermoneutral for the $\text{ROO}^\bullet + \text{LOOH}$ reaction and exothermic by ca. 11 kcal/mol for the $\text{ROO}^\bullet + \alpha\text{-ToCH}$ reaction. LOOH is a stronger H-bond donor to water than $\alpha\text{-ToCH}$ ($\alpha_2^{\text{H}}(\alpha\text{-ToCH}) = 0.370$; $\alpha_2^{\text{H}}(\text{tert-butyl hydroperoxide}) = 0.442$, where α_2^{H} is a measure of the relative ability of a solute to donate a H-bond to a hydrogen bond acceptor such as water.).²² Therefore, LOOH with its hydroperoxyl group at the lipid-water interface will also be intrinsically less reactive towards radical attack than $\alpha\text{-ToCH}$ with its phenolic hydroxyl group at this interface. Probably those few particles of vitamin E depleted LDL in which initiation actually does occur will be extensively oxidized but those in which initiation does not will be unaffected. As a consequence there is little overall peroxidation and the *apparent* chain length is small. In the experiments done by Neuzil *et al.*, LDL was freshly prepared from a *FIVE* patient and contained not just no α -tocopherol but also no lipid hydroperoxides. In the absence of both $\alpha\text{-ToCH}$ and LOOH, $\text{ROO}^\bullet$ cannot transfer its radical character from the aqueous phase into the LDL particle and therefore the lipids are highly resistant to oxidation.

Although $\text{O}_2^{\bullet-}$ was unable to oxidize vitamin E-depleted LDL, $\text{O}_2^{\bullet-}$ was capable of oxidizing PLPC/cholate mixed micelles. Both SOD and CAT showed a protective effect on the oxidation of PLPC/cholate mixed micelles by $\text{O}_2^{\bullet-}$. These results

suggest that the protein portion of LDL may play an important role in the oxidative modification this lipoprotein.

It is well known that $O_2^{\bullet-}$ is simply not reactive enough to initiate lipid peroxidation by hydrogen atom abstraction from LH or α -ToCH. The oxidation of PLPC/cholate mixed micelles by SOTS-1-derived $O_2^{\bullet-}$ was found to be pH dependent. At physiological pH, there is a sufficient amount of $HOO^{\bullet}$ to initiate lipid peroxidation.

APPENDIX 2A.

Tables of Data From Experiments Described in Chapter 2.

Tables 2A.1. Reaction of SOTS-1-derived $O_2^{\bullet -}$ (0.57 mM SOTS-1) with LDL (0.4 μ M) and inhibitory effects of SOD (25 units) and CAT (4 560 units) and DEF (100 μ M), incubated for 3 hours in 50 mM phosphate-buffered saline (pH 7.4) at 37°C.

Sample	Ch18:2OOH (nmol)
LDL	0
LDL + SOTS-1	2.4
LDL + SOTS-1 (100 μ M DEF)	1.2
LDL + decomposed SOTS-1	0
LDL + SOTS-1 + SOD	0.06
LDL + SOD	0
LDL + SOTS-1 + denatured SOD	2.0
LDL + denatured SOD	0
LDL + SOTS-1 + CAT	0.23
LDL + CAT	0.15
LDL + denatured CAT	0.20
LDL + CAT (100 μ M DEF)	0.007

Tables 2A.2. Reaction of LDL (0.3 μM) with $\text{O}_2^{\bullet-}$ generated by the acetaldehyde/xanthine oxidase system (AA/XO, 20 mM AA, 1.08×10^{-3} units XO) and inhibition by SOD (25 units) and CAT (4 560 units) and DEF (100 μM), incubated for 3 hours in 50 mM phosphate-buffered saline (pH 7.4) at 37°C.

Sample	Ch18:2OOH (nmol)
LDL	0
LDL + AA + XO	1.0
LDL + AA + XO + SOD	0.55
LDL + AA + XO + CAT	0.61
LDL + AA	0.028
LDL + XO	0.69
LDL + XO (100 μM DEF)	0.26

Table 3A.3. Reaction of LDL (3.7 μM) with SOTS-1-derived $\text{O}_2^{\cdot -}$ (200 μM SOTS-1) and ABAP-derived $\text{ROO}^{\cdot}$ (10 mM ABAP), incubated for 80 minutes in 50 mM phosphate-buffered saline (pH 7.4) at 37°C. R_p is the maximal rate of LOOH accumulation. R_g is the radical generation rate ($R_g(\text{ABAP}) = 1.4 \times 10^{-6} [\text{ABAP}] \text{ s}^{-1}$; $R_g(\text{SOTS-1}) = 2 e (1.46 \times 10^{-4} \text{ s}^{-1}) [\text{SOTS-1}]$, $e = 0.20$). The apparent chain length is $\chi = R_p/R_g$. $R_{\alpha\text{-TocH}}$ is the rate of consumption of $\alpha\text{-TocH}$. The rate of initiation is $R_i = 2 R_{\alpha\text{-TocH}}$. The radical chain length χ' is R_p/R_i .

Sample	R_p (10^9 M/s)	R_g (10^8 M/s)	χ (R_p/R_g)	$R_{\alpha\text{-TocH}}$ (10^9 M/s)	R_i (10^9 M/s)	χ' (R_p/R_i)
LDL + ABAP	5.6	1.4	4.0	3.7	7.4	7.6
LDL + SOTS-1	0.64	0.12	0.6	0.93	1.8	3.5

Table 2A.4. Size distribution of PLPC/cholate mixed micelles measured by PCS in 50 mM phosphate-buffered saline (pH 7.4) at 23°C.

Diameter (nm)	Relative Number	Percent (%)
35.0	2.2	0.6
41.0	11.5	3.1
48.0	37.5	10.2
56.3	77.1	20.9
66.0	100.0	27.1
77.3	81.7	22.1
90.7	42.1	11.4
106	13.7	3.7
125	2.8	0.8
146	0.4	0.10

Table 2A.5. Reaction of SOTS-1-derived $O_2^{\bullet -}$ (0.47 mM SOTS-1) with PLPC/cholate mixed micelles (0.76 mM PLPC, 0.97 mM sodium cholate) and inhibition by SOD (8 units) and CAT (3 890 units) and DEF (100 μ M), incubated for 80 minutes in 50 mM phosphate-buffered saline (pH 7.4) at 37°C.

Sample	PCOOH (nmol)
PLPC	0.00087
PLPC + SOTS-1	0.22
PLPC + SOTS-1 (100 μ M DEF)	0.18
PLPC + SOTS-1 + SOD	0.0079
PLPC + SOD	0.00080
PLPC + SOTS-1 + CAT	0.012
PLPC + CAT	0.00034

Table 2A.6. Reaction with SOTS-1-derived $O_2^{\bullet -}$ (0.52 mM SOTS-1) with vitamin E-enriched PLPC/cholate mixed micelles (0.76 mM PLPC, 0.97 mM sodium cholate, 180 μ M α -TocH), incubated for 80 minutes in 50 mM phosphate-buffered saline (pH 7.4) at 37°.

Sample	PCOOH (nmol)
PLPC	0.0014
PLPC + α -TocH	0.00093
PLPC + SOTS-1	0.11
PLPC + SOTS-1 + α -TocH	0.0057

Table 2A.7. Size distribution of LDL in 50 mM phosphate-buffered saline at pH 7.4 measured by PCS at 23°C.

Diameter (nm)	Relative Number	Percent (%)
8.8	1.5	0.4
10.3	7.6	1.8
12.1	25.8	6.3
14.2	59.7	14.5
16.7	93.8	22.8
19.5	100.0	24.3
22.9	72.4	17.6
26.8	35.6	8.6
31.4	11.9	2.9
36.8	2.7	0.7
43.2	0.4	0.1

Table 2A.8. Size distribution of LDL in 50 mM phosphate-buffered saline at pH 9.0 measured by PCS at 23°C.

Diameter (nm)	Relative Number	Percent (%)
4.0	92.9	11.4
4.7	98.6	12.0
5.5	100.0	12.2
6.4	97.0	11.9
7.5	90.1	11.0
8.8	80.0	9.8
10.4	67.9	8.3
12.1	55.1	6.7
14.2	42.8	5.2
16.7	31.8	3.9
19.6	22.6	2.8
22.9	15.3	1.9
26.9	10.0	1.2
31.5	6.2	0.8
36.9	3.7	0.5
43.3	2.1	0.3
50.7	1.1	0.1
59.4	0.6	0.07
69.7	0.3	0.04

Table 2A.9. Size distribution of LDL in 50 mM phosphate-buffered saline at pH 6.0 measured by PCS at 23°C.

Diameter (nm)	Relative Number
16.9	57.2
18.7	100.0
20.8	96.3
23.1	47.5
28.5	13.5

Table 2A.10. pH dependence of the oxidation of PLPC/cholate mixed micelles (0.72 mM PLPC, 0.97 mM sodium cholate) with SOTS-1-derived $O_2^{\bullet -}$ (0.46 mM SOTS-1), incubated in sodium phosphate buffer (0.1 M, pH 6.0, 7.0 and 7.8) or sodium borate buffer (0.1 M, pH 9.0 and 10.0) for 80 minutes at 37°C. The PLPC/cholate mixed micelles contained ~ 0.002 nmol PCOOH before incubation with SOTS-1.

pH	PCOOH (nmol)
6.0	0.41
7.0	0.28
7.8	0.15
9.0	0.032
10.0	0.0026

3. The Effect of Electrostatic Charge on Alkylperoxyl Radicals Used to Initiate the Oxidation of LDL

3.1. Introduction

The zeta-potential of an LDL particle is negative. It is therefore possible that the oxidation of LDL will depend on the electrostatic charge of the initiating alkylperoxyl radical. Azo initiators having different electrostatic charges (positive, negative and neutral) were therefore examined in relation to their ability to oxidize LDL. The neutral (ABMHP) and positively charged (ABAP) initiators were commercially available, but the negatively charged initiator (ABCBS) had to be synthesized. The chemical structures of these azo initiators are shown in Figure 3.1.

These azo initiators thermally decompose, cleaving the two C-N bonds to give the carbon-centered radical ($R^\bullet$) with the release of N_2 . Under aerobic conditions, $R^\bullet$ reacts with O_2 (near encounter controlled) to form a peroxyl radical ($ROO^\bullet$) (reaction 3.1.). The reactivity of tertiary alkylperoxyl radicals is believed to be independent of the nature of the tertiary alkyl group, R. In equation 3.1., e^{23} is the efficiency of escape of the geminate radicals from the solvent cage in which they are produced. Typically, the efficiency of cage escape e is ≈ 0.5 .^{23, 24}

3.2. Hydrolysis of ABAP

3.2.1. Introduction

ABAP (positive, 2,2'-azobis(2-amidinopropane) dihydrochloride):

ABCBS (negative, 3,3'-azobis(3-cyano-1-butanefulfonic acid) disodium salt):

ABMHP (neutral, 2,2'-azobis[2-methyl-N-(2-hydroxyethyl)propionamide]):

Figure 3.1. Structures of azo initiators with different electrostatic charges.

ABAP is one of the most popular thermolabile azo compounds used by biologists to initiate the oxidation of biological targets. However, kinetic studies on the thermal decomposition of ABAP have shown that it undergoes a pH dependent hydrolysis to the corresponding amide.²⁵

This amide product is considerably more stable thermally (37°C and pH ~ 7) than the amidine from which it is derived. For the quantitative investigation of the abilities of alkylperoxyl radicals with different electrostatic charges to initiate LDL peroxidation, it is obvious that the LDL should be treated with approximately equal fluxes of these radicals. For radical flux matched experiments using ABAP, the rate of hydrolysis and the amount of ABAP which undergoes hydrolysis has to be known. These were determined by monitoring the decomposition of ABAP by 1) UV-visible spectroscopy and 2) ¹H NMR.

Another azo initiator which decomposes to form positively charged peroxy radicals is 2,2'-azobis(*N,N*-dimethylene-isobutyramidine) dihydrochloride (ABDBA).

This compound undergoes hydrolysis much more slowly than ABAP. Therefore, the ability of ABDBA to oxidize LDL was also examined.

3.2.2. Results

The thermal decomposition of ABAP (50 mM) at pH 7.4 and 37°C was monitored using UV-visible spectroscopy. The azo functionality (N=N) has an absorption maximum λ_{max} at ~ 365 nm and the extinction coefficient was calculated to be 20 M⁻¹ cm⁻¹. This value is in good agreement with the extinction coefficients expected for other azo compounds: ϵ = 14 for the *trans*-t-butyl analogue and ϵ = 16 for the 1-adamantanyl analogue.²⁶ After 30 days (4 half-lives) the residual intensity of the azo band of ABAP was 27 % of its initial intensity (Table 3A.1.). However, it was expected that after 30 days, the residual intensity of the azo band would be only 5 % of its initial intensity. Assuming that the extinction coefficients of ABAP and the corresponding amide are equal, 22 % of ABAP underwent hydrolysis. As indicated in Table 3A.1., after 4 days (0.6 half-lives), only 3 % of ABAP has undergone hydrolysis. Therefore, over the period of 24 to 36 hours (i.e. the incubation time for the oxidation of LDL by ABAP), the hydrolysis can simply be neglected.

The thermal decomposition of ABAP in D₂O and at 37°C was monitored by ¹H NMR (400 MHz) over a period of 4 days. The ¹H NMR kinetic data for the thermal decomposition of ABAP showed a decrease in intensity of the signal due to the CH₃ group of ABAP (δ = 1.4 ppm) and the grow-in of several signals in the

methyl region, where one of these signals would correspond to the amide product. Figure 3.2. shows a stacked plot of these spectra.

To identify which peak corresponded to the amide product, the base-catalysed hydrolysis of ABAP in D₂O at room temperature was monitored by ¹H NMR. Measurements were taken at 30 minute intervals for the first 6 hours and then at 1 hour intervals thereafter for a total time of 16 hours. Figure 3.3. shows a stacked plot of these spectra. Figure 3.3. shows the disappearance of the methyl protons of ABAP at $\delta = 1.35$ ppm and the grow-in of the methyl protons of the amide product at $\delta = 1.30$ ppm.

From the kinetic data of the thermal decomposition of ABAP, the rate constant for the hydrolysis of ABAP at 37°C in D₂O was determined to be $k = 2.4 \times 10^{-7} \text{ s}^{-1}$. Under physiological conditions, 78 % ABAP undergoes homolysis and only 22 % of ABAP hydrolysed to the stable amide product. This result is in good agreement with the yield of the amide product obtained by UV-visible spectroscopy.

3.3. Rate of Decomposition of the Initiators by ¹H NMR

3.3.1. Introduction

For the planned, quantitative investigation of the abilities of the four water-soluble alkylperoxyl radicals (ABAP, ABCBS, ABMHP, ABDBA) to initiate lipid peroxidation in LDL, it was important to be able to treat LDL with known and

Figure 3.2. Stacked plot showing the decomposition of ABAP at 37°C in D₂O (pH = 7). The time interval between successive spectra was approximately 1 day.

Figure 3.3. Stacked plot showing the base-catalysed hydrolysis of ABAP at 25°C (pH = 12). The spectra shown here were taken at times 0, 1, 2, 4, 8 and 16 hours.

approximately equal fluxes of these peroxy radicals. The rates of thermal decomposition of the three azo initiators which are commercially available (ABAP, ABDBA and ABMHP) are known. However, since it was necessary to measure the rate of decomposition of ABCBS, I decided to measure the rates for all four of these azo compounds by ^1H NMR.

3.3.2. Results

3.3.2.1. Thermal Decomposition of ABAP

The thermal decomposition of ABAP was measured at 37°C in D_2O using ^1H NMR (400 MHz). Measurements were taken daily over a period of 4 days. The rate constant and half-life for ABAP decomposition were calculated from the rate of disappearance of the methyl protons. The decay obeyed first-order kinetics and the rate constant for the decomposition of ABAP at pH 7 and 37°C was calculated to be $k = 1.1 \times 10^{-6} \text{ s}^{-1}$ which corresponds to a half-life of 7 days (Figure 3.4.).

3.3.2.2. Thermal Decomposition of ABCBS

The kinetics of the decomposition of ABCBS in D_2O at 37°C were monitored using ^1H NMR (400 MHz). Spectra were measured at random over a period of 18 days. The rate constant and half-life for ABCBS decomposition were calculated from the decrease in intensity of the signal due to the CH_3 groups ($\delta = 1.8 \text{ ppm}$). Figure 3.5. shows the first-order decay of the methyl protons of

Figure 3.4. Decomposition of ABAP at 37°C and pH 7, determined from the integrated intensity of the methyl protons.

Figure 3.5. Decomposition of ABCBS at 37°C and pH 7, determined from the integrated intensity of the methyl protons.

ABCBS. The rate constant for the thermal decomposition of ABCBS was calculated to be $k = 3.5 \times 10^{-7} \text{ s}^{-1}$ at 37°C and pH 7 ($t_{1/2} = 23$ days).

3.3.2.3. Thermal Decomposition of ABDBA

The kinetics of the thermal decomposition of ABDBA at 37°C in D₂O were measured by ¹H NMR (400 MHz). Measurements were recorded at random intervals over a period of 70 hours. The disappearance of the methyl protons ($\delta = 1.42$ ppm) was plotted over time and obeyed first-order kinetics (Figure 3.6.). The rate constant for ABDBA decomposition was calculated to be $k = 5.2 \times 10^{-6} \text{ s}^{-1}$, corresponding to a half-life of 37 hours at pH 7 and 37°C.

3.3.2.4. Thermal Decomposition of ABMHP

The thermal decomposition of ABMHP was monitored for 10 days at 37°C in D₂O using ¹H NMR. The disappearance of the methyl protons of ABMHP ($\delta = 1.27$ ppm) is shown in Figure 3.7. The decay obeyed first-order kinetics and the rate constant for ABMHP decomposition was calculated to be $k = 1.8 \times 10^{-7} \text{ s}^{-1}$, which corresponds to a half-life of 45 days at pH 7 and 37°C.

The kinetic data for decomposition of the four azo initiators are summarized in Table 3A.2.

Figure 3.6. Decomposition of ABDBA at 37°C and pH 7, determined from the integrated intensity of the methyl protons.

Figure 3.7. Decomposition of ABMHP at 37°C and pH 7, determined from the integrated intensity of the methyl protons.

3.4. Concentration Dependence of LDL Oxidation

3.4.1. Introduction

The conventional picture of α -tocopherol shows it as an antioxidant, suppressing lipid peroxidation (see Figure 1.6.). For α -TocH inhibited peroxidation, the overall kinetic rate law is

$$R_p = R_g (k_p/2k_{inh}) [LH]/[\alpha\text{-TocH}] \quad (3.7.)$$

In this case, the rate of peroxidation, R_p , is directly proportional to the rate of generation of the initiating radicals, R_g , and hence to the concentration of the initiator. An increase in the applied radical flux would lead to the faster formation of LOOH.

However, for tocopherol-mediated peroxidation, the following expression holds true for the rate of peroxidation

$$R_p^{inh} = k_{TMP} [LH] [\alpha\text{-TocH}] (k_i/2k_{ter}) \quad (3.8.)$$

Accordingly, Bowry *et al.*¹³ found that the rate of peroxidation (R_p) was independent of the rate of radical generation over a rather large concentration range of the initiator (0.5 to 55 mM ABAP). This has since become an important characteristic of tocopherol-mediated peroxidation which describes the kinetics of LDL peroxidation.

To study the relative abilities of charged alkylperoxyl radicals to initiate lipid peroxidation, it was important to check that R_p was independent of R_g over a reasonable concentration range for each initiator.

3.4.2. Results

3.4.2.1. TMP Range of ABAP

Freshly prepared LDL (1.6 μM) from a normal donor was incubated at pH 7.4 and 37°C with three different concentrations of ABAP (1 mM, 10 mM and 55 mM) for 80 minutes (1 half-life for SOTS-1). The concentrations of ABAP were chosen to match the concentration range given by Bowry *et al.*¹³ The initial rates of formation of positively charged alkylperoxyl radicals, $^+\text{ROO}^*$, and the total radical fluxes (based on $e = 0.5$)²³⁻²⁵ for each concentration of ABAP are shown in Table 3A.3. As shown in Figure 3.8., the rate of peroxidation, R_p , was independent of the concentration of ABAP. After 80 minutes, nearly all of the α -TocH in the LDL was consumed by the reaction with 10 mM ABAP but only a small amount of the α -TocH in the LDL was consumed by the reaction with 1 mM ABAP (0.05 nmol α -TocH, 32 %). After 20 minutes, the LDL was depleted of α -TocH by the reaction with 55 mM ABAP and the LDL was no longer undergoing tocopherol-mediated peroxidation. This is shown in Figure 3.8. where after 20 minutes, the rate of peroxidation of LDL increased in the reaction with 55 mM ABAP.

The apparent chain length decreased with increasing concentration of ABAP, as expected (Table 3A.3.). In the presence of α -TocH, R_p remains constant for all concentrations of ABAP, however R_g increases with increasing concentration of ABAP.¹⁵ The reaction of LDL with 1 mM ABAP was propagated by a radical chain ($\chi_{\text{inh}} = 3.1$ where χ_{inh} is the chain length in the “ α -TocH-inhibited” phase, i.e. in the presence of α -TocH) whereas the chain length in the reaction of LDL with

Figure 3.8. Reaction of LDL ($1.6 \mu\text{M}$) with 3 different concentrations of ABAP (1 mM $\circ$, 10 mM $\triangle$ and 55 mM $\square$), incubated for 80 minutes in 50 mM phosphate-buffered saline (pH 7.4) at 37°C . The arrow indicates the time where all of the α -TocH has been consumed in the reaction with 55 mM ABAP.

10 mM ABAP was < 1 ($\chi_{inh} = 0.21$). In the reaction of LDL with 55 mM ABAP, χ_{inh} was smaller than in the chain length in the “uninhibited” phase (i.e. after the consumption of all antioxidants), χ_{uninh} ($\chi_{inh} = 0.056$, $\chi_{uninh} = 0.47$). The chain lengths in the α -ToCH-inhibited phase and in the uninhibited phase depend on R_g .¹⁵ For a radical generation rate $R_g > 2 \text{ nM s}^{-1}$ (as in the case of 55 mM ABAP), it was expected that $\chi_{inh} < \chi_{uninh}$ and the reverse was expected for $R_g < 2 \text{ nM s}^{-1}$.¹⁵

3.4.2.2. TMP Range of SOTS-1

Freshly prepared LDL (1.5 μM) was incubated at pH 7.4 and 37°C with three concentrations of SOTS-1 (200 μM , 450 μM and 750 μM) for 80 minutes (1 half-life for SOTS-1). The concentration range for SOTS-1 was limited by its solubility of ca. 1.5 mM at pH 7 and 25°C. One concentration of SOTS-1 (200 μM) was approximately matched to ABAP (10 mM) in terms of total radical flux at 45 μM $\text{O}_2^{\bullet-}$ for the 80 minute experiment and the initial rate of formation of $\text{O}_2^{\bullet-}$ was 11 nM/s. The total radical fluxes and the initial rates of formation of $\text{O}_2^{\bullet-}$ for each concentration of SOTS-1 are given in Table 3A.4. As shown in Figure 3.9., the rate of formation of LOOH was independent of the concentration of SOTS-1.

The chain length χ_{inh} decreased with increasing concentration of SOTS-1 (see Table 3A.4.). For all SOTS-1 concentrations, the apparent chain length was < 1 , indicating that the oxidation of LDL by SOTS-1-derived $\text{O}_2^{\bullet-}$ may not be a chain reaction. This is not unexpected since it was shown previously that in matched

Figure 3.9. Reaction of LDL (1.5 μM) with 3 different concentrations of SOTS-1 (200 μM Δ , 450 μM $\times$ and 750 μM $\bullet$), incubated for 80 minutes in 50 mM phosphate-buffered saline (pH 7.4) at 37°C.

experiments, $O_2^{\cdot-}$ is not as efficient as $ROO^{\cdot}$ at oxidizing LDL. Possibly the chains are terminated by the reaction:

3.4.2.3. TMP Range of ABCBS

Freshly isolated LDL (1.4 μM) from a normal donor was incubated at pH 7.4 and 37°C with three concentrations of ABCBS (0.45 mM, 3.9 mM and 39 mM) for 160 minutes. LDL had to be incubated longer with ABCBS than in the ABAP and SOTS-1 experiments because of the relatively slow decomposition of this azo compound. It was found that at the lowest concentration of ABCBS (0.45 mM), there was a delay before R_p reached its maximum value. This build-up period corresponds to the time required to establish the steady-state concentration of $\alpha\text{-Toc}^{\cdot}$ in the LDL particles. Therefore, to allow for the “grow-in” of $\alpha\text{-Toc}^{\cdot}$, LDL peroxidation by ABCBS was monitored over 160 minutes rather than over 80 minutes as in the ABAP and SOTS-1 experiments. Two concentrations of ABCBS (3.9 mM and 39 mM) were approximately matched to ABAP (1 mM and 10 mM) in terms of initial rate of formation of $ROO^{\cdot}$ (1.2 $\mu\text{M/s}$ and 12 $\mu\text{M/s}$ of negatively charged alkylperoxyl radicals, $^{\cdot}ROO^{\cdot}$, respectively). However, due to the different incubation periods, the amounts of ABCBS were not matched in terms of total radical flux to the concentrations of ABAP. In this experiment, 13 μM $^{\cdot}ROO^{\cdot}$ was formed from 3.9 mM ABCBS whereas in the 80 minute experiment with 1 mM ABAP, only 5 μM $^{\cdot}ROO^{\cdot}$ was formed. Initial rates of

production of $\text{ROO}^\bullet$ and the total radical fluxes for each ABCBS concentration based on $e = 0.5$ are shown in Table 3A.5.

The results are shown in Figure 3.10. After 80 minutes, all of the $\alpha\text{-TocH}$ in the LDL was consumed by the reaction with 39 mM ABCBS and the LDL was no longer undergoing TMP. In the presence of $\alpha\text{-TocH}$, 3.9 mM and 39 mM ABCBS initiated the peroxidation of LDL at the same rate. However, the peroxidation of LDL initiated by 0.45 mM ABCBS was slower. As shown in Figure 3.10., the rate of formation of LOOH was increasing over time and it appears that the steady-state concentration of $\alpha\text{-Toc}^\bullet$ was not established. Bowry *et al.*¹³ have shown that LDL undergoes tocopherol-mediated peroxidation when the concentration of ABAP is in the range of 0.5 mM to 55 mM. In these matched experiments, 0.45 mM ABCBS corresponds to 0.1 mM ABAP and this concentration is too small to initiate TMP at a steady rate in LDL within the time over which these experiments were run.

The apparent chain lengths for the reaction of LDL with each concentration of ABCBS were calculated as shown in Table 3A.5. As expected, the chain lengths decreased with increasing concentration of ABCBS. The oxidation of LDL by 0.45 mM and 3.9 mM ABCBS was propagated by a radical chain with chain lengths χ_{inh} of 22 and 9.7, respectively. In the presence of $\alpha\text{-TocH}$, LDL peroxidation initiated by 39 mM ABCBS was not formally a chain reaction ($\chi_{\text{inh}} =$

Figure 3.10. Reaction of LDL (1.4 μ M) with 3 concentrations of ABCBS (0.45 mM $\blacktriangle$, 3.9 mM $\circ$ and 39 mM $\triangle$), incubated for 160 minutes in 50 mM phosphate-buffered saline (pH 7.4) at 37°C. The arrow indicates the time where all of the α -Toch has been consumed in the reaction with 39 mM ABCBS.

1.2). After the consumption of α -Toch, LDL oxidation was propagated by a radical chain ($\chi_{\text{uninh}} = 4.5$). As expected for a radical generation rate $R_g > 2 \text{ nM s}^{-1}$, $\chi_{\text{inh}} < \chi_{\text{uninh}}$.¹⁵

3.4.2.4. TMP Range of ABDBA

Freshly prepared LDL (1.5 μM) from a normal donor was incubated at pH 7.4 and 37°C with three concentrations of ABDBA (0.026 mM, 0.26 mM and 2.8 mM) for 160 minutes. Two concentrations of ABDBA (0.26 mM and 2.8 mM) were matched in terms of total radical flux and initial rate of formation of $\text{ROO}^{\bullet}$ to ABCBS (3.9 mM and 39 mM) and are therefore also matched to 1 mM and 10 mM ABAP, it being assumed that $e = 0.5$ for all these initiators. In this time, 13 μM $\text{ROO}^{\bullet}$ would have been formed by ABDBA (0.26 mM) and the initial rate of formation was 1.3 nM/s $\text{ROO}^{\bullet}$. The total radical fluxes and initial rates of $\text{ROO}^{\bullet}$ production from ABDBA are shown in Table 3A.6.

LDL peroxidation initiated by both 0.26 mM and 2.8 mM ABDBA occurred at the same rate in the presence of α -Toch (Figure 3.11.). After 60 minutes, all of the α -Toch in the LDL was consumed by the reaction with 2.8 mM ABDBA and the rate of peroxidation was no longer independent of radical flux. The rate of LOOH formation was slower in the reaction with 0.026 mM ABDBA, as expected. In these matched experiments, 0.026 mM ABDBA corresponds to 0.1 mM ABAP and is smaller than the minimum concentration required to initiate TMP at a steady rate in LDL within the time these experiments were run. As shown in

Figure 3.11. Reaction of LDL ($1.5 \mu\text{M}$) with 3 different concentrations of ABDBA (0.026 mM +, 0.26 mM $\circ$ and 2.8 mM $\triangle$), incubated for 160 minutes in 50 mM phosphate-buffered saline ($\text{pH } 7.4$) at 37°C . The arrow indicates the time where all of the α -TocH has been consumed in the reaction with 2.8 mM ABDBA.

Figure 3.11., the rate of peroxidation was increasing with time and therefore, the α -Toc $^{\bullet}$ in the LDL had not attained a steady-state concentration.

The apparent radical chain lengths were calculated and decreased with increasing concentration of ABDBA (Table 3A.6.). LDL peroxidation initiated by 0.026 mM ABDBA and 0.26 mM ABDBA was propagated by a radical chain with chain lengths χ_{inh} of 64 and 14 respectively. As expected¹⁵ for $R_p > 2 \text{ nM s}^{-1}$, the chain length in α -TocH-inhibited phase was smaller than in the uninhibited phase ($\chi_{inh} = 1.1$, $\chi_{uninh} = 3.6$).

3.4.2.5. TMP Range of ABMHP

Freshly isolated LDL (1.5 μM) from a normal donor was incubated at pH 7.4 and 37°C with three concentrations of ABMHP (1.1 mM, 6.6 mM and 56 mM) for 160 minutes. The solubility of ABMHP is limited to ca. 70 mM at pH 7 and 25°C in buffer solution. One concentration of ABMHP (6.6 mM) was matched to both ABCBS (3.9 mM) and ABDBA (0.26 mM) in terms of total radical flux based on the assumption that the efficiency of cage escape of the initiating radicals e was the same from ABMHP as from the other (charged) azo initiators, i.e. 0.5. This assumption turned out to be incorrect for ABMHP though it would appear to be approximately valid for the three charged azo initiators (see below). The apparent total radical flux was set at 13 $\mu\text{M ROO}^{\bullet}$ and the apparent initial rate of formation of $\text{ROO}^{\bullet}$ at 1.2 nM/s. The initial rates of formation of $\text{ROO}^{\bullet}$ and the total radical fluxes for each concentration of ABMHP are given in Table 3A.7.

The results are shown in Figure 3.12. In these experiments, it became obvious that ABMHP was much less efficient at oxidizing LDL than the charged azo-initiators. After 160 minutes, only ~ 70 % of the α -TocH in the LDL was consumed by the reaction with 56 mM ABMHP implying that e is considerably less than 0.5 for this initiator. As shown in Figure 3.12., the initial rate of LOOH accumulation increases over time and therefore a longer time is required to establish the steady state concentration of α -Toc $^{\bullet}$ and to attain the maximal R_p .

The apparent chain lengths were calculated for the reaction of LDL with each concentration of ABMHP. As shown in Table 3A.7., the chain lengths decreased with increasing concentration of ABMHP. The peroxidation of LDL by 1.1 mM ABMHP and 6.6 mM ABMHP was propagated by a radical chain ($\chi = 6.1$ and $\chi = 4$, respectively). The oxidation of LDL by 56 mM ABMHP was not a chain reaction ($\chi = 0.79$).

3.5 Effect of Electrostatic Charge in Experiments Matched for Total Radical Flux

3.5.1. Results

Freshly prepared LDL (2.0 μ M) was incubated at pH 7.4 and 37°C with ABAP (1.0 mM), ABCBS (4.2 mM) and ABMHP (7.3 mM) for 24 hours. The amounts of ABAP, ABCBS and ABMHP were matched in terms of their *apparent* radical

Figure 3.12. Reaction of LDL (1.5 μ M) with 3 different concentrations of ABMHP (1.1 mM $\diamond$, 6.6 mM $\circ$ and 56 mM $\triangle$), incubated for 160 minutes in 50 mM phosphate-buffered saline (pH 7.4) at 37°C.

fluxes. These were set at 0.1 mM ROO[•] for the 24 hour experiments and the initial rate of formation of ROO[•] was 1.3 nM/s (based on the assumption that $e = 0.5$ in all cases). In matched experiments, the neutral alkylperoxyl radicals were much less efficient at oxidizing LDL than charged ROO[•] (Figure 3.13).

All of the α -ToCH in the LDL was consumed by the reaction with ABAP and ABCBS and was consumed at approximately the same rate (~ 0.3 nM/s) and in approximately the same time (~ 10 hours) (see Figure 3.13. and Table 3A.8.). This demonstrates that e is about the same for these two initiators. Since it is known that $e = 0.5$ for ABAP,²⁵ e must be about 0.5 for the azo compound yielding negatively charged alkyl radicals (and alkylperoxyl radicals). After incubation of the LDL with ABMHP for 24 hours, the LDL was still undergoing TMP with only 70 % of the α -ToCH consumed. In the reaction of LDL with ABMHP, α -ToCH was consumed at a much slower rate of ~ 0.08 nM/s. From this I concluded that the efficiency of cage escape e was ≈ 0.15 for ABMHP.

The apparent chain lengths ($\chi = R_p/R_g$) were calculated for both the α -ToCH-inhibited phase and the uninhibited phase (see Table 3A.8.). Both α -ToCH-inhibited and uninhibited LDL peroxidation by ABAP, ABCBS and ABMHP were propagated by a radical chain. As expected for a radical generation rate $R_g < 2$ nM s⁻¹, $\chi_{inh} > \chi_{uninh}$ for LDL oxidation initiated by both ABAP and ABCBS.¹⁵ Since these azo initiators reside entirely or almost entirely in the aqueous phase, R_g is

Figure 3.13. Reaction of LDL (2.0 μ M) with ABAP (1 mM, ■), ABCBS (4.2 mM, ▲) and ABMHP (7.3 mM, ●) incubated for 24 hours in 50 mM phosphate-buffered saline (pH 7.4) at 37°C.

assumed to be independent of species present in LDL. However, previous studies in micelles and liposomes have shown that the rate of initiation R_i in lipid dispersions is only 20 % to 60 % of the known R_g .²⁸ This is consistent with the values of the efficiency of initiation of lipid peroxidation, R_i/R_g , shown in Table 3A.8. Since R_i is only a fraction of the known R_g , the apparent chain lengths are lower than the true chain lengths χ' ($\chi' = R_p/R_i$) calculated from the rate of α -ToCH consumption, $R_{\alpha\text{-ToCH}}$ ($R_i = 2 R_{\alpha\text{-ToCH}}$). The chain length χ' can only be calculated for α -ToCH-inhibited peroxidation because R_i cannot be measured in the uninhibited phase of LDL oxidation.

The rate of initiation R_i by ABMHP is only ca. 12 % of the known R_g (Table 3A.8.). ABMHP has only limited solubility in aqueous solution (~ 70 mM at pH 7 and 25°C). It therefore appeared plausible that ABMHP had sufficient lipid solubility to dissolve in the LDL and initiate oxidation from within the lipid particles.

3.6. Does ABMHP Dissolve in LDL Particles?

3.6.1. Introduction

In their studies of TMP, Bowry *et al.*¹³ showed that the efficiency of initiation of LDL peroxidation by peroxy radicals derived from a lipid-soluble azo compound, AMVN (2,2'-azobis(2,4-dimethylvaleronitrile)), was lower than the efficiency of initiation by peroxy radicals derived from a water-soluble azo compound (ABAP).¹³ With AMVN, initiation relies on the escape of one radical from the

geminate pair of radicals formed in the LDL particle by the decomposition of the AMVN. The low efficiency of initiation by AMVN is due to the competition between “escape” of one radical from LDL and radical recombination, which destroys the two radicals so that no chain is started. The high microviscosity inside the LDL also reduces the efficiency of cage escape of one radical of the geminate pair, favoring radical/radical destruction (Figure 3.14.).

To check whether ABMHP dissolves in LDL, the partitioning of ABMHP from the aqueous solution into an organic solvent was studied.

3.6.2. Results

A saturated solution of ABMHP (55 mM) was prepared in millipore water and the UV-visible spectrum was recorded ($A_{365} \sim 1.7$). An aliquot of this solution was extracted into an aliquot of equal volume of toluene, chloroform, hexane, diethyl ether and olive oil. Olive oil consists mostly of triglycerides and it was chosen because it is chemically more similar to LDL than the simple organic solvents. After extraction, the aqueous layers were removed and the UV-visible spectra were recorded. After extraction into each of the organic solvents mentioned above, the residual intensity of the azo band of ABMHP was 100% of its initial value (Table 3A.9.). Therefore, it would appear from these results that ABMHP remains in aqueous solution and does not partition into any of the organic solvents studied and therefore presumably does not partition into LDL particles.

Figure 3.14. LDL peroxidation by a lipid-soluble azo initiator. Initiation of oxidation depends on the competition between “escape” and radical recombination.¹³

3.7. LDL Peroxidation by ABMHP

3.7.1. Introduction

As shown in section 3.6., ABMHP does not have sufficient lipid solubility to dissolve in LDL and therefore, it must be assumed that ABMHP initiates LDL oxidation from the aqueous phase. However, with ABMHP LDL peroxidation occurred with a lower than expected rate of initiation (R_i) as judged by the rate of vitamin E consumption when compared to oxidation by the other water-soluble azo initiators (ABAP and ABCBS). The kinetics of LDL oxidation by neutral $\text{ROO}^\bullet$ from ABMHP were further investigated by incubating the LDL with ABMHP at ca. 80 % of its solubility limit (~ 70 mM at pH 7 and 25°C).

3.7.2. Results

Freshly isolated LDL ($1.9\ \mu\text{M}$) was incubated at pH 7.4 and 37°C with ABMHP (57 mM) for 8 hours. In this experiment, all of the $\alpha\text{-Toch}$ in the LDL was consumed after 4 hours (see Figure 3.15.). The $\alpha\text{-tocopherol}$ was consumed at a rate of $0.71\ \text{nM/s}$, corresponding to a R_i of $1.4\ \text{nM/s}$. The time at which the LDL was depleted of $\alpha\text{-Toch}$ in this experiment is consistent with that predicted by the oxidation of LDL by $7.3\ \text{mM}$ ABMHP (Figure 3.13.). In that experiment, it was predicted by the rate of consumption of $\alpha\text{-Toch}$ that at ~ 30 hours, all of the $\alpha\text{-Toch}$ in the LDL would be consumed by the reaction with $7.3\ \text{mM}$ ABMHP. Therefore, increasing the concentration of ABMHP by a factor of 8 (57 mM), the time at which the LDL was depleted of $\alpha\text{-Toch}$ decreased by a factor of ~ 8 (to 4 hours, as shown in Figure 3.15.). The LOOH yield versus time plot also

Figure 3.15. Reaction of LDL (1.9 μ M) with ABMHP (57 mM), incubated for 8 hours in 50 mM phosphate-buffered saline (pH 7.4) at 37°C.

displayed a curve shape consistent with TMP. If the sample had been incubated for a longer time, I would expect to observe LDL oxidation continuing in the absence of α -ToCH, giving rise to the sigmoid-shaped curve typical of TMP. This experiment served to confirm that for ABMHP e is only ca. 0.15.

3.8. Effect of Electrostatic Charge in Experiments Matched for the Rate of Initiation

3.8.1. Introduction

In (apparently) total radical flux matched experiments, the neutral alkylperoxyl radicals were much less efficient at oxidizing LDL than the charged peroxyl radicals (Figure 3.13.). To further investigate the abilities of alkylperoxyl radicals with different electrostatic charges to initiate the peroxidation of LDL, the concentrations of the initiators were matched according to the rates at which they consumed α -ToCH in LDL.

3.8.2. Results

Freshly isolated LDL (4.4 μ M) was incubated at pH 7.4 and 37°C with ABAP (1.0 mM), ABCBS (3.8 mM), ABDBA (0.21 mM) and ABMHP (31 mM) for 30 hours. The amounts of ABAP, ABCBS and ABDBA were matched in terms of their calculated radical fluxes. These were set at 0.1 mM ROO $^{\bullet}$ for the 30 hour experiments and the initial rate of formation of ROO $^{\bullet}$ was 1.3 nM/s (based on the assumption that $e = 0.5$). The amount of ABMHP was matched to the concentration of ABAP, ABCBS and ABDBA according to the rate of α -ToCH consumption (~ 0.6 nM/s). In matched experiments, both charged and neutral

alkylperoxyl radicals oxidized LDL with the same efficiency. As shown in Figure 3.16., both charged and neutral $\text{ROO}^\bullet$ consumed $\alpha\text{-TocH}$ at the same rate and after 10 hours, all of the $\alpha\text{-TocH}$ in the LDL was consumed.

In these genuinely matched experiments, $^\bullet\text{ROO}^\bullet$ from both ABDBA and ABAP, $^\bullet\text{ROO}^\bullet$ from ABCBS and neutral $\text{ROO}^\bullet$ from ABMHP consumed $\alpha\text{-TocH}$ at the same rate (Figure 3.16.) and their LOOH yield versus time plots displayed the sigmoid shape consistent with TMP. (These results indicate that the hydrolysis of ABAP is negligible over the period of 30 hours, as predicted previously by the studies of the thermal decomposition of ABAP, section 3.2.2.). The apparent chain lengths were calculated for both the $\alpha\text{-TocH}$ -inhibited and uninhibited phases of LDL oxidation (see Table 3A.10.). LDL peroxidation by all azo initiators was propagated by a radical chain. As expected for a radical generation rate $R_g < 2 \text{ nM s}^{-1}$, $\chi_{\text{inh}} > \chi_{\text{uninh}}$ for LDL oxidation initiated by all four azo compounds.

3.9. Summary and Conclusions

Previous kinetic studies on the thermal decomposition of ABAP have shown that it undergoes hydrolysis to the corresponding amide (reaction 3.5.).²⁶ ABAP has been commonly used to initiate the oxidation of several biological target molecules but its hydrolysis has usually been ignored. For the treatment of LDL

Figure 3.16. Reaction of LDL ($4.4 \mu\text{M}$) with ABAP (1.0 mM, □), ABCBS (3.8 mM, △), ABDBA (0.21 mM, ◇) and ABMHP (31 mM, ○), incubated for 30 hours in 50 mM phosphate-buffered saline (pH 7.4) at 37°C .

with matched amounts of each azo initiator, the rate of hydrolysis and the amount of ABAP that underwent hydrolysis were determined. From both UV-visible spectroscopy and ^1H NMR studies, it was concluded that 22 % of ABAP hydrolysed to the amide with a rate constant $k = 2.4 \times 10^{-7} \text{ s}^{-1}$ under physiological conditions (pH 7 and 37°C). The hydrolysis of ABAP can simply be neglected over the incubation time for the studies of LDL oxidation by ABAP. The relative unimportance of hydrolysis was further confirmed by the comparison of LDL oxidation by ABAP and ABDBA. In total radical flux matched experiments, ABAP and ABDBA consumed α -Toch at the same rate and their LOOH yield versus time plots were nearly identical (see Figure 3.16.).

For the study of the abilities of positively charged, negatively charged and neutral alkylperoxyl radicals to initiate LDL peroxidation, it was important to treat the LDL with approximately equal fluxes of these radicals. The rates of thermal decomposition of the azo initiators were measured by ^1H NMR by monitoring the decrease in the intensity of the methyl protons. Decay of these azo compounds followed clean first-order kinetics in all cases and the derived first-order rate constants were: ABAP $k = 1.1 \times 10^{-6} \text{ s}^{-1}$; ABCBS $k = 3.5 \times 10^{-7} \text{ s}^{-1}$; ABDBA $k = 5.2 \times 10^{-6} \text{ s}^{-1}$; ABMHP $k = 1.8 \times 10^{-7} \text{ s}^{-1}$.

In their studies of tocopherol-mediated peroxidation, Bowry *et al.*¹³ showed that the rate of peroxidation (R_p) was independent of the rate of radical generation (R_g) over a rather large concentration range of the initiator (0.5 to 55 mM

ABAP).¹³ To check whether LDL peroxidation by SOTS-1, ABCBS, ABDBA and ABMHP also followed TMP kinetics, LDL oxidation by a range of concentrations of these initiators was studied. As shown in section 3.4., the rate of LOOH accumulation was independent of the concentration of the initiator (SOTS-1, ABCBS, ABDBA and ABMHP) in the “ α -ToCH-inhibited” phase. The apparent chain lengths of oxidation in the α -ToCH-inhibited phase and in the uninhibited phase were calculated for each concentration of each initiator. In the presence of α -ToCH, R_p is independent of the concentration of the initiator however R_g increases with concentration. Therefore, the apparent chain length of oxidation ($\chi = R_p/R_g$) decreased with increasing initiator concentration.

In *apparently* total radical flux matched experiments, the neutral alkylperoxyl radicals from ABMHP were found to be much less efficient at oxidizing LDL than the charged alkylperoxyl radicals from the other azo compounds (Figure 3.13.). My results showed that the rate of initiation by ABMHP was only ca. 12 % of the measured rate of decomposition of ABMHP, rather than the expected ca. 50 % (Table 3A.8.). From these results, it appeared possible that the neutral azo initiator (ABMHP) had sufficient lipid-solubility to partition into the LDL particles and initiate oxidation from within these particles, which would lead to a low e value and hence a lower than otherwise expected rate of initiation (see Scheme 3.1.). However, my experiments showed that upon extraction of an aqueous solution of ABMHP into a series of organic solvents, ABMHP remained in the aqueous phase. I therefore concluded that the efficiency of cage escape is lower

for neutral radicals ($e \approx 0.15$). Escape of the geminate radical pair from the water solvent cage might be expected to be greater (i.e. ~ 0.5) for charged radicals (because of electrostatic repulsion between the like charges) than for neutral radicals. To investigate the effects of electrostatic charge on the abilities of radicals $\text{ROO}^\bullet$ to initiate LDL peroxidation, the amounts of each azo initiator were matched in terms of their rates of α -tocopherol consumption (and hence in terms of R_i). In these genuinely matched experiments, the charged and neutral alkylperoxyl radicals oxidized LDL with the same efficiencies and all showed sigmoid plots of lipid hydroperoxides versus time which are characteristic of TMP. I conclude that the initiation of LDL peroxidation by water-soluble alkylperoxyl radicals is probably independent of the nature (charge) of the alkylperoxyl radical. However, superoxide (the simplest peroxyl radical) is much less efficient at initiating LDL peroxidation. It probably initiates via its conjugate acid $\text{HOO}^\bullet$ and then is more efficient at terminating TMP chains than water-soluble alkylperoxyl radicals thanks to a reaction between the superoxide anion and the α -tocoperoxyl radical (reaction 3.9.).

APPENDIX 3A.

Tables of Data From Experiments Described in Chapter 3.

Table 3A.1. The thermal decomposition of ABAP (50 mM) in 50 mM phosphate-buffered saline (pH 7.4) at 37°C monitored by UV-visible spectroscopy.

Time (days)	Absorbance ($\lambda = 365$ nm)	% ABAP Remaining (calculated from absorbance)	% ABAP Remaining (calculated from $A_t = A_0 e^{-kt}$)
0	1.20	100	100
4	0.83	69	66
20	0.39	33	13
30	0.32	27	5

Table 3A.2. Rate constants and half-lives of the thermal decomposition of ABAP, ABCBS, ABDBA and ABMHP, determined by the decay of the methyl protons at 37°C in D₂O using ¹H NMR (400 MHz).

Initiator	Rate constant k (10⁶ s⁻¹)	Half-life t_{1/2} (days)	Literature k ²⁹ (10⁶ s⁻¹)
ABAP	1.1	7	1.4
ABCBS	0.35	23	-
ABDBA	5.2	1.5	11.3
ABMHP	0.18	45	0.22

Table 3A.3. Reaction of LDL (1.6 μM) with 3 concentrations of ABAP (1 mM, 10 mM and 55 mM), incubated for 80 minutes in 50 mM phosphate-buffered saline (pH 7.4) at 37°C. R_g is the initial rate of $^{\bullet}\text{ROO}^{\bullet}$ production based on the assumption that $e = 0.5$ ($R_g = 1.1 \times 10^{-6} [\text{ABAP}] \text{ s}^{-1}$). R_p^{inh} is the maximum rate of LOOH accumulation in the presence of $\alpha\text{-TocH}$. R_p^{uninh} is the maximum rate of LOOH accumulation in the absence of $\alpha\text{-TocH}$. The apparent radical chain length χ is R_p/R_g .

[ABAP] (mM)	R_g (10^9 M/s)	Total Radical Flux (μM)	R_p^{inh} (10^9 M/s)	R_p^{uninh} (10^9 M/s)	χ_{inh}	χ_{uninh}
1.0	1.1	5.3	4.4	-	3.1	-
10	11	53	2.9	-	0.21	-
55	61	290	4.3	3.6	0.056	0.47

Table 3A.4. Reaction of LDL (1.5 μM) with 3 concentrations of SOTS-1 (200 μM , 450 μM and 750 μM), incubated for 80 minutes in 50 mM phosphate-buffered saline (pH 7.4) at 37°C. R_g is the initial rate of $\text{O}_2^{\bullet-}$ formation ($R_g = 2e(1.46 \times 10^{-4} \text{ s}^{-1}) [\text{SOTS-1}]$; $e = 0.20$). R_p^{inh} is the maximum rate of LOOH accumulation in the presence of $\alpha\text{-TocH}$. The apparent radical chain length is $\chi = R_p/R_g$.

[SOTS-1] (μM)	R_g (10^9 M/s)	Total Radical Flux (μM)	R_p^{inh} (10^9 M/s)	χ_{inh}
200	12	40	2.5	0.22
450	26	91	3.2	0.12
750	44	150	3.1	0.07

Table 3A.5. Reaction of LDL (1.4 μM) with 3 concentrations of ABCBS (0.45 mM, 3.9 mM and 39 mM), incubated for 160 minutes in 50 mM phosphate-buffered saline (pH 7.4) at 37°C. R_g is the initial rate of $\text{ROO}^\bullet$ production based on the assumption that $e = 0.5$ ($R_g = 3.5 \times 10^{-7}$ [ABCBS] s^{-1}). R_p^{inh} is the maximum rate of LOOH accumulation in the presence of $\alpha\text{-Toch}$. R_p^{uninh} is the maximum rate of LOOH accumulation in the absence of $\alpha\text{-Toch}$. The apparent radical chain length χ is R_p/R_g .

[ABCBS] (mM)	R_g (10^9 M/s)	Total Radical Flux (μM)	R_p^{inh} (10^9 M/s)	R_p^{uninh} (10^9 M/s)	χ^{inh}	χ^{uninh}
0.45	0.16	1.5	3.4 ^a	-	22	-
3.9	1.4	13	13	-	9.7	-
39	14	130	17	62	1.2	4.5

^aSteady state not reached in 160 minutes, see Figure 3.10.

Table 3A.6. Reaction of LDL (1.5 μM) with 3 concentrations of ABDBA (0.026 mM, 0.26 mM and 2.8 mM), incubated for 160 minutes in 50 mM phosphate-buffered saline (pH 7.4) at 37°C. R_g is the initial rate of $^{\bullet}\text{ROO}^{\bullet}$ production based on the assumption that $e = 0.5$ ($R_g = 5.2 \times 10^{-6}$ [ABDBA] s^{-1}). R_p^{inh} is the maximum rate of LOOH accumulation in the presence of $\alpha\text{-Toch}$. R_p^{uninh} is the maximum rate of LOOH accumulation in the absence of $\alpha\text{-Toch}$. The apparent radical chain length χ is R_p/R_g .

[ABDBA] (mM)	R_g (10^9 M/s)	Total Radical Flux (μM)	R_p^{inh} (10^9 M/s)	R_p^{uninh} (10^9 M/s)	χ_{inh}	χ_{uninh}
0.026	0.14	1.3	8.7 ^a	-	64	-
0.26	1.4	13	19	-	14	-
2.8	15	140	17	52	1.1	3.6

^aSteady state not reached in 160 minutes, see Figure 3.11.

Table 3A.7. Reaction of LDL (1.5 μM) with 3 concentrations of ABMHP (1.1 mM, 6.6 mM and 56 mM), incubated for 160 minutes in 50 mM phosphate-buffered saline (pH 7.4) at 37°C. R_g is the initial rate of $\text{ROO}^\bullet$ formation based on the assumption that $e = 0.5$ ($R_g = (1.8 \times 10^{-7} \text{ s}^{-1}) [\text{ABMHP}]$). R_p^{inh} is the maximum rate of LOOH accumulation in the presence of $\alpha\text{-Toch}$. The apparent radical chain length is $\chi = R_p/R_g$.

[ABMHP] (μM)	R_g (10^9 M/s)	Total Radical Flux (μM)	R_p^{inh} (10^9 M/s)	χ_{inh}
1.1	0.20	1.9	1.2 ^a	6.1
6.6	1.2	11	4.8	4.0
56	10	97	8.0	0.79

^aSteady state not reached in 160 minutes, see Figure 3.12.

Table 3A.8. Reaction of LDL (2.0 μM) with ABAP (1.0 mM), ABCBS (4.2 mM) and ABMHP (7.3 mM), incubated for 24 hours in 50 mM phosphate-buffered saline (pH 7.4) at 37°C. R_g is the initial rate of $\text{ROO}^\bullet$ production based on the assumption that $e = 0.5$ (R_g (ABAP) = 1.1×10^{-6} [ABAP] s^{-1} , R_g (ABCBS) = 3.5×10^{-7} [ABCBS] s^{-1} , R_g (ABMHP) = $(1.8 \times 10^{-7} \text{ s}^{-1})$ [ABMHP]). R_p^{inh} is the maximum rate of LOOH accumulation in the presence of $\alpha\text{-TocH}$. R_p^{uninh} is the maximum rate of LOOH accumulation in the absence of $\alpha\text{-TocH}$. $R_{\alpha\text{-TocH}}$ is the rate of consumption of $\alpha\text{-TocH}$. The rate of initiation is $R_i = 2 R_{\alpha\text{-TocH}}$. The apparent radical chain length χ is R_p/R_g . The radical chain length of oxidation χ' is R_p/R_i . The efficiency of lipid initiation is termed as R_i/R_g .

	LDL + ABAP	LDL + ABCBS	LDL + ABMHP
Total Radical Flux (μM)	91	120	110
R_g (10^9 M/s)	1.1	1.5	1.3
R_p^{inh} (10^9 M/s)	2.6	3.9	0.97
R_p^{uninh} (10^9 M/s)	1.2	1.0	-
$R_{\alpha\text{-TocH}}$ (10^{10} M/s)	2.9	2.7	0.78
R_i (10^{10} M/s)	5.8	5.4	0.16
χ^{inh} (R_p^{inh}/R_g)	2.4	2.7	0.74
χ^{uninh} (R_p^{uninh}/R_g)	1.1	0.69	-
χ' (R_p^{inh}/R_i)	4.5	7.2	6.2
R_i/R_g	0.53	0.37	0.12

Table 3A.9. Extraction of an aqueous solution of ABMHP (55 mM) into toluene, chloroform, hexane, diethyl ether and olive oil. The initial absorbance of the solution of ABMHP in water was 1.55 at $\lambda = 365$ nm.

Organic Solvent	Absorbance of Water Phase After Extraction ($\lambda = 365$ nm)	Amount of ABMHP in Water Phase (%)
Toluene	1.52	98
Chloroform	1.48	95
Hexane	1.54	99
Diethyl ether	1.48	95
Olive oil	1.54	99

Table 3A.10. Reaction of LDL (4.4 μM) with ABAP (1.0 mM), ABCBS (3.8 mM), ABDBA (0.21 mM) and ABMHP (31 mM), incubated for 30 hours in 50 mM phosphate-buffered saline (pH 7.4) at 37°C. R_g is the initial rate of $\text{ROO}^\bullet$ production based on the assumption that $e = 0.5$ ($R_g(\text{ABAP}) = 1.1 \times 10^{-6} [\text{ABAP}] \text{ s}^{-1}$, $R_g(\text{ABCBS}) = 3.5 \times 10^{-7} [\text{ABCBS}] \text{ s}^{-1}$, $R_g(\text{ABDBA}) = 5.2 \times 10^{-6} [\text{ABDBA}] \text{ s}^{-1}$). The total radical flux and R_g for ABMHP were calculated assuming $e = 0.15$ ($R_g = 2e (1.8 \times 10^{-7} \text{ s}^{-1}) [\text{ABMHP}]$). R_p^{inh} is the maximum rate of LOOH accumulation in the presence of $\alpha\text{-Toch}$. R_p^{uninh} is the maximum rate of LOOH accumulation in the absence of $\alpha\text{-Toch}$. $R_{\alpha\text{-Toch}}$ is the rate of consumption of $\alpha\text{-Toch}$. The rate of initiation is $R_i = 2 R_{\alpha\text{-Toch}}$. The apparent radical chain length χ is R_p/R_g . The radical chain length of oxidation χ' is R_p/R_i .

	LDL + ABAP	LDL + ABCBS	LDL + ABDBA	LDL + ABMHP
Total Radical Flux (μM)	110	140	90	180
R_g (10^9 M/s)	1.1	1.3	1.1	1.7
R_p^{inh} (10^8 M/s)	1.5	2.0	1.7	1.4
R_p^{uninh} (10^9 M/s)	9.3	5.4	5.8	2.9
$R_{\alpha\text{-Toch}}$ (10^{10} M/s)	7.0	6.3	6.7	5.0
R_i (10^9 M/s)	1.4	1.3	1.4	1.0
χ_{inh} (R_p^{inh}/R_g)	14	15	15	8.2
χ_{uninh} (R_p^{uninh}/R_g)	8.5	4.1	5.3	1.7
χ' (R_p^{inh}/R_i)	11	15	12	14

4. Experimental Procedures

Materials. Microperoxidase (MP-11), cholesterol, *tert*-butyl hydroperoxide (70% in water), Chelex 100, sodium tetraborate, isoluminol, cholesteryl benzoate, superoxide dismutase, catalase, deferoxamine mesylate and xanthine oxidase were purchased from Sigma and used as received. Acetaldehyde, sodium cholate and α -tocopherol were purchased from Aldrich. Acetaldehyde was distilled before use. 2,2'-Azobis(2-amidinopropane) dihydrochloride, 2,2'-azobis[2-methyl-*N*-(2-hydroxyethyl)-propionamide] and 2,2'-azobis(*N,N'*-dimethylene-isobutyramidine) dihydrochloride were obtained from Wako Chemicals. 1-Palmitoyl-2-linoleoyl-*sn*-glycero-3-phosphocholine was purchased from Avanti Polar Lipids. All HPLC analytical columns, guard columns and Sephadex PD-10 columns were purchased from Supelco. SOTS-1 was prepared by Ms. S. Lin according to our published procedure.³ 3,3'-Azobis(3-cyano-1-butanefulfonic acid) disodium salt was synthesized by Dr. T. Paul. Methanol and isopropyl alcohol were used at their HPLC grade and stored over molecular sieves and Chelex and filtered through a 0.2 micron nylon Millipore membrane before use. All glassware was sterilized by autoclaving before use.

Preparation of Phosphate-Buffered Saline Solution. PBS buffer (250 mM) was prepared (48.3 g $\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$, 11.0 g $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$, 43.8 g NaCl in 1 L H_2O) using Millipore water and stored over Chelex. PBS (50 mM) was prepared by a 1:5 dilution of 250 mM PBS. The pH was adjusted to pH 7.4 and the buffer was

sterilized by filtration over a 0.2 micron nylon Millipore membrane into a sterile container.

Isolation of Human LDL. Venous blood from a non-fasting male donor was collected in eight 7 mL lithium heparin Vacutainers. The blood samples were centrifuged at maximum speed for 10 minutes in a clinical centrifuge. The plasma (total volume approximately 17 mL) was decanted using sterilized Pasteur pipettes, transferred to a sterilized plastic tube and kept on ice. The density of the plasma was adjusted to 1.21 g/mL using spectrograde KBr (326.5 mg/mL). Quick seal centrifuge tubes (12 mL volume) were prepared by adding 7.25 mL of 50 mM PBS to each tube. The tubes were centrifuged at 55 000 rpm for 4 hours using a 70.1Ti rotor in a Beckman preparative ultracentrifuge. After centrifugation, the LDL band was visualized as a yellow layer near the middle of the tube. The tubes were pierced just below the band using a 5 mL sterile syringe and the LDL layer was slowly drawn into the syringe. The LDL (total volume approximately 7 mL) was transferred to a sterile tube and stored at 5°C.

Experiments with LDL. The LDL was purified (removal of KBr) and separated from water-soluble antioxidants (ascorbate, urate) by passing it through a Sephadex PD-10 size exclusion column. The LDL was incubated in PBS (50 mM, pH 7.4) with the radical initiator at 37°C. Prior to analysis by HPLC, the polar lipids were separated from the non-polar lipids by extraction. Extraction mixtures containing 2 mL methanol with 0.02% acetic acid, 50 μ L of 170 μ M

cholesteryl benzoate (internal standard) in chloroform and 10 mL hexane were prepared in Kimax glass centrifuge tubes. The hexane was previously stored over Millipore water (3:1 hexane:water). A 0.2 mL aliquot of oxidized LDL solution was added to the extraction mixture and the mixture was vortexed for approximately 1 minute. The extraction tubes were then centrifuged at maximum speed in a clinical centrifuge for approximately 15 minutes. An aliquot of 8 mL of the hexane layer was removed and evaporated to dryness under a stream of N₂. The residue was dissolved in 0.2 mL of 1:1 methanol:isopropanol.

Preparation of PLPC/chole mixed micelles. In a glovebag purged with N₂, PLPC (25.0 mg) was dissolved in chloroform (1.0 mL). An aliquot (170 µL) was removed and the CHCl₃ was evaporated under a stream of N₂. PBS buffer (50 mM, 281 µL) and sodium cholate solution in PBS (30 mg/mL, 109 µL) were added to the lipid film and the mixture was vortexed. The solution was passed through a polycarbonate membrane (pore diameter = 100 nm) 25 times in a hand-held extrusion apparatus. The size of the PLPC/chole mixed micelles was measured using the Nicomp Submicron Particle Sizer Autodilute Model 370. After incubation of the PLPC/chole mixed micelles with the initiator, a 0.2 mL aliquot of the sample was removed and placed in methanol (1 mL). The methanol was evaporated under a stream of N₂ and the residue was dissolved in 0.2 mL methanol.

HPLC with Chemiluminescence Detection (CL-HPLC). Chromatographs were obtained using a Hewlett Packard Series II 1090 Liquid Chromatograph equipped with a Shodex CL-2 Chemiluminescence Detector and a Neslab RTF-8 Refrigerated Circulating Bath. Samples were stored in the sample compartment at 4°C prior to analysis. Data were compiled on a PC using HP software. The HPLC was equipped with a C18 guard column and a LC18DB reverse phase column. LDL experiments were performed with 1:1 methanol:isopropanol as solvent at a flow rate of 1 mL/min. Under these conditions, Ch18:2OOH eluted at approximately 9.1 minutes. PLPC/cholate mixed micelles experiments were performed with 100% methanol as eluent at a flow rate of 1 mL/min. PCOOH eluted at approximately 6.3 minutes. Chemiluminescence was detected in the range of 0 to 1 000 mV.

Preparation of isoluminol/microperoxidase solution. Sodium borate buffer (100 mM, pH 10) was prepared (76.3 g $\text{Na}_2\text{B}_4\text{O}_7 \cdot 10\text{H}_2\text{O}$, 6.3 g NaOH in 2 L) using Millipore water and stored over Chelex. Methanol (500 mL) and borate buffer (500 mL) were filtered through a 0.2 micron nylon Millipore membrane. Isoluminol (350 mg) was dissolved in 500 mL of methanol and 500 mL borate buffer. Microperoxidase (5.0 mg) was dissolved in the solution. This solution was used for postcolumn HPLC chemiluminescence detection.

HPLC with Electrochemical Detection (EC-HPLC). Chromatographs were obtained using a Hewlett Packard 1090 Liquid Chromatograph equipped with a

Figure 4.1. Schematic diagram of the HPLC set-up for the separation of lipid oxidation products.

Hewlett Packard 1049A Programmable Electrochemical Detector. Data were compiled on a PC using HP software. Experiments were performed with 55% methanol with 2 mM LiCl and 45% isopropanol with 2 mM LiCl as solvent at a flow rate of 1 mL/min using a C18 guard column and a LC18DB reverse phase column. Electrochemical detection was measured in the range -0.4 V to 1.4 V with an applied potential of 0.6 V.

Determination of the extraction efficiency. The efficiency of the extraction of the non-polar core lipids of LDL into the hexane layer was monitored using cholesteryl benzoate as an internal standard (see Experiments with LDL section). Due to the small volume of solvent (0.2 mL) used to dissolve the hexane residue, some of the non-polar lipids may be lost on the sides of the vessel.

A stock solution of cholesteryl benzoate (50 μ M) was prepared in 1:1 methanol:isopropanol. The following injection volumes were made on the HPLC: 1 , 5 , 10 , 15 , 20 and 25 μ L using 1:1 methanol:isopropanol as eluent. At a flow rate of 1 mL/min, cholesteryl benzoate eluted at 10.7 minutes (234 nm). In each LDL experiment the area of the Ch18:2OOH peak was corrected for the extraction efficiency according to the calibration curve for cholesteryl benzoate shown in Figure 4.2.

Calibration for *tert*-butyl hydroperoxide (tBuOOH). A calibration curve for *tert*-butyl hydroperoxide was performed on CL-HPLC before each experiment to

Figure 4.2. Calibration curve for cholesteryl benzoate monitored at 234 nm using 1:1 methanol:isopropanol as eluent. The error bars correspond to ± 3 standard deviations.

account for changes in the chemiluminescence detection due to differences in the reactivity of the isoluminol/microperoxidase solution. A stock solution of *t*-butyl hydroperoxide (1.0×10^{-4} M) was prepared in methanol and stored at -20°C . The following injection volumes were made in triplicate on CL-HPLC: 1, 5, 10 and 15 μL using methanol as eluent. At a flow rate of 1 mL/min, tBuOOH eluted at approximately 3.2 minutes. An exemplary calibration curve for tBuOOH is shown in Figure 4.3.

Yamamoto *et al.*¹¹ have shown that the reactivity of the isoluminol/microperoxidase cocktail depends on the hydroperoxide. Therefore, Yamamoto *et al.*¹¹ established calibration factors to account for the different sensitivities of hydroperoxides towards isoluminol/microperoxidase. The relative sensitivities of tBuOOH, PCOOH and Ch18:2OOH are 0.39, 1.44 and 0.75 respectively. These numbers were used to calculate the concentration of Ch18:2OOH from the tBuOOH calibration curve.

Determination of the concentration of LDL. The concentration of LDL was determined by HPLC separation with UV detection of free cholesterol. The concentration of LDL is equal to the concentration of free cholesterol divided by 550.¹³ A stock solution of cholesterol (1.2 mM) was prepared in 1:1 methanol:isopropanol. The following injection volumes were made in triplicate on HPLC: 1, 5, 10, 15, 20 and 25 μL using 1:1 methanol:isopropanol as eluent. At a flow rate of 1 mL/min, cholesterol eluted at approximately 5.6 minutes (205 nm).

Figure 4.3. Calibration curve for tBuOOH monitored by chemiluminescence detection using 1:1 methanol:isopropanol as eluent. The error bars correspond to ± 3 standard deviations.

Figure 4.4. Calibration curve for free cholesterol monitored at 205 nm using 1:1 methanol:isopropanol as eluent. The error bars correspond to ± 3 standard deviations.

Determination of the concentration of Vitamin E. A stock solution of vitamin E (38 μ M) was prepared in 1:1 methanol:isopropanol with 2 mM LiCl. The following injection volumes were made in triplicate on EC-HPLC: 1, 5, 10, 15, 20 and 25 μ L using 55% methanol and 45% isopropanol with 2 mM LiCl as eluent. At a flow rate of 1 mL/min, α -tocopherol eluted at approximately 5.2 minutes.

Extraction of ABMHP into organic solvents. A solution of ABMHP (0.56 g) was prepared in millipore water (35 mL). The UV-vis spectrum of the ABMHP solution was recorded. An aliquot of the ABMHP solution (5 mL) was added to glass centrifuge tubes containing 5 mL aliquots of toluene, chloroform, diethyl ether, hexane and olive oil. "Blank" tubes were also prepared by adding 5 mL of millipore water to 5 mL aliquots of the organic solvents mentioned above. The blank samples were prepared to assure that there were no water-soluble contaminants in the organic solvents that could be extracted into the water phase and absorb at the same wavelength as ABMHP. The centrifuge tubes were vortexed for approximately 30 seconds and spun at maximal speed in a clinical centrifuge for 5 minutes. The water phase was removed from each tube and the UV-visible spectrum recorded.

Figure 4.5. Calibration curve for α -tocopherol monitored by electrochemical detection using 55% methanol and 45% isopropanol with 2 mM LiCl as eluent. The error bars correspond to ± 3 standard deviations.

5. References and Notes

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