AN INVESTIGATION INTO THE ANTIOXIDANT CAPACITY OF L-ARGININE AND L-CITRULLINE IN RELATION TO THEIR VASCULAR PROTECTIVE PROPERTIES

By

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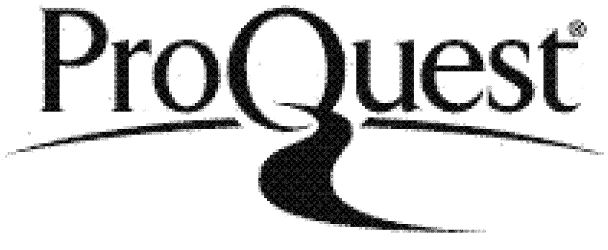

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This thesis is dedicated to my Parents; Keith and Nancy Coles and not forgetting my Brother, Thomas

Also to Gethin Pugh

And in memory of Alfred and Lily Hall, my Grandparents

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Summary

Oxidative stress plays a prominent role in the development of endothelial dysfunction. L-arginine and L-citrulline have been demonstrated to have beneficial effects in vascular disease and this thesis aimed to determine whether this protective effect is mediated via an antioxidant mechanism. With evidence to suggest L-citrulline may have antioxidant properties in plants, and the current contradictions that exist concerning the antioxidant properties of L-arginine in cells and in humans, a study aimed at fully characterising the antioxidant properties of both molecules was needed.

The ability of L-arginine and L-citrulline to scavenge both superoxide and hydroxyl radicals were investigated with an antioxidant effect only demonstrated against hydroxyl radicals. The mechanism of antioxidant action was shown to be two fold: 1) through inhibition of hydroxyl radical production and 2) through direct scavenging of the hydroxyl radical. Three model systems were developed to test the effect of endogenous and exogenous ROS on biological function. This allowed investigation into whether the antioxidant properties demonstrated *in vitro* were transferable to physiological and pathophysiological systems. Both amino acids were able to regulate endogenous ROS in platelets but this was shown to have little effect on overall platelet function. In terms of exogenous radicals, L-citrulline but not L-arginine, was able to protect against superoxide mediated endothelial dysfunction in vessels however this was deemed to be through a ROS independent effect. Both amino acids inhibited hydroxyl radical mediated lipid peroxidation at concentrations of amino acid found in the plasma. Taken together, these studies suggest that the improvement in vascular function seen upon administration of L-arginine and L-citrulline is in part mediated through an ability to scavenge hydroxyl radicals. However, their inability to directly affect superoxide levels draws into question whether significant antioxidant effects occurs *in vivo* and would suggest that a certain proportion of their protective effect on the vascular system is mediated via an antioxidant independent mechanism.

Abbreviations

μ	Micro; 10^{-6}
ACh	Acetylcholine
ADMA	Asymmetric dimethylarginine
ADP	Adenosine diphosphate
ANOVA	Analysis of variance
Ascorbic acid	AA
ATP	Adenosine triphosphate
AU	Arbitrary Units
AUC	Area Under Curve
Ca^{2+}	Calcium
$[Ca^{2+}]i$	Intracellular calcium
$[Ca^{2+}]e$	Extracellular calcium
$CaCl_2$	Calcium Chloride
CAD	Coronary artery disease
cAMP	Cyclic adenosine 3',5-monophosphate
CAT	Cationic Amino Acid transporter
cGMP	Cyclic guanosine 3'5'-monophosphate
CNP	C-natriuretic peptide
CO_2	Carbon dioxide
COX	Cyclooxygenase
Cu	Copper
Cu/ZnSOD	Copper zinc superoxide dismutase
DAG	Diacylglycerol
DEPMPO	5-(Diethoxyphosphoryl)-5-methyl-1pyrroline-N-oxide
DMSO	Dimethylsulphoxide
DNA	Deoxyribonucleic acid
DTPA	Diethylenetriaminepentaacetate
DTS	Dense tubular system
EC_{50}	Effective concentration that gives 50% of the maximum response
ecSOD	Extracellular superoxide dismutase
EDTA	Ethylene-diamine-tetra-acetic acid
EETS	Epoxyeicosatrienoic acids
eNOS	Endothelial nitric oxide synthase

EPR	Electron paramagnetic resonance
FAD	Flavin adenine nucleotide
FMN	Flavin mononucleotide
GPVI	Glycoprotein VI
GPx	Glutathione peroxidase
GR	Glutathione reductase
GSH	Reduced glutathione
GSSG	Oxidised glutathione pair
GSNO	S-nitrosoglutathione
GTP	Guanosine triphosphate
GTPase	Guanosine triphosphatase
H_2O_2	Hydrogen peroxide
HCl	Hydrochloric acid
HPLC	High performance liquid chromatography
HX	Hypoxanthine
ICAM	Intercellular adhesion molecule
iNOS	Inducible nitric oxide synthase
IP_3	Inositol 1,4,5-triphosphate
KCl	Potassium chloride
KH_2PO_4	Potassium dihydrogen orthophosphate
LA	L-arginine
L-Arg	L-arginine
LC	L-citrulline
L-Cit	L-citrulline
LDL	Low density lipoprotein
L-NAME	n-nitro-L-arginine-methyl ester
LOX	Lipoxygenase
m	Milli; 10^{-3}
M	Molar
MBF	Myocardial blood flow
MDA	Malondialdehyde
MGD	N-(Dithiocarbamoyl)-N-Methyl-D-glucamine
$MgSO_4.7H_2O$	Magnesium sulphate
MgSOD	Manganese superoxide dismutase
MPO	Myeloperoxidase
n	nano; 10^{-9}
Na^+	Sodium

NaCl	Sodium chloride
NADH	Nicotinamide adenine dinucleotide
NADPH	Nicotinamide adenine dinucleotide phosphate
$NaHCO_3$	Sodium hydrogen carbonate
NaOH	Sodium hydroxide
NHE	Sodium / hydrogen exchanger
nNOS	Neuronal nitric oxide synthase
NO	Nitric oxide
NO_2	Nitrogen dioxide
NOA	Nitric oxide Analyser
NOS	Nitric oxide synthase
O_2	Oxygen
O_2^-	superoxide
$OH^\bullet$	Hydroxyl radical
$ONOO^-$	Peroxynitrite
PAF	Platelet activating factor
PBN	N-t-Butyl-α-phenylnitrone
PBS	Phosphate buffered saline
PC	Phosphatidylcholine
PE	Phenylephrine
pGC	Particulate guanylate cylcase
PGI_2	Prostacyclin
PIP_2	Phosphatidylinositol bisphosphate
PKC	Protein kinase C
PLA_2	Phospholipase A_2
PLC	Phospholipase C
PRP	Platelet Rich Plasma
Q.D.	Every day
R_{MAX}	Maximal relaxation
ROS	Reactive oxygen species
sGC	Soluble guanylate Cylcase
SOD	superoxide dismutase
TBA	Thiobarbituric acid
TBAR	Thiobarbituric acid reactive substance
TID	Three times a day
TMP	Tetramethoxypropane
TxA_2	Thromboxane A_2

U	Units
VCAM	Vascular cell adhesion molecule
VSMC	Vascular smooth muscle cell
XO	xanthine oxidase

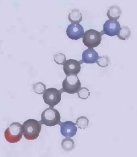
Note on Unit definitions

The term unit (U) refers to enzyme activity which is defined independently for each enzyme. The following lists these definitions for the enzymes used within this thesis.

Xanthine Oxidase: One unit will convert 1μmole of xanthine to uric acid per minute at pH 7.5 at 25°C.

Superoxide dismutase: Amount of enzyme that inhibits the rate of reduction of ferricytochrome C by 50% at pH 7.8 at 25°C.

Catalase: One unit will decompose 1μmole of hydrogen peroxide per minute at pH 7.0 at 25°C.



Introduction

Chapter One

to these amino acids and the role of free radicals in endothelial dysfunction. It will also provide a comprehensive review of all studies to date investigating this topic and explain how this thesis is adding to the growing body of work concerning alternative roles of L-arginine and L-citrulline.

1.1 Background to L-arginine

1.1.1 History of L-arginine

L-arginine (2-amino-5-(diaminomethylidene amino)pentanoic acid) is one of the most metabolically versatile amino acids. It has been studied for over 100 years and was first discovered by Schulze in 1886, when it was isolated from Lupin seedlings. The majority of advances in elucidating L-arginine metabolism were made in the 20th century as summarised chronologically in Table 1.

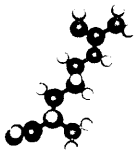
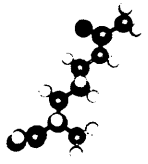


Table 1 History of L-arginine metabolism

Time period	Finding	Reference Number
1886	Isolation of L-arginine from Lupin seedlings.	[325]
1895	L-arginine found to be a constituent of mammalian protein	[140]
1932	L-arginine found to be involved in the urea cycle	[184]
1939	L-arginine found to be necessary for the synthesis of creatine	[102]
1980's	L-arginine discovered as the precursor of NO	[161,274,320]
1990's	The enzyme arginine decarboxylase discovered in mammalian cells, which was shown to convert L-arginine into agmatine.	[214]

1.1.2 Physical and chemical properties

L-arginine is a colourless solid at room temperatures with a melting point of 244°C. It is an α-amino acid with an asymmetric carbon. For this reason two enantiomers exist, of which Laevo is the natural form. The presence of three ionisable groups (the carboxyl group, α-amino group and side-chain) gives rise to three pKa values; 1.82, 8.99 and 12.18 for each respective group. At physiological pH, L-arginine is positively charged and so lies on the outside of a protein's quaternary structure due to its hydrophilic nature.



1.1.3 L-arginine metabolism

1.1.3.1 L-arginine synthesis

The majority of endogenous L-arginine synthesis occurs via the intestinal-renal axis. The epithelial cells of the small intestine produce citrulline primarily from glutamate and glutamine. The proximal tubules of the kidney then extract citrulline from the circulation and convert it into L-arginine, which is subsequently returned to the circulation.

The overall concentration of L-arginine found in human adult plasma is 70-150μM [12] .Although *de novo* synthesis of L-arginine from citrulline occurs mainly in the kidney, it also occurs in other cells types; specifically those that contain NOS [138,406]. Several factors that are known to stimulate iNOS, such as cytokines, also stimulate conversion of citrulline into L-arginine, suggesting that citrulline may provide an alternative source of L-arginine in situations where there is a high turnover of NO. This is known as the citrulline-arginine pathway Figure .

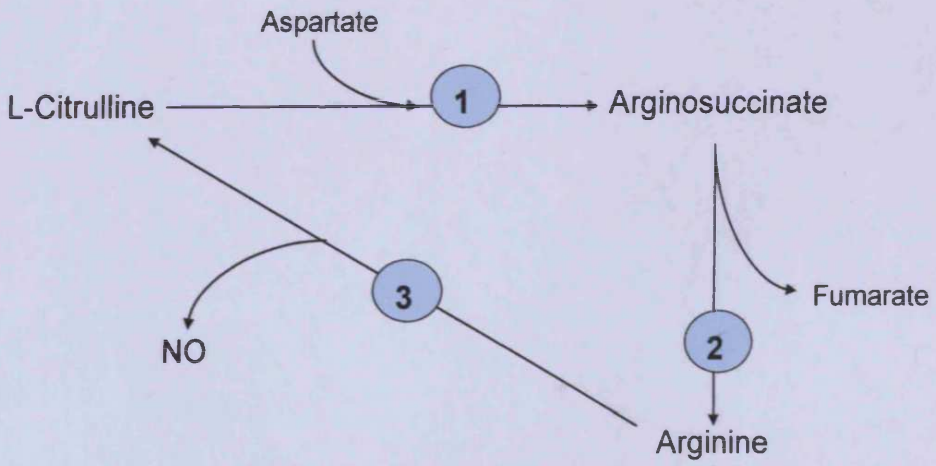
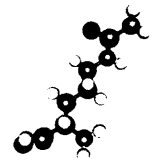


Figure 1 Arginine-Citrulline cycle. 1=Arginosuccinate synthase, 2=Arginosuccinase, 3=Nitric oxide synthase.

1.1.3.2 Uptake and transport of L-arginine

L-arginine is a semi-essential amino acid. This means that in healthy adults the conversion of endogenous L-citrulline to L-arginine is sufficient to meet the body's needs. In certain cases however, such as infants, growing children, adults with dysfunctional kidneys or small intestine and adults under catabolic stress, endogenous synthesis of L-arginine can no longer meet the body's demands and so exogenous intake of L-arginine becomes essential.

In most mammalian cells, arginine requirements are met by the uptake of extracellular arginine via specific transporters, including: y^+, b^{o+}, B^{o+}, or y^+L [183,368]. The distribution of these transporters varies between cells as does the activity which can be regulated in response to stimuli such as bacterial endotoxin and inflammatory cytokines [227]. The y^+ transporter appears to be the dominant transporter involved in the uptake of L-arginine in mammalian cells. The y^+ transporter is a high affinity, Na^+ independent transporter of arginine, lysine and ornithine [405].



1.1.3.3 L-arginine catabolism and the biological significance of catabolism products

L-arginine is catabolized by several enzymes, however even the catabolism of L-arginine leads to the production of several biologically significant molecules (Figure).

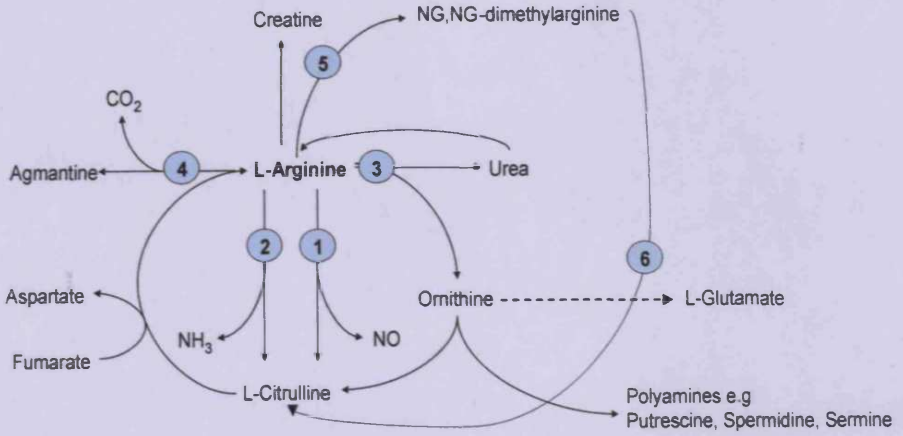


Figure 2 Schematic representation of L-arginine metabolism. 1=NOS, 2=Arginine deaminase, 3=Arginase, 4=Arginine decarboxylase, 5=Arginine N-methyltransferase

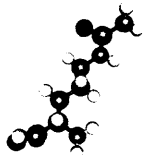
L-arginine is the substrate of NOS, which catalyses the conversion of L-arginine into NO and L-citrulline. Other direct catabolism products include urea, ornithine and agmatine. L-arginine is also involved in the synthesis of creatine, polyamines and glutamate. The biological role of these molecules is summarised in Table 2.

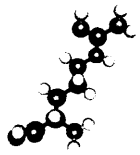
Table 2 **Function of L-arginine breakdown products**

Molecule	Function
Polyamines	Modulate ion channel function (NMDA + AMPA receptors) Potentiates cell proliferation and differentiation
Creatine	Aids in energy supply to muscle cells
Glutamate	Excitatory neurotransmitter in mammalian nervous system GABA precursor
Nitric oxide	Part of immune response Involved in maintaining a healthy endothelium
Agmantine	Putative neurotransmitter – binds to α_2-adrenoceptors and imidazoline receptors evoking clonidine like effects on blood pressure Blocks NMDA receptors and other action ligand gated channels

1.1.3.4 Other roles of L-arginine

L-arginine is also involved in the regulation of gene expression, a field of science known as argenomics. This is complex field which does not come under the scope of this thesis. Of interest is however the fact that increased levels of arginine suppress arginosuccinate synthase and lyase activity and increased levels of citrulline increase the activity of these enzymes [317,318] . Reductions in L-arginine availability are also known to decrease iNOS expression [88,206] but to increase CAT-1 (Cationic Amino acid Transporter 1) expression [14,94-96].





1.2 Background to L-citrulline

1.2.1 History of L-citrulline

L-citrulline (2-amino-5-(carbamoylamino)pentanoic acid) takes it name from the Latin for watermelon, *Citrullus vulgaris*, due to the high quantities of L-citrulline found in watermelons. There has been little interest in citrulline because it is a non-protein amino acid. Recent studies however have highlighted the importance of citrulline in metabolism, specifically its ability to synthesise L-arginine [406].

1.2.1.1 Physical and chemical properties

Like L-arginine, L-citrulline is a colourless solid at room temperature. It has a melting point of 222°C and exists as two enantiomers, of which the L-form is the most commonly found.

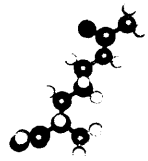
L-citrulline has 2 ionisable groups and the two corresponding pKa values are 2.43 and 9.69 for the α-carboxyl and α-amino groups respectively. At physiological pH L-citrulline is therefore uncharged.

1.2.2 L-citrulline metabolism

1.2.2.1 Synthesis of L-citrulline

L-citrulline is a non-essential amino acid, meaning the body can obtain necessary citrulline levels from glutamine conversion in enterocytes [401]. Plasma concentrations of L-citrulline are thought be roughly 30-100μM [404]. L-citrulline can also be obtained exogenously from food [285] and also from glutamate, proline and arginine (Figure).

3



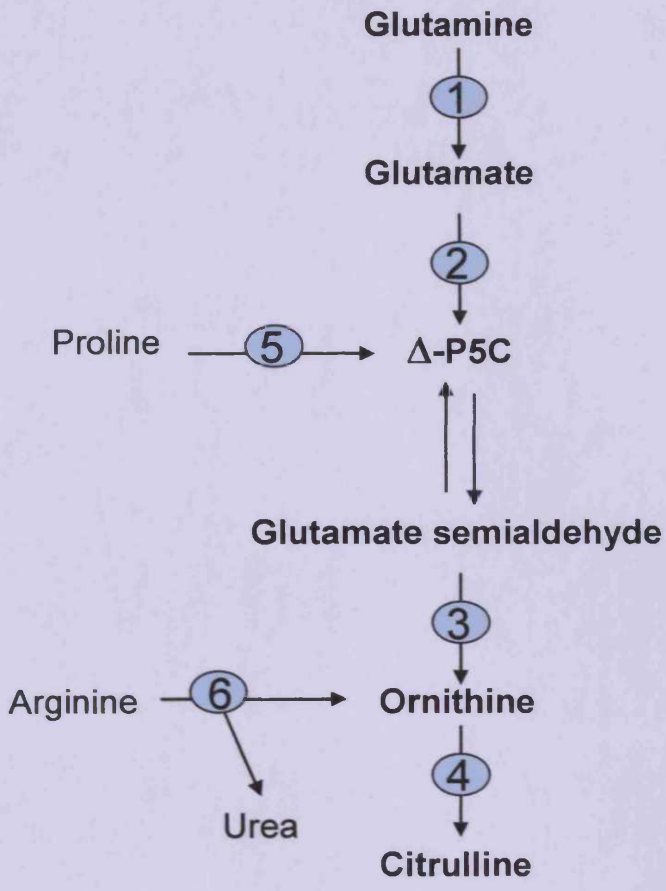
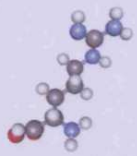


Figure 3 Metabolic pathway of Citrulline synthesis. 1= Glutaminase, 2=P5C synthase, 3=Ornithine aminotransferase, 4=Ornithine carbamoyltransferase, 5=proline oxidase, 6=Arginase



1.2.2.2 Uptake and transport of L-citrulline.

Uptake of L-citrulline into the cell occurs via different transporters, dependent on the cell type in question. Table 3 summarises citrulline transport in various cell types.

Table 3 Summary of L-citrulline transport

Cell type	Transport available	Reference
Cells in the nervous system e.g. microglial cells, astrocytes and neurons	Transport mechanism yet to be fully characterised but thought to use the L-amino acids carrier system	[319]
Endothelial aortic cells	Transport proceeds through a transport system that can also carry arginine but is independent to the y^+system.	[145]
Rat aortic smooth muscle cells	Transport is partially Na^+ dependent and pH insensitive. Transporter is distinct from those used by L-arginine.	[393]
Macrophages	Two transport systems co-exist. One is a saturable system for neutral amino acid transport, while the other competes with L-arginine	[23]
Enterocytes	Suggested to be a transporter from the B° system.	[74]
Kidney	No transporter identified but evidence for its existence.	[272]
Intracellular exchange: mitochondria: cytosol	Ornithine-citrulline exchanger, localised to inner wall of mitochondria	[286]

1.2.2.3 L-citrulline catabolism and the biological significance of catabolism products

Within mammals there is only one enzyme capable of catabolising L-citrulline, arginosuccinate synthase. Although L-citrulline does not produce as many biologically significant metabolic products as L-arginine, one very important function of L-citrulline is to act as a precursor for NO. The majority of cells that contain NOS are able to uptake L-citrulline and through a truncated urea cycle synthesise L-arginine which can then go on to produce NO [406]. This truncated urea cycle is cyclical in nature as the production of NO from L-arginine leads to the production of L-citrulline, which in turn is recycled back to L-arginine Figure 4

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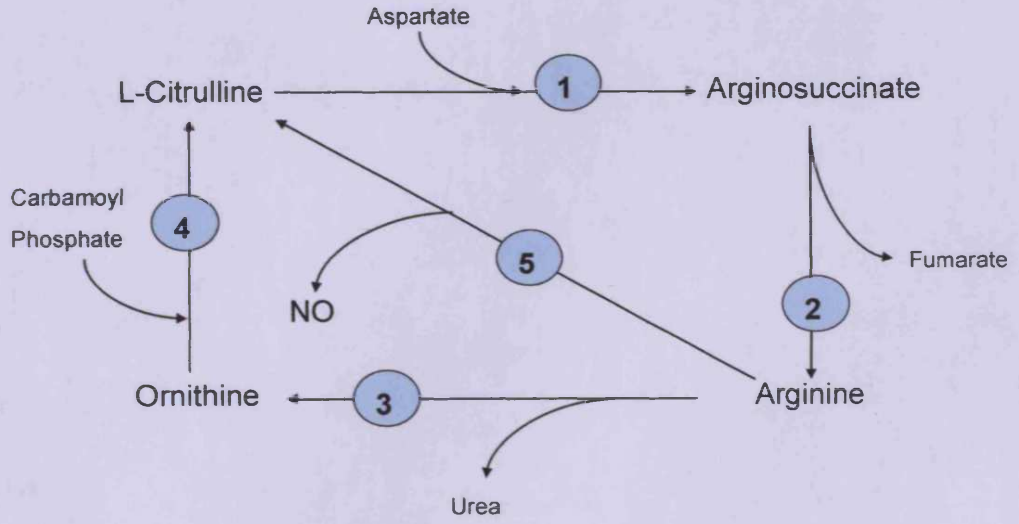


Figure 4 Arginine-Citrulline cycle. 1=Arginosuccinate synthase, 2=Arginosuccinase, 3=Arginase, 4=Carbamoyl phosphate synthase 5=Nitric oxide synthase.

In activated macrophages, citrulline recycling accounts for up to 20% of NO produced [250]. Synthesis of L-arginine from L-citrulline is cell specific however, and does not occur in cell types such as vascular smooth muscle cells [393] . It is important for this cycle to be controlled as excess NO production can lead to damage to the surrounding cells. Increased levels of arginine suppress Arginosuccinate synthase and lyase and increased levels of citrulline increase the activity of these enzymes [317,318]. Increased levels of NO are also known to suppress this Arginosuccinate synthase [134]. In this way whenever the product or substrate of NOS becomes too high, NOS expression is reduced, thus preventing the formation of toxic levels of NO.

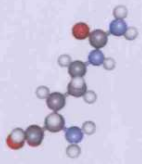
1.3 Free radicals

1.3.1 Introduction

A free radical is defined as a species capable of independent existence that contains one or more unpaired electrons. Any species with an unpaired electron is paramagnetic and highly reactive, although chemical reactivity does vary between species. As this thesis investigates the antioxidant properties of two molecules, L-arginine and L-citrulline, some time shall be spent discussing aspects of Reactive Oxygen Species (ROS).

1.3.2 Mechanisms of radical initiation, propagation and termination

The chemical reactions of a radical can be split into 3 main stages: Initiation, in which there is a net increase in the number of free radicals; propagation in which the net number of free radicals remains constant, and termination in which there is a net decrease in the number of free radicals.



1.3.2.1 Initiation

Radicals are formed via numerous mechanisms as summarised in Table 4.

Table 4 **Summary of radical generation**

Radical Generation process		Associated reaction / radical production process
Physical generation of radicals	U.V Light	$A—B + h\nu \longrightarrow A^{\bullet} + B^{\bullet}$ $A—B + h\nu \longrightarrow AB^{\bullet +} e^{-}$
	Ionising radiation	$A—B \longrightarrow AB^{\bullet +} + e^{-}$ $A—B + e^{-} \longrightarrow AB^{\bullet -}$ $AB^{\bullet +} + e^{-} \longrightarrow AB^{*}$ $AB^{*} \longrightarrow A^{\bullet} + B^{\bullet}$
	Water hydrolysis	$H_2O \longrightarrow H_2O^{\bullet +} + e^{-}$ $H_2O + H_2O^{\bullet -} \longrightarrow {}^{\bullet}OH + H_3O^{+}$
	Mechanical production	Bond breaking when linear or cross chain polymers are subjected to: Shear stress, bending, cutting, breaking or grinding. Tribo-electric effect: electrons are transferred from one molecule to another during flow.
Chemical generation of radicals	Thermolysis	$A—B + \text{heat} \longrightarrow A^{\bullet} + B^{\bullet}$
	Redox processes	$A + B \longrightarrow A^{\bullet +} + B^{\bullet -}$

1.3.2.2 Propagation

Propagation occurs when a free radical interacts with a non-radical molecule producing a new radical species. These reactions can repeat and initiate what is known as a chain reaction. One of the most important examples of this is lipid peroxidation.

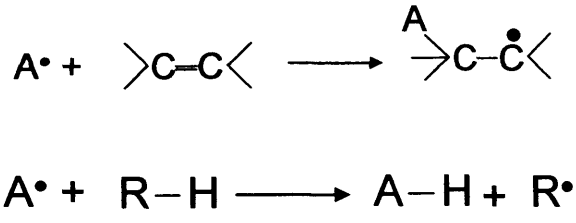


Figure 5 Examples of radical propagation reactions

1.3.2.3 Termination

After numerous propagation steps, a free radical will eventually encounter another radical resulting in the formation of a stable, non-radical species. This effectively terminates all further radical reactions.

Below is an example of free radical chemistry, illustrating the initiation, propagation and termination steps Figure 5.

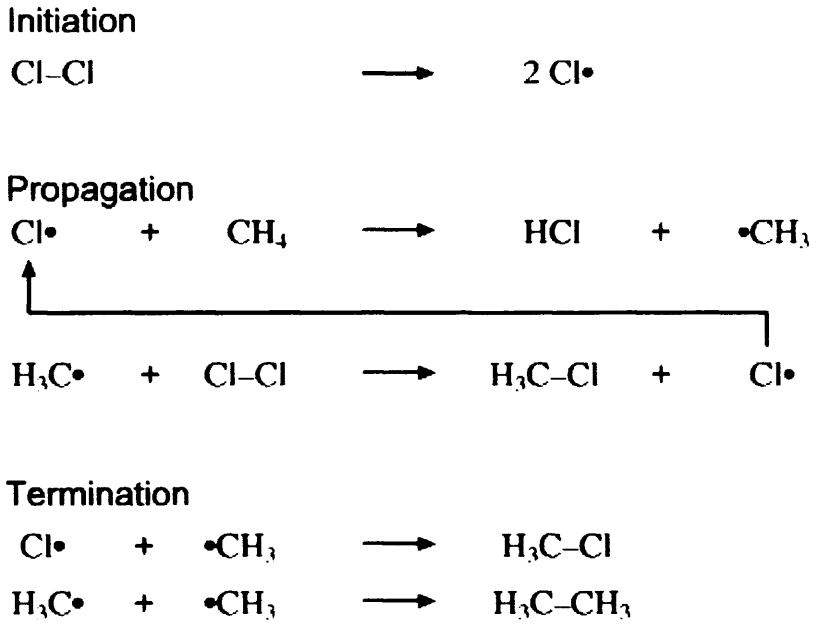


Figure 6 Example of the three stages of radical interactions. Taken from http://www.meta-synthesis.com

1.3.3 Reactive Oxygen Species

One of the major sources of radicals in the body is molecular oxygen. Although a free radical itself, molecular oxygen itself is relatively unreactive and under normal conditions undergoes a four electron reduction to form two molecules of water. Intermediate reductions can also occur resulting in the formation of superoxide, hydrogen peroxide and hydroxyl radicals (Figure).

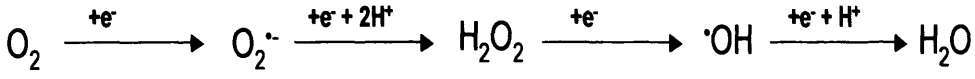


Figure 7 Reduction of molecular oxygen

The following section aims to give a brief introduction to the types of ROS.

1.3.3.1 Superoxide Radical

Superoxide is formed via a one-electron reduction of molecular oxygen

$$O_2 + e^- \rightarrow O_2^{\bullet-} \qquad \textbf{Equation 1}$$

Several enzymes exist capable of catalysing this reduction including xanthine oxidase, NADPH oxidase and uncoupled NOS. superoxide is unstable in aqueous solution and so is lost through a dismutation reaction. Dismutation is spontaneous however in the presence of superoxide dismutase, the reaction rate significantly increases to $5x10^5$ M^{-1} s^{-1} [150]. This reaction allows superoxide levels to be kept low.

$$O_2^{\bullet-} + O_2^{\bullet-} + 2H^+ \rightarrow H_2O_2 + O_2 \qquad \textbf{Equation 2}$$

Superoxide is poorly membrane permeable and is therefore restricted to the compartment in which it is produced.

Superoxide can react with other molecules in 3 main ways:

Reducing agent

e.g. $CytC(Fe^{3+}) + O_2^{\bullet-} \rightarrow CytC(Fe^{2+}) + O_2$ **Equation 3**

Oxidising agent

e.g. $Fe^{2+} + 2H^+ + O_2^{\bullet-} \rightarrow Fe^{3+} + H_2O_2$ **Equation 4**

Nucleophile

e.g. $CCl_4 + O_2^{\bullet-} \rightarrow CCl_3O_2^{\bullet-} + Cl^-$ **Equation 5**

1.3.3.2 Hydroperoxyl and peroxyl radicals

The hydroperoxyl radical is the protonated form of superoxide. Superoxide acts as a Bronsted-Lowry base meaning it accepts a hydrogen ion. This results in the formation of a hydroperoxyl radical ($HO_2^{\bullet}$).

$O_2^{-\bullet} + H^+ \leftrightarrow HO_2^{\bullet}$ **Equation 6**

The hydroperoxyl radical is a more powerful reducing agent than superoxide but the pK_a value of the above reaction, 4.8, results in there being very little hydroperoxyl radical present at neutral pH.

The hydroperoxyl radical is the simplest form of what are known as peroxyl

radicals – $RO_2^{\bullet}$. Peroxyl radicals are formed when molecular oxygen reacts with an allyl radical. The allyl radical is in turn formed from the reaction of a peroxyl radical with an alkene (Figure 8). This chain reaction continues until the supply of substrate e.g. oxygen, runs out. This reaction is the main cause of lipid peroxidation.

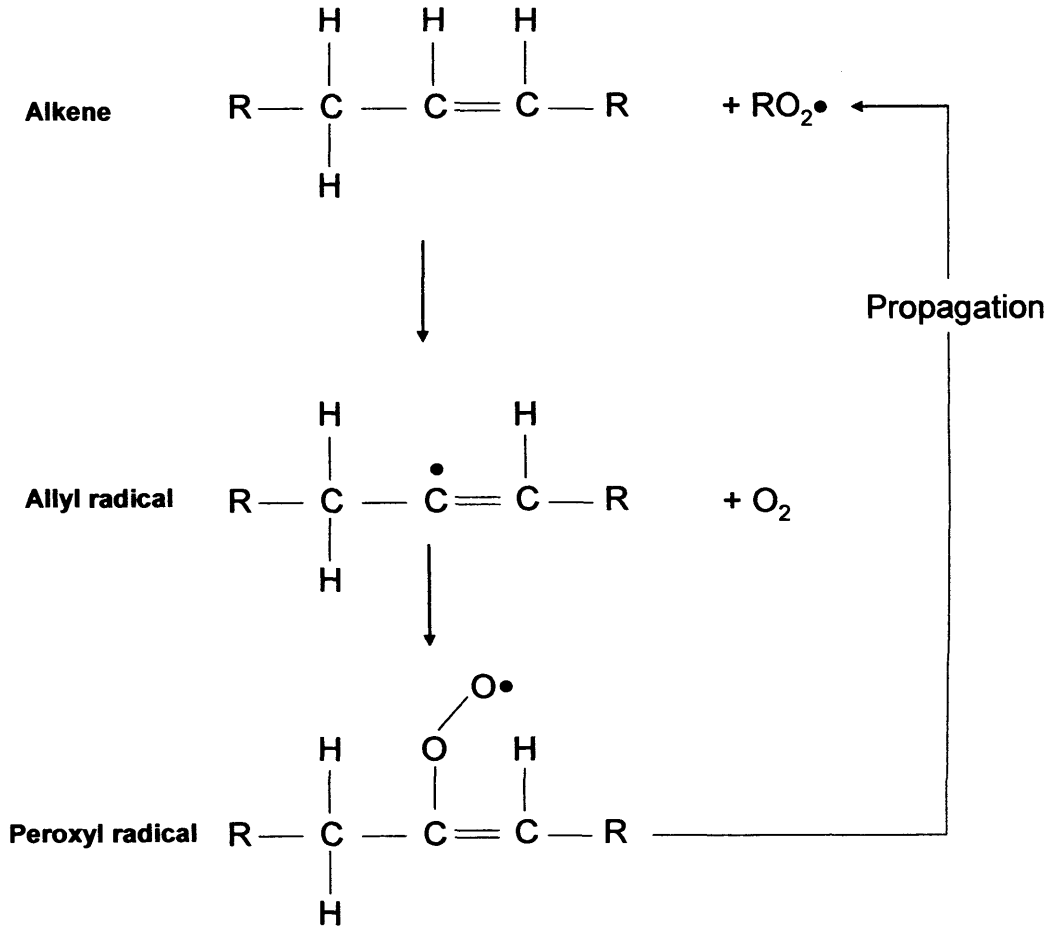


Figure 8 Peroxyl radical formation and propagation

1.3.3.3 Hydrogen peroxide

Hydrogen peroxide is generated in any system containing superoxide via the dismutation reaction discussed above. It is also generated from enzymes including glycolate oxidase, D-amino acid oxidase and urate oxidise, located in microsomes, peroxysomes and the mitochondria. Hydrogen peroxide is a weak oxidising agent and is known to exert deleterious effects in cells. There is also some evidence to suggest that the toxic effects of hydrogen peroxide are partly mediated via its conversion into the hydroxyl radical [299,355].

1.3.3.4 Hydroxyl Radical

The hydroxyl radical is synthesised from hydrogen peroxide through bond homolysis or electron transfer (Figure).

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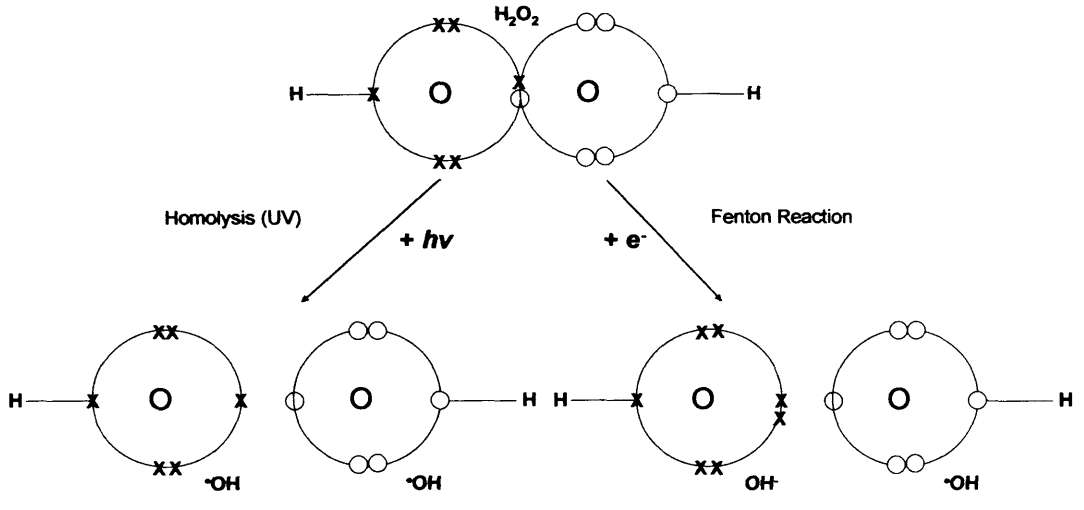
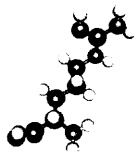


Figure 9 Production of hydroxyl radicals via homolysis and electron transfer



Electron transfer usually involves transfer of an electron from either a transition metal (Fenton chemistry) or from superoxide (Haber-Weiss reaction). These two reactions act synergistically to propagate continuous hydroxyl radical production.

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + {}^{\bullet}OH + OH^- \qquad \textbf{Equation 7}$$

$$^{\bullet}OH + H_2O_2 \rightarrow H_2O + O_2^{\bullet-} + H^+ \qquad \textbf{Equation 8}$$

$$O_2^{\bullet-} + H_2O_2 \rightarrow O_2 + {}^{\bullet}OH + OH^- \qquad \textbf{Equation 9}$$

The hydroxyl radical is known to react at diffusion rates making it one of the most aggressive ROS in the vasculature. Hydroxyl radicals react with neighbouring molecules via three main mechanisms;

1. Hydrogen abstraction
2. Addition
3. Electron transfer.

Although hydroxyl radicals are highly reactive they are not as dangerous as the superoxide radical due to their rapid reaction rate. They therefore react with the closest available molecule, whatever that may be, leading to widespread but indiscriminate damage.

1.3.4 Formation of reactive oxygen species

The theory of radical formation has been briefly touched on, however some time will be taken to discuss the production of ROS in biology, specifically the cardiovascular system. Superoxide is thought to be the most commonly generated ROS. Reactions such as dismutation, Haber-Weiss and Fenton chemistry however mean that wherever superoxide is produced hydrogen peroxide and the hydroxyl radical are most certainly generated.

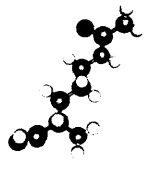


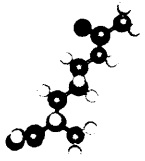
Table 5 Generation of ROS

Radical Generation process		**Associated reaction / radical production process**
Chemical generation of ROS	superoxide	Univalent reduction of molecular oxygen
	Hydrogen peroxide	Dismutation of superoxide
	Hydroxyl radical	Haber Weiss reaction
		Fenton chemistry
Biological generation of ROS		NAD(P)H oxidase
		Xanthine oxidase [105]
		Cytochrome P450 [40]
		Nitric oxide synthase [388]
		Mitochondrial electron transport chain
		Arachadonic acid metabolism [189]
		Intracellular organelles e.g. Endoplasmic reticulum and peroxisomes [324]

This thesis is specifically interested in formation of ROS in the cardiovascular system. To this end, several radical producing systems have been implicated in radical production in both endothelial cells and vascular smooth muscle cells. The following gives a broad overview of these systems in relation to the cardiovascular system.

1.3.4.1 Xanthine oxidase

Xanthine oxidoreductase is an enzyme involved in purine metabolism and converts hypoxanthine and xanthine into urate. It exists in two forms as either a dehydrogenase or an oxidase. Both catalyse the conversion of hypoxanthine and xanthine into urate [242] however the dehydrogenase converts $NAD(P)^+$ into NAD(P)H [135], the oxidase converts oxygen into superoxide [242].



Xanthine dehydrogenase is present in higher amounts that the oxidase form, however conversion of xanthine dehydrogenase into xanthine oxidase can occur via a reversible oxidation of sulphydryl residues of xanthine dehydrogenase or via irreversible proteolytic cleavage of xanthine dehydrogenase in the presence of pro-inflammatory mediators [242,349]. Xanthine oxidase is thought exist mainly in three locations, cytosolic, bound to the outer surface of endothelial cells [390], and released into circulating blood – where it is mostly in the oxidase form. It is activated by pro-inflammatory mediators such as TNF-α, interleukins and complement 5a and Lipopolysaccharide.

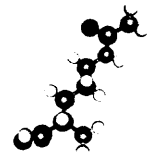
1.3.4.2 NADP(H) oxidase

NAD(P)H oxidase is most commonly described in phagocytes where it is involved in the oxidative burst response to invading pathogens. NAD(P)H binds to the enzyme and releases two electrons which are passed to two molecules of oxygen to form superoxide.

Neutrophil oxidase components are present in endothelial cells [25,114,172,216-218] and this enzyme is thought to be the major source of superoxide radicals in the vasculature [200]. There are some differences between endothelial and the neutrophil NAD(P)H oxidase including:

1. It continuously generates superoxide at low levels even in unstimulated cells although its activity can be induced by several agonists.
2. A substantial proportion of superoxide generated is intracellular whereas neutrophil oxidase mainly occurs in the extracellular department.

Activity is regulated by cytokines, hormones and mechanical forces and is known to be involved in the pathogenesis of vascular disease. Stimulation of vascular smooth muscle cells with angiotensin II, thrombin, platelet derived growth factor, tumour growth factor-α (levels of which are thought to increase during endothelial dysfunction), all increase vascular ROS formation and



NAD(P)H oxidase activity [29,77,118,149,232].

1.3.4.3 Cytochrome P450

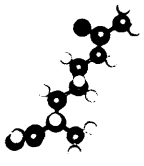
Cytochrome P450 is a haem containing hepatic endoplasmic reticular flavoenzyme. It has been shown to oxidise, peroxidise and/or reduce cholesterol, vitamins, steroids and many other compounds in an oxygen and NADPH-dependent manner [101]. Cytochrome P450 has also been found to be expressed in the cardiovasculature system. These enzymes metabolise arachidonic acid and are therefore implicated in vascular regulation through the generation of vasodilator and vasoconstrictor metabolites [101]. They also generate superoxide, hydrogen peroxide and hydroxyl radicals during the enzyme reaction cycle when electrons for the reduction of the central haem iron are transferred to the activated bound oxygen molecule in an NADPH-dependent reaction [101].

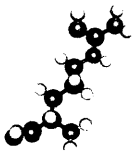
1.3.4.4 Uncoupled NOS

NOS is a cytochrome P450 like enzyme. NADP is an electron donor from which an electron is transported via flavin mediated electron transport to a prosthetic haem group. This is then passed onto the guanidinium group of L-arginine resulting in NO formation nitric oxide synthase is said to be uncoupled when the electron is passed on to molecular oxygen as opposed to L-arginine resulting in superoxide formation [141,144,182,249,287,333,370]. Uncoupled NOS is detrimental as not only is superoxide produced but NO bioavailability is also decreased, as less is being synthesised and also peroxynitrite is formed.

Mechanisms by which NOS are thought to become uncoupled include:

- Oxidation of tetrahydrobiopterin [196,201]
- Oxidation of zinc thiolate clusters leading to enzyme monomerisation [421]
- Decreased L-arginine bioavailability [141,287]
- NOS can be partly uncoupled so NO and superoxide are produced, effectively turning NOS into a peroxynitrite generator.

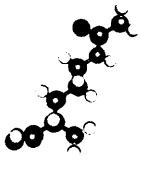




1.3.4.5 Mitochondrial respiratory chain

Mitochondria are essential energy producing organelles found in all eukaryotic cells. Enzymes found in the inner mitochondrial space use electron transfer to generate a proton gradient used by ATP synthetase to generate ATP. Under normal conditions, 1-4% of the electrons react with molecular oxygen resulting in the formation of superoxide and hydrogen peroxide. Under normal conditions ROS levels are controlled by MnSOD and uncoupling proteins [54].

Under conditions where superoxide production is increased, for example when the mitochondria undergoes oxidative damage from other sources, the antioxidant mechanisms are insufficient to keep ROS levels low and so superoxide and hydrogen peroxide are free to react with other molecules such as transition metals resulting in the production of hydroxyl radicals [207].



1.3.5 Physiological and pathophysiological roles of oxygen derived free radicals.

For many years it was generally assumed that ROS were only involved in pathophysiological processes. In the cardiovascular system alone ROS have been linked to cardiac failure, hypertension, cardiac hypertrophy, ischaemic heart disease, atherosclerosis, hypertension and diabetes to name but a few disease states (Table 9). In recent years this way of thinking has been questioned, with several studies presenting a role for ROS in vital cellular processes such as cell signaling, induction of gene expression and control of enzyme activity.

It would appear that the concentration of ROS dictates whether a physiological or pathophysiological action will be seen. Lower concentrations of ROS, as maintained by antioxidants, would appear to contribute to healthy cellular function whereas higher concentrations lead to oxidative stress resulting in cell damage and potential cell death. Table 6 summarises the physiological and pathophysiological roles of ROS.

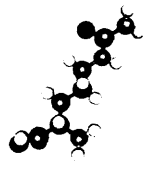
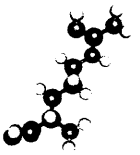
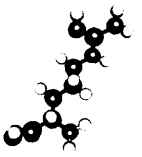


Table 6 Physiological and Pathophysiological roles of ROS

Physiological and pathophysiological role	**Action**	**Biological function**
Physiological	Redox modification of thiol groups [273]	Activation of serine/threonine kinases [354]
		Regulation of tyrosine phosphatase activity [19]
		Induction of gene expression via activation of growth factors and transcription factors[19].
		Regulation of ion channels leading to electrophysiological instability in the myocardium resulting in development of arrhythmias [169,181,313]
	Other effects	Required for platelet derived growth factor mediated cell growth [348]
		Mediate inflammatory responses [16]
		Thyroid hormone synthesis [87]
		Insulin signaling [185,247]
		Hydrogen peroxide may account for EDHF activity in murine and human mesenteric arteries and human coronary arterioles
		ROS may be involved in the physiological control of endothelial cell growth, migration, proliferation and survival [219]
		Involved in the microvasculature inflammatory response to pathogens
Pathophysiological	Oxidation of iron sulphur clusters	Inactivation of enzymes e.g dehydratases [190] Release of iron leading to conversion of hydrogen peroxide into the hydroxyl radical.



	Oxidation of short chain sugars	Leads to creation of toxic dicarbonyls which are toxic via their reactivity with amino groups of proteins and nucleic acids [267]
	Oxidation of aromatic amino acids and sulphur containing amino acids	Damage to proteins. Inactivation of enzymes if amino acid contained in active centre of enzyme .
	Damage to DNA (via oxidation of nucleic acids, abstraction of electrons from sugar or base nucleotides)	Cellular mutations leading to mutagenesis, impaired cell function and/or cell death.
	Lipid peroxidation	Damage to cell membrane leading to impaired cell function and/or cell death.



1.3.6 Antioxidant defence mechanisms

The body has evolved a number of mechanisms involved in tightly controlling ROS levels. As mentioned previously, this allows ROS to carry out their physiological function whilst preventing oxidative stress. The body utilises a large variety of intra- and extracellular molecules collectively termed antioxidants. Examples include transferrin, α-tocopherol, ascorbic acid, superoxide dismutase, catalase, glutathione peroxidase and mannitol.

1.3.6.1 Transferrin

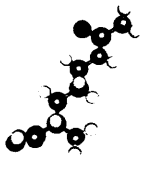
Apotransferrin is found in human plasma and binds Fe(III) and is involved in transferring Fe(III) to cells. Through the binding of iron, its interaction with hydrogen peroxide is inhibited thus preventing iron-catalysed reactions e.g. Fenton chemistry.

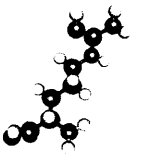
1.3.6.2 Albumin

Albumin is a protein found in human plasma that is capable of binding copper(II) and to a lesser extent iron. Binding of these metals does not prevent them interacting with hydrogen peroxide and so albumin is termed a sacrificial antioxidant. This means that the hydroxyl radicals formed are targeted to specific sites on the albumin protein. The oxidation of albumin is preferred to the oxidation of surrounding molecules such as lipids and DNA as the damaged albumin protein can be quickly replaced. As such, albumin is actually a target or replacement substrate.

1.3.6.3 Ascorbic acid

Ascorbic acid, more commonly known as vitamin C, is provided through the diet in the majority of species. Its most striking chemical property is its ability to act as a reducing agent. The levels of ascorbic acid found *in vivo* have been shown to





exert antioxidant effects with concentrations in plasma in the range of 30-100μM. It is found in higher concentrations in cerebrospinal fluid, aqueous humour of the eye, gastric juice and lung lining fluid where it is thought to be in millimolar amounts. Direct evidence that ascorbic acid can actually act as an antioxidant *in vivo* is limited, but it is known to scavenge $O_2^{\bullet-}$ and $^{\bullet}OH$ with rate constants of $>10^5$ M^{-1} s^{-1} and 10^9 M^{-1} s^{-1} respectively *in vitro*. Ascorbic acid is also thought to act synergistically with α-tocopherol.

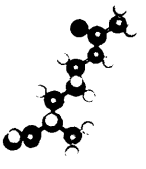
1.3.6.4 α-tocopherol

α-tocopherol, otherwise known as vitamin E, is the most important lipid soluble antioxidant in biological fluids [252]. It binds to lipoproteins in cell membranes thus placing it in the perfect position to prevent lipid oxidation. Radicals preferentially react with vitamin E rather than other lipid molecules thus the lipid chain reaction is terminated. The resultant α-tocopherol radical formed is less reactive than the initial free radical. Ascorbic acid acts synergistically with α-tocopherol as the α-tocopherol radical formed migrates to the cell surface where ascorbic acid reduces it back to α-tocopherol [42].

1.3.6.5 Superoxide dismutase

Superoxide dismutase catalyses the dismutation of two molecules of superoxide into hydrogen peroxide [235]. Three mammalian forms of SOD have been identified to date and these are:

SOD 1 – copper/Zinc SOD (Cu/ZnSOD) – localised in cytosol and nucleus of all cell types [400].
SOD 2 – Manganese SOD (MnSOD) – localised in mitochondria [339,387]
SOD 3 - extracellular SOD (ecSOD) – localised in the extracellular fluids such as lymph, synovial fluid, connective tissue and plasma [231]



1.3.6.6 Glutathione peroxidase

Glutathione peroxidase removes hydrogen peroxide from mammalian cells and is expressed in all eukaryotic cells. Glutathione is a tri-peptide consisting of glycine, cysteine and glutamic acid. The presence of cysteine makes GSH readily oxidisable and so is utilised by the glutathione peroxidase to reduce hydrogen peroxide to water.

$$GSH + H_2O_2 \rightarrow GS\text{-}SG + 2H_2O \qquad \textbf{Equation 10}$$

In order to maintain sufficient levels of GSH, the enzyme glutathione reductase catalyses the conversion of GS-SG back to GSH.

$$GS\text{-}SG + NADPH \rightarrow GSH + NADP^+ \qquad \textbf{Equation 11}$$

1.3.6.7 Catalase

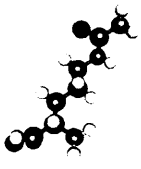
Catalase is a haem-containing enzyme that catalyses the conversion of hydrogen peroxide into water and oxygen via a two step reaction.

$$H_2O_2 + Fe(III)\text{-}E \rightarrow H_2O + O{=}Fe(IV)\text{-}E \qquad \textbf{Equation 12}$$

$$H_2O_2 + O{=}Fe(IV)\text{-}E \rightarrow H_2O + Fe(III)\text{-}E + O_2 \qquad \textbf{Equation 13}$$

Where Fe-E represents the ion centre of the haem group of catalase

It is located in peroxisomes of all eukaryotic cells and in the cytoplasm of erythrocytes [234]. Under normal conditions, glutathione peroxidase is able to maintain low concentrations of hydrogen peroxide. If the concentration of hydrogen peroxide exceeds glutathione peroxidases capacity, the action of catalase becomes important [128].



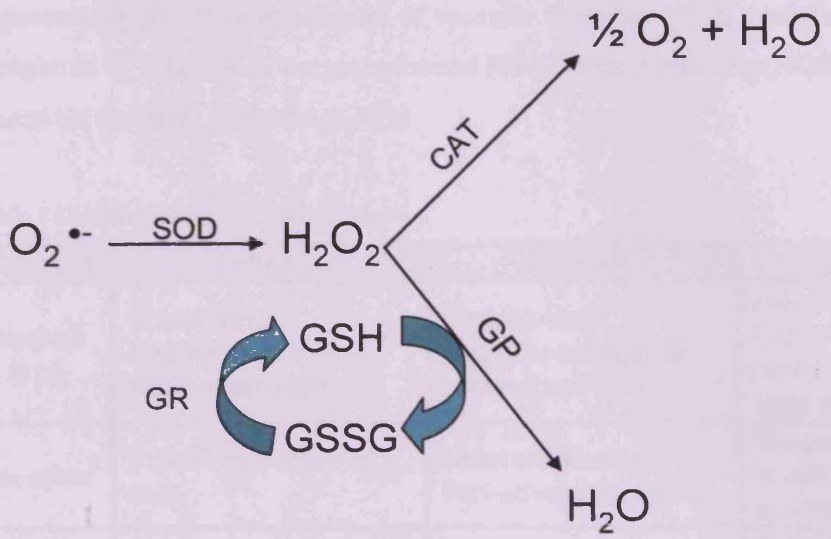
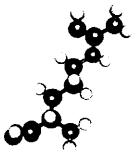


Figure 10 Co-operative activity between antioxidant systems. GR=Glutathione reductase ; GP= Glutathione Peroxidase ; CAT = catalase



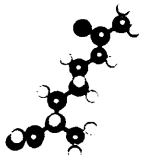
1.3.7 Nitric oxide

Nitric oxide is a nitrogen-based ROS that is thought to be one of the most important mediators in the control of vascular function. NO is synthesised from L-arginine by a family of enzymes termed Nitric oxide Synthases (NOS). Table 7 illustrates the main isoforms of NOS.

Table 7 Characterisation of NOS isoforms

	eNOS	nNOS	iNOS
Enzyme type	Constitutive. Requires calcium to become activated	Constitutive. Requires calcium to become activated	Inducible. Constantly produces basal level of $NO^{\bullet}$
Location	Vascular endothelial cells	Neuronal tissue. Peri-adventitial nerves	Generally found in cells such as macrophages
Stimulus	Acetylcholine, Bradykinin, ATP, Substance P and shear stress [244]	Activation of synaptic pathways.	Cytokines e.g. (IL 1). Tumour necrosis factor (TNF) [245]
Role of $NO^{\bullet}$ produced	-Vasodilatation of vascular smooth muscle cells [297] - Inhibits growth factors within the vessel wall [106] - Inhibits platelet aggregation and adhesion to endothelium [292,293] - Inhibits synthesis and expression of cytokines and cell adhesion molecules [187]	- Neurotransmitter. - May play a role in blood vessel tone when present in peri-adventitial nerves	Released from activated macrophages as a toxic mediator as part of extracellular defence against pathogens which they cannot ingest, such as parasitic worms

NOS is known as a redox enzyme as it contains both a reductase and an oxygenase domain. The reductase domain contains the binding sites for; NADPH, FAD and FMN and the oxygenase sub unit contains binding sites for; L-arginine, Oxygen and BH_4 as well as containing the Haem group, iron protoporphyrin IX. The haem group is considered to be the catalytic site.



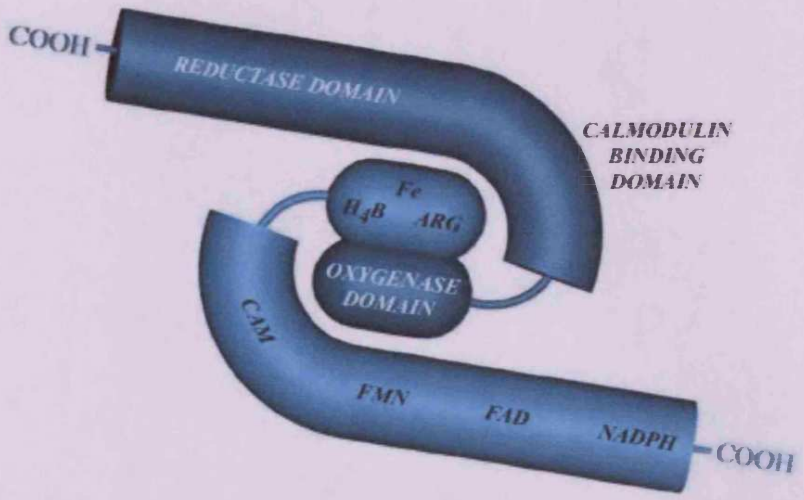


Figure 11 Structure of eNOS. Taken from http://biology.kenyon.edu

NOS catalyses the 1:1 conversion of L-arginine into NO and L-citrulline. The reaction proceeds as follows:

1.3.7.1 Reductase domain:

1. NADPH acts as the source of electrons. These electrons are shuttled from NADPH by the flavins, FAD and FMN to the neighbouring iron haem.

1.3.7.2 Oxygenase domain

1. NOS binds L-arginine so that its guanidinium group is adjacent to the haem group.
2. The haem group binds molecular oxygen, which upon electron transfer from NADPH, becomes activated.
3. The activated molecular oxygen is then cleaved, with one oxygen atom released as water and the other incorporated into one of the terminal guanidino nitrogens of L-arginine yielding N^G-hydroxy-L-arginine.
4. A further molecule of oxygen is activated facilitating the further oxidation of N^G-hydroxy-L-arginine to $NO^{\bullet}$ and citrulline.

$NO^{\bullet}$ is highly lipophilic, so once produced, it diffuses along a concentration

gradient to adjacent smooth muscle and also into the blood stream where it forms numerous metabolites.

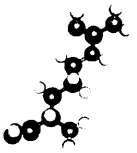
$NO^{\bullet}$ in the vascular smooth muscle binds to the haem component of soluble guanylate cyclase (sGC), resulting in activation of sGC and a corresponding increase in cyclic guanosine monophosphate (cGMP) levels [175]. The increase in cGMP levels are concomitant with decreases in intracellular calcium brought about via several mechanisms including activation of calcium pumps [374] and inhibition of calcium channels [31]. This decrease in calcium results in dephosphorylation of myosin light chain and subsequent relaxation of the smooth muscle.

1.3.8 The endothelium

The endothelium comprises a single layer of cells that line blood vessels in the human body. Their small size belies their important role in vascular biology as these cells maintain vascular homeostasis via regulation of;

1. Inflammatory response
2. Haemostasis
3. Myocardial function
4. Angiogenesis
5. Vascular tone and permeability

Endothelial cells have evolved mechanisms that allow detection of changes in haemodynamic forces, oxygen levels and also local blood borne signals such as agents released from the autonomic and sensory nerves, circulating hormones, autocoids and cytokines. Thus the endothelium is able to dynamically interact with its environment by detecting and responding to changes occurring in the cardiovascular system. For example changes in blood pressure are met with a change in vessel tone or if the integrity of the vessel is damaged then the endothelium regulates the formation of a platelet plug to prevent leakage in and out of the vessel. No less than 14 active mediators are known to be released that



control vascular tone. These can be broadly categorised as contracting and relaxing factors and are summarised in Table 8.

Table 8 Endothelial derived factors involved in control of vessel tone

Constricting Factors	**Relaxing Factors**
Thromboxane A2	Adenosine
Isoprostanes	Prostacyclin
20-hydroxy eicosatetraenoic acid	Nitric oxide
superoxide	Hydrogen peroxide
Hydrogen peroxide	Epoxyeicosatrienoic acids (EETS)
Endothelin 1	C-natriuretic peptide (CNP)
Angiotensin II	
Uridine adenosnie tetraphosphate	

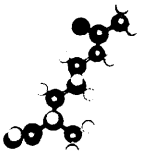
Endothelial cells are also able to control the expression of surface proteins such as adhesion molecules that interact with other molecules [219].

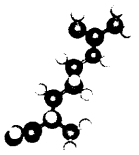
1.3.9 Endothelial dysfunction

Maintenance of vascular homeostasis is a tightly controlled process in which the balance between vasoconstriction and dilation, inhibition and promotion of smooth muscle cell proliferation and migration, inhibition and stimulation of platelet aggregation and thrombogenesis and fibrinolysis must be carefully controlled [75]. The disruption of this balance leads to endothelial dysfunction.

Endothelial dysfunction has been associated with a large number of both physiological and pathophysiological processes including:

1. Diabetes I and II
2. Ageing
3. Obesity
4. Hypercholesterolaemia
5. Hyperhomocystinaemia





6. Sepsis
7. Rheumatoid arthritis
8. Smoking
9. Nitrate tolerance
10. Hypertension
11. Coronary artery disease
12. Heart Failure

Diseases associated with endothelial dysfunction are thought to be risk factors for cardiovascular disease. In fact endothelial dysfunction, as assessed by flow mediated dilatation in brachial arteries, has been shown to predict long term cardiovascular disease in patients with coronary artery disease, hypertension, heart failure or atherosclerosis [56,97,112,142,278,314,350].

Endothelial dysfunction can be generally summarised as:

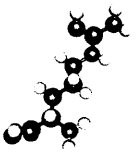
1. Altered anticoagulant properties
2. Altered anti-inflammatory properties
3. Impaired modulation of vascular growth
4. Altered vascular remodelling
5. Impairment of endothelium dependent vasorelaxation

There exists a large body of work linking the generation of ROS to the development of cardiovascular disease [47,116,117]. Nearly every risk factor for cardiovascular disease leads to enhanced ROS production and it therefore seems likely that oxidative stress is a common pathway as it is present in several unrelated cardiovascular risk factors. Increased levels of reactive oxygen species are linked to up-regulation or increased activity of enzymes such as NADP(H) oxidase, xanthine oxidase, cytochrome P450 and uncoupled nitric oxide synthase. It remains to be seen whether ROS are causative, or indeed just a consequence of endothelial dysfunction.Table 9 briefly summarises evidence for the role of ROS in several cardiovascular risk factors.

Table 9 Source of free radicals in various disease states

Disease State	Source of free radicals	Experimental model	Reference
Atherosclerosis and coronary artery disease	NAD(P)H oxidase	• Hypercholesterolaemic rabbits • Atherosclerotic lesions in mice • Plaques in human coronary arteries • Coronary artery bypass surgery • Coronary artery disease	[20,124,136,341,342,382]
	xanthine oxidase	• Hypercholesterolemia • Heavy Smokers • Coronary artery disease	[47,49,72,265] [123] [342]
	Uncoupled NOS	• Hypercholesterolemia • Heavy Smoker	[143]
	Cytochrome P450	• Hypercholesterolaemia	[219]
Diabetes	NAD(P)H oxidase	• Streptozotocin-treated rats • Porcine model of Streptozotocin induced diabetes	[147,415]
	Uncoupled NOS	• Streptozotocin-treated rats • Insulin resistant rates	[147] [332]
	Mitochondria	• Diabetic *in vitro* model	[85,260]
Hypertension	NAD(P)H oxidase	• Angiotensin II induced hypertension • Renovascular hypertension • Genetic hypertension • DOCA-salt hypertension	[195,220,248,360,413,416]
	xanthine oxidase	• Spontaneously hypertensive rats	[254,351]
	Uncoupled NOS	• Spontaneously hypertensive rats • DOCA-salt hypertension in mice	[67]
	Cytochrome P450	• Hypertension	[219]

Heart failure	NAD(P)H oxidase	• Heart failure induced by myocardial infarction in rats • Experimental pressure overload cardiac hypertrophy and failure	[21] [226]
	xanthine oxidase	• Chronic heart failure	[92]
Sepsis	NAD(P)H oxidase	• Sepsis	[17,119] 682,683
	xanthine oxidase	• Sepsis	[242]



1.3.10 Effect of ROS on the endothelium

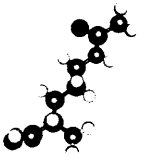
Superoxide is the main product of nearly all the enzymes discussed above. However where superoxide is produced, the formation of hydrogen peroxide and hydroxyl radicals is highly likely due to Fenton chemistry and the Haber-Weiss reaction. Therefore although the above enzymes do not all synthesise hydrogen peroxide and hydroxyl radicals directly, this does not mean these radical species do not contribute to endothelial dysfunction.

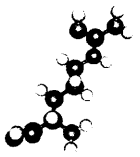
ROS exert numerous deleterious effects on the endothelium and these can be broadly split into nitric oxide dependent and independent effects.

1.3.10.1 Nitric oxide dependent effects

Nitric oxide is thought to inhibit smooth muscle proliferation, inhibit leukocyte adhesion and platelet aggregation and to control arterial tone. Therefore anything that reduces the bioavailability of nitric oxide will push the endothelium towards a dysfunctional state. A large body of work has shown that decreased NO bioavailability may be related to increased levels of oxidative stress. Several reactive oxygen species have been shown to react with nitric oxide including superoxide, hydroxyl radicals and lipid radicals. The detrimental effects caused by these reactions can be divided into:

1. Decreased NO production
    a. Oxidised LDL impairs receptor induced endothelial NO production [188]
2. Increased NO degradation via interactions with
    a. superoxide
    b. Hydroxyl radicals [282]
    c. Lipid radicals e.g. $LO^{\bullet}$ or $LOO^{\bullet}$ [262]
3. Production of toxic bi-products peroxynitrite
    a. $O_2^{\bullet-} + NO^{\bullet} \rightarrow ONOO^-$ (rate constant = $7x10^9$ mol l^{-1} s^{-1})





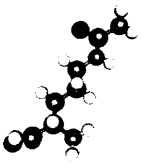
Peroxynitrite, is a highly toxic molecule that has numerous detrimental effects on the endothelium including:

1. Inhibition of soluble guanylate cyclase [386]
2. Oxidation of zinc thiolate clusters and tetrahydrobiopterin leading to NOS monomerisation and therefore eNOS uncoupling [196,201,421]
3. Inactivation of prostacyclin synthase [422]
4. Inhibition of superoxide dismutase

1.3.10.2 Nitric oxide independent effects

ROS also react directly with endothelial components to cause endothelial damage. Such interactions include:

1. Oxidation of low density lipoproteins (LDL)
    a. Adhesion of leukocytes to the endothelium and migration into the sub-endothelial space [256]
    b. Endothelial cell toxicity ([257]
2. Oxidative damage to macromolecules such as membranes and DNA
3. Interaction with connexins 37, 40 and 43 [120] leading to decreased electrotonic signaling.
4. Activation of metalloproteinases
5. Adverse regulation of genes involved in controlling the formation of
    a. Adhesion molecules
    b. Chemotactic substances
    c. Antioxidant enzymes
6. Promotion of contraction of smooth muscle by increasing calcium mobilisation and increasing sensitivity of contractile proteins to calcium [83,170,352]
7. superoxide mediated stretch and agonist induced endothelium dependent contractions in canine cerebral arteries [176]
8. Activation of endothelial enzymes e.g. Cyclooxygenase (COX) which



releases endothelial derived contracting factors [18,167].

9. Free radical-catalysed peroxidation of arachidonic acid leading to the formation of prostaglandin F2 like compounds [246].
10. Hydrogen peroxide can cause vasoconstriction and depolarisation

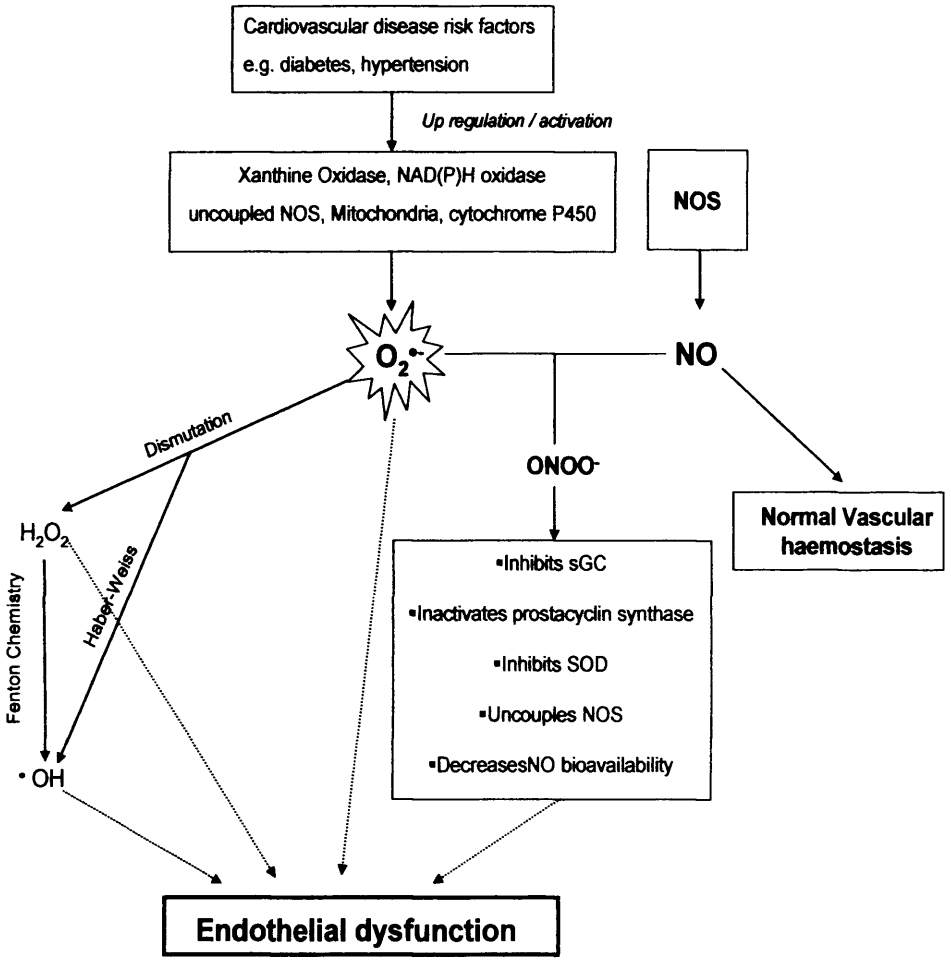


Figure 12 Role of ROS in endothelial dysfunction

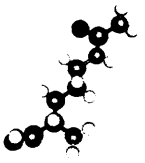
1.3.10.3 Interaction between different ROS sources

As well as directly reacting with endothelial components to cause endothelial dysfunction, ROS can also interact with other reactive oxygen sources to cause further ROS generation. This highlights how small changes in ROS levels can be amplified and or modulated through interaction of different enzyme systems. Table 10 summarises the phenomenon of ROS induced ROS release.

Table 10 Interaction between ROS from various sources

ROS system affected	Mechanism	Reference
Mitochondria	Oxidative damage to mitochondria results in increased superoxide production via decreased activity of respiratory enzymes and mitochondrial membrane potential	[207]
Xanthine oxidase	Superoxide increases the conversion of xanthine dehydrogenase into the oxidase form. Implicated in oscillatory sheer stress	[237]
NOS	Oxidative degradation of tetrahydrobiopterin leads to NOS uncoupling and thus increased superoxide production	[219]
NADPH oxidase	Mitochondrial ROS generation can lead to NAD(P)H oxidase activation	[219]
	Exposure of smooth muscle cells or fibroblasts to hydrogen peroxide causes NAD(P)H oxidase activation	[219]

This importance of ROS both in the cause and development of endothelial dysfunction is clear and this section highlights the need for further work into ways to reduce ROS levels in the cardiovascular system.

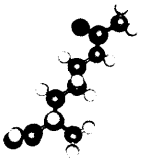


1.4 Platelets and Reactive Oxygen Species

1.4.1 Introduction

Platelets are involved in both physiological and pathophysiological control of the vessel wall via the release of growth factors [90], lipid mediators [177] and cytokines [35]. Platelets have been shown to be involved with thrombus formation, angiogenesis, vascular tone [7], inflammation and atherothrombotic disease [154] [30]. Modulation of platelets *in vivo* is therefore vital if a healthy ordered vascular system is to be maintained. If platelet regulation breaks down pathophysiological disease occurs. Platelet function is mediated via numerous factors, both cell and blood borne. Platelet activation occurs in conditions of endothelial damage where underlying collagen is exposed or upon exposure to platelet derived agonists such as thromboxane A2 (TxA_2), adenosine diphosphate (ADP), thrombin or platelet activating factor (PAF). This thesis has focused on the effects of collagen on platelet function and what follows describes signaling events specific to collagen.

Collagen is a well known agonist for platelet activation mainly through activation of a series of receptors mainly GPVI and $\alpha 2\beta 1$ receptor. Activation of the latter leads to activation of phospholipase A_2. Activation of GPV1 leads to stimulation of PLC and a cascade of signaling events ultimately leading to an increase in intracellular calcium as illustrated in Figure (adapted from [301]). The increase in cytosolic calcium is thought to be from an extracellular source (70%) and internal stores (30%). Resting calcium is thought to be roughly 100nM and is maintained by the leak of calcium in and out of the platelets via the plasma membrane and accumulation in intracellular stores. Calcium levels are also maintained by the sodium-calcium exchanger (NCX) which under normal conditions pumps calcium out of the cell and sodium in against their concentration utilising ATP [302]. Collagen leads to the formation of thromboxane, which is known to lead to increases in intracellular sodium levels. The Na^+/H^+ exchanger (NHE) also increases intracellular sodium levels but to a



lesser extent. This increase in sodium is enough to reverse the NCX resulting in an increase in calcium through this mechanism. This mechanism is thought to account for the majority of calcium influx [300]. Other sources of increased calcium include release from intracellular stores via the actions of IP_3 on the dense tubular system. The increase in calcium mediates shape change, granule secretion and aggregation.

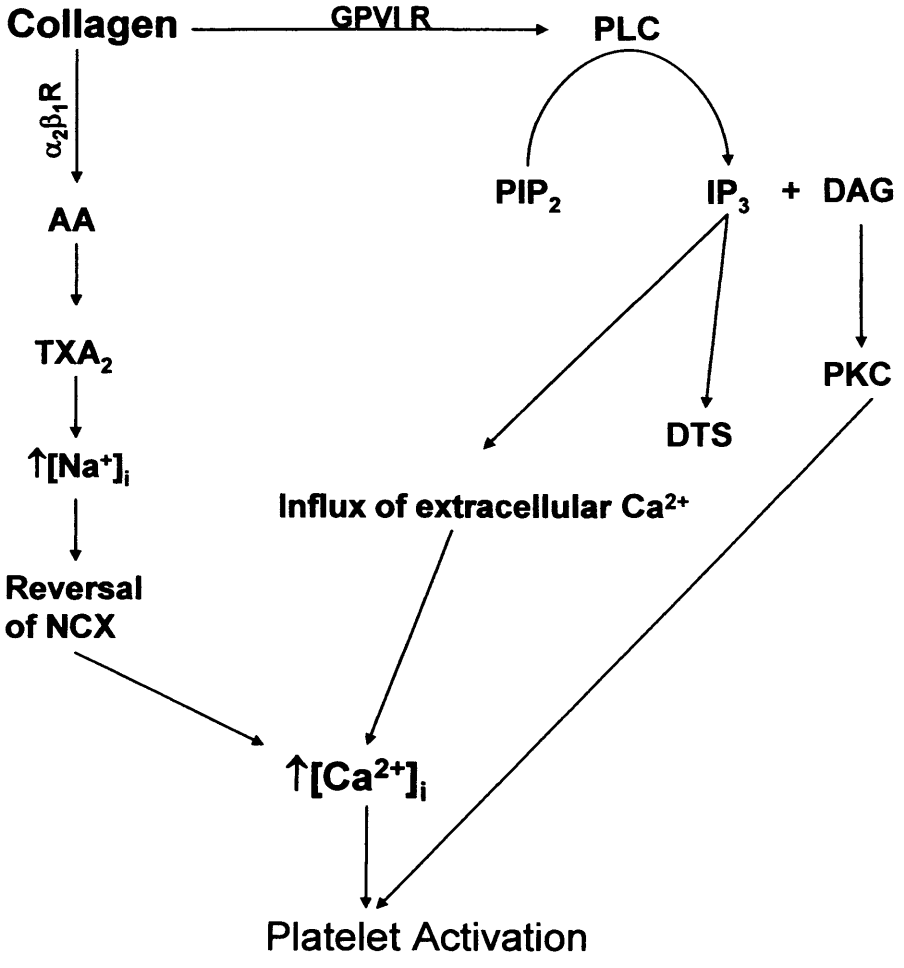
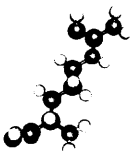


Figure 13 Collagen signaling cascade. NCX=Na^+/Ca^{2+} transporter; DTS=dense tubular system.

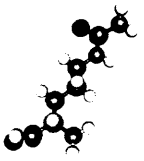


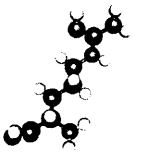
1.4.2 Relationship between collagen activation and nitric oxide synthase (NOS)

Radomski *et al* [294] demonstrated that collagen induced activation of platelets was accompanied by an increase in the cyclic nucleotide cyclic Guanosine monophosphate (cGMP). This lead to the conclusion that upon activation of platelets by collagen, the enzyme NOS was activated leading to subsequent production of nitric oxide. NO was then suggested to go on to activate Soluble guanylate cyclase (sGC) resulting in increased cGMP levels. More recently it has been shown that platelets contain both iNOS and cNOS [239,251]. *et al* [295] also demonstrated that NO formation was dependent on the concentration of free calcium in the cytosol over the range of 0.1-3.0μM. This level of calcium is through to be induced upon collagen activation and has been shown to activate nNOS in the brain [381]. It was therefore suggested that collagen caused NOS activation via increases in intracellular calcium levels. Further support for this comes from Lantoine *et al* [198] who demonstrated that in low calcium media nitric oxide production is reduced.

There can be no doubt therefore that nitric oxide regulates platelet function to some degree but this may not represent the main source of NO. Under physiological conditions NO is thought to be mainly released from the endothelium, where it can go on to interact with circulating platelets and prevents thrombosis [334], aggregation [66,344] and adhesion of platelets to the vessel wall [41,76]. This is supported by the fact that increased platelet activation is seen in diseases associated with endothelial dysfunction such as heart failure [315], hypercholesterolaemia [259]and diabetes [395]. In such cases where the endothelium becomes damaged, the role of platelet NO may become more pronounced, in terms of both platelet recruitment and vascular tone.

The amount of NO produced from platelets is thought to be 1000 times less than from endothelial cells [15,322,363,417]. However this may be enough to regulate platelet function and inhibit further platelet recruitment (negative feedback



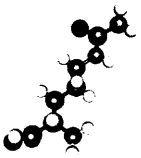


mechanism). Platelet derived nitric oxide exerts several functions in the platelet including:

1. Inhibition of dense granule secretion by recruited platelets.
2. Inhibition of alpha granule secretion leading to decreased p-selectin expression on the platelet surface [103]. Inhibition of p-selectin expression has been shown to reduce leukocyte accumulation and subsequent fibrin deposition.
3. Interference with enzyme phosphorylation [60].
4. Modulation of haemprotein of enzymes which govern the formation of eicosanoid mediators e.g. thromboxane A_2 and PGI_2 [61].
5. Activation of soluble guanylate cyclase leading to increases in cGMP and subsequent activation of PKG [68] .
6. PKG mediated phosphorylation of TXA_2 receptors [69].
7. PKG mediated phosphorylation of proteins involved in calcium signaling pathways [70].
8. cGMP independent role including acceleration of Sarco/Endoplasmic reticulum Ca^{2+}-ATPase (SERCA) dependent refilling of internal stores and inhibition of agonist induced TXA_2 synthesis via s-nitrosocysteine [71].

1.4.3 Modulation of platelet function by ROS

Of late, the role of ROS in platelet regulation has been highlighted. ROS can regulate platelet function directly or indirectly. Indirect regulation means that ROS interact with a mediator known to effect platelet function thus changing its effect on platelets e.g. NO. ROS will reduce the bioavailabilty of NO, thus increasing platelet reactivity. ROS can also directly regulate platelet function when they are produced within the platelet itself. Platelets were first observed to release superoxide during activation by Marcus *et al* 1977 [229]. Later studies went on to show that both resting and activated platelets could synthesise a wide range of ROS including superoxide, hydroxyl radicals and hydrogen peroxide [45,99,375]. Platelets therefore have the capacity to exert both a paracrine and



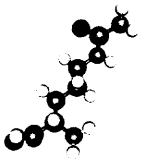
autocrine role via production of ROS. To date the following sources of ROS have been found in platelets:

1. xanthine oxidase
2. Uncoupled eNOS
3. NAD(P)H oxidase [158,238,283,330,419]
4. Cyclooxygenase 1
5. Mitochondiral respiratory chain complex I leakage
6. PI_3 Kinase (via membrane translocation of platelet $p67^{phox}$) [316]
7. PKC (via activation of NAD(P)H oxidase) [329,377,418]
8. Membrane depolarisation
9. PLA2 dependent AA release [44]

Reactive oxygen species known to modulate platelet function include superoxide [98,160,208,311], the hydroxyl radical [159,372]) and the non radical species hydrogen peroxide [80,151,266].

Superoxide has also been shown to reduce the threshold to collagen, thrombin, ADP and arachidonic acid and to even cause spontaneous aggregation [78,131,312,328]. Superoxide has long term effects on thrombus degradation due to its ability to prevent ADP degradation via endonucleosidases. This results in increased levels of ADP and decreased formation of adenosine, an inhibitor of platelet activation/recruitment. Superoxide also decreases NO bioavailability which results in increased aggregation. This is due to the extensive role of NO in the control of platelet function. Superoxide has also been demonstrated to increase serotonin release and thus increase platelet activation [132].

Hydrogen peroxide has also been implicated in the modulation of platelet activity and is thought to exert its effects via activation of phospholipase C [266], activation of COX [213] and inhibition of arachidonyl-Co A synthetase, leading to increased levels of arachidonic acid [151]. Hydrogen peroxide has also been linked to hydroxyl radical production through Fenton chemistry [159]. Hydroxyl radicals have been shown to lead to platelet aggregation, serotonin release, thromboxane production [159] and PKC production [288]. It has also been demonstrated that



hydroxyl radical scavengers inhibit ADP, collagen, arachidonic acid, PAF-induced platelet aggregation, and platelet cyclooxygenase pathway activation [372].

ROS also modulate platelet activity via their ability to shift the intracellular redox balance within the platelet. Although it is not known if the following occurs in cells, superoxide oxidises reduced glutathione (GSH) to oxidised glutathione (GSSG) [396,397]. This changes the extracellular ratio of GSH:GSSG (normally 100:1). This change results in decreased levels of reduced thiol groups which results in increased sensitivity to activation via platelet agonists [366]. One receptor thought to be affected by this change in redox balance is the GPIIb/IIIa. Other receptors also thought to be effected by the redox balance in the platelet are the $\alpha_{IIb\beta 3}$ receptor [91,193,264], $\alpha_{2\beta 1}$ integrin [194] and the P_2Y_{12} receptor ([81]). Independent to this is oxidative control of the short intracellular tail of β_3 [202].

Reactive oxygen species are also linked to the stimulation of phospholipase A_2 [159,160,243]. The following diagram sumarises the effects of ROS on platelet enzyme activity.

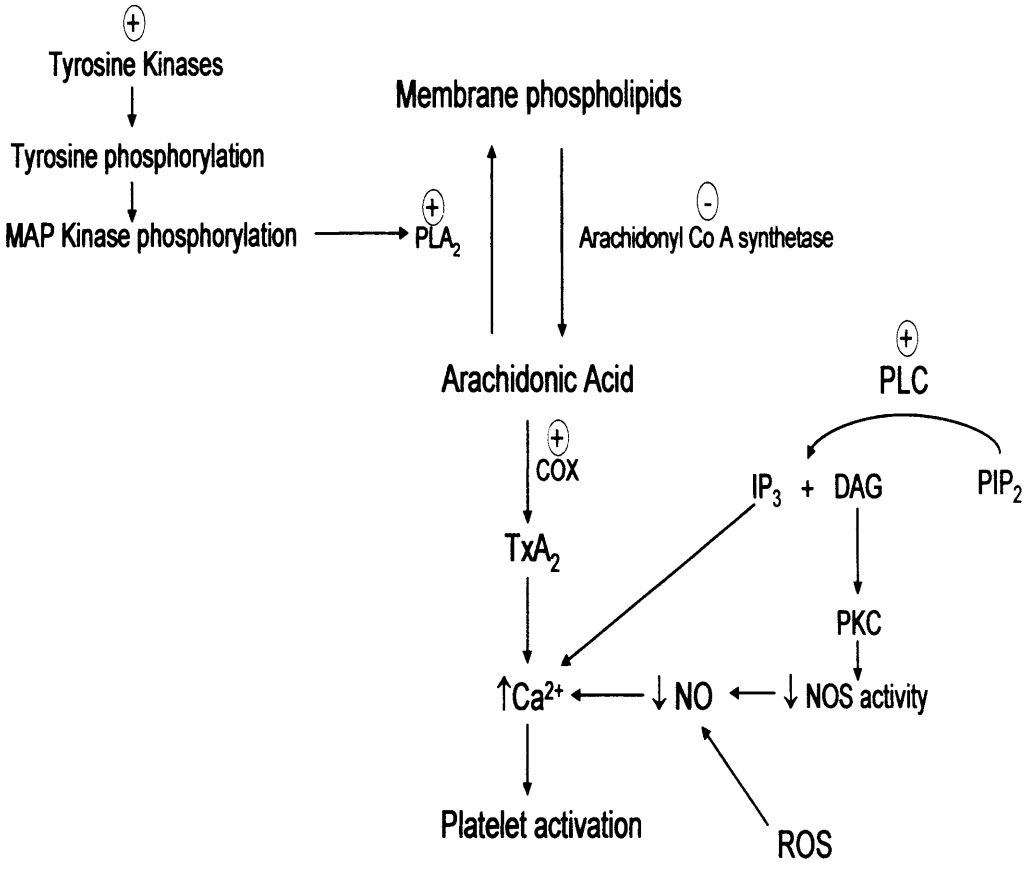


Figure 14 Effect of ROS on platelet enzyme activity. (+) represents an increase in enzyme activity (-) represents a decrease in enzyme activity

1.5 Arginine supplementation

Interest in L-arginine as a potential treatment of cardiovascular disease originated from the hypothesis that any molecule capable of increasing NO bioavailability would improve endothelial function. As the substrate for NOS, L-arginine was an obvious choice. In 1992, two seminal experiments were carried out demonstrating a beneficial role of L-arginine supplementation on endothelial function. Creager *et al* [73] demonstrated that supplemental L-arginine improved endothelial dependent NO mediated forearm vasodilator response in hypercholesterolaemic subjects. In the same year, Dubois-Rande *et al* [86] demonstrated that infusion of L-arginine improved endothelial vasodilator function in atheromatous left anterior descending coronary arteries. Since these initial findings there exists a wealth of information on the beneficial effects of exogenous L-arginine. The following tables aim to summarize studies on the effects of L-arginine. Although not exhaustive, the most significant research studies have been included.

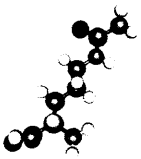


Table 11 Clinical studies of L-arginine supplementation

Disease State	Ref	Subjects	Study design	Measured outcomes	Result	Conclusions
Healthy Subject	[2]	12 Healthy men	Oral supplementation with L-arginine 7g TID x 3 days	Platelet aggregation, brachial artery dilation	+/-	Inhibition of platelet aggregation but no effect on endothelium-dependent dilation.
	[209]	26 Healthy subjects	Oral supplementation with L-arginine 20g/day x 28 days	Coronary blood flow, endothelin levels.	+	Improvement in coronary small vessel endothelial function and reduced endothelin levels.
	[63]	26 healthy males	20g/day L-arginine or placebo administered orally for 28 days.	Endothelium function assessed by venous occlusion plethysmography.	-	No effect on endothelial function.
	[345]	Healthy human patients	Blood taken from healthy patients and 5-100mM L-arginine added.	Haemostasis	+	Inhibition of haemostasis activation.
	[11]	Healthy Male volunteers	Blood taken from healthy patients and 50-1000μM L-arginine added.	Platelet aggregation, cGMP levels	+	Inhibition of platelet aggregation.
Coronary Artery Disease	[5]	10 men with CAD	Oral supplementation with L-arginine 1g TID x 3 days	Brachial artery dilation, monocyte adhesion	+	Improved endothelial dependent dilation and reduced monocyte adhesion

	[33]	29 men with CAD, one woman with CAD	Oral supplementation with L-arginine 9g QD x 28 days	Brachial artery dilation ICAM-1, VCAM-1, E-selectin	-	No improvement in NO bioavailability
	[359]	8 patients with Coronary artery disease and stable angina.	Atrial pacing performed during intracoronary infusions of normal saline and L-arginine (150μmol/min).	Luminal diameter of epicardial coronary arteries as assessed by quantitative angiography.	+	Significant increase in diameter of all coronary segments and stenoses.
	[411]	31 stable CAD patients	Oral L-arginine (10g) daily for 4 weeks	Brachial artery endothelial function studies, serum concentrations of lipid and inflammatory markers. Susceptibility of LDL particles to oxidation.	+	Increased brachial artery flow-mediated dilatation and decreased susceptibility of LDL to oxidation. No effect on inflammatory mediators.
Hypercholesterolaemia	[65]	27 Hypercholesterol-aemic subjects	Oral supplementation with L-arginine 7g TID x 30days	Brachial artery dilation	+	Improved endothelium dependent dilation
	[55]	24 hypercholesterola-emic patients and 18 controls	Oral supplementation with L-arginine. 14 or 21g/day x 3 months	ADMA and L-arginine; mononuclear and T-Cell adhesiveness	+	Plasma L-arginine:ADMA ratio inversely correlated with mononuclear cell adhesiveness
	[356]	20 hypercholesterola-emic patients, 12 normal controls	Oral supplementation with L-arginine 8.4/day x 2 weeks	Mononuclear cell adhesiveness	+	Increased MNC adhesiveness reversed by L-arginine
	[398]	23 patients with hypercholesterola-emia 14 normal controls	Oral supplementation with L-arginine 8.4/day x 2 weeks	Platelet aggregation	+	Modest attenuation of increased platelet activity.

Congestive Heart Failure	[129]	40 patients with sever CHF randomized	Patients randomized to an L-arginine group, L-arginine + forearm training group, L-arginine + training group, control group.	Endothelial vasodilatation in internal radial artery diameter.	+	Improved endothelium-dependent vasodilatation
	[64]	20 CHF patients, 7 controls	Oral supplementation with L-arginine 20g/day x 28 days	Forearm blood flow	-	No influence on endothelial function
Smoking	[3]	Eight smokers, eight non-smoking controls	One time dose of 7g	Monocyte/endothelial ell adhesion, ICAM-1, VCAM-1, E-selectin	+	Inhibition of monocyte-endothelial cell adhesion.
	[48]	11 Healthy Smokers, 12 healthy non-smokers, both with no other coronary risk factors	Myocardial blood flow was measured at rest and, after intravenous infusion of L-arginine (30 g as 10% arginine hydrochloride) for 45 minutes at a rate of 6.67 ml/min.	Non-invasive measurements of myocardial blood flow (MBF)	-	No effect on resting MBF in smokers or non-smokers.
Diabetes Mellitus	[51]	20 type II diabetic patients	I.V. infusion of L-arginine (3g in 10ml saline) 5, 10, 30 and 60 minutes after the assessment of baseline values.	Plasma homocysteine concentrations NO_x, Sulphydryl groups (markers of oxidative stress)	+	Decreased plasma homocysteine levels, decreased oxidative stress and increased NO bioavailabilty
	[224]	Thirty patients with diabetes mellitus	Patients received placebo or 2g L-arginine orally given as 2x1g dose per day for 3 months	Lipid peroxidation as assessed by malondialdehyde (MDA).	+	Reduced AMDA levels.
Ageing	[57]	34 patients with atypical chest pain and no coronary risk factors	Intracoronary infusion of L-arginine (160 μmol/min for 20 min). immediately prior to endothelial function assessment	Endothelial function as assessed by coronary blood flow was measured with an intracoronary Doppler catheter	+	Restoration of endothelium-dependent coronary microvascular function

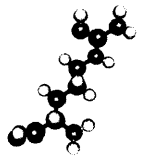
Hypertension	[275]	12 normal control subjects and 14 hypertensive patients	L-arginine infused at 40 μmol/min into brachial artery immediately before start of experiment	Endothelial function as assessed by the effect of acetylcholine on forearm vasculature as measured by strain gauge plethysmography.	-	No change in basal blood flow or vascular resistance in either group
	[353]	essential hypertensive patients (n = 34) and normotensive subjects (n = 30)	L-arginine infused into the brachial artery - 1μmol.100 $mL^{-1}.min^{-1}$	Endothelial function as assessed by the effect of acetylcholine on forearm vasculature as measured by strain gauge plethysmography	+	Improved vascular relaxation
Misc	[32]	10 post-menopausal women without additional risk factors for atherosclerosis.	9g L-arginine or placebo daily for one month.	Nitric oxide levels in Serum, endothelial function, markers for inflammation	-	No effect on NO levels, flow mediated dilation or soluble cell adhesion molecules.
	[121]	22 patients with peripheral arterial obstructive disease (PAOD)	Patients received 60mmol L-arginine infusions, each lasting 3 hours, daily for seven consecutive days.	Pain free and maximum walking distances, ADP/Collagen induced platelet aggregation, cGMP levels	+	Elongation of pain free and maximum walking distances, aggregation inhibited and cGMP levels increased
	[165]	32 patients with atherosclerotic peripheral artery disease	Patients split into two groups receiving 3x2g/day and 3x4g/day for 28 days.	Total antioxidant status Nitric oxide concentration.	+	Increased NO levels and increased total antioxidant status.

Table 12 Experimental studies of L-arginine supplementation

	Ref	Subjects	Study design	Measured outcomes	Result	Conclusions
Hypercholesterolaemia	[37]	Rabbits- 18 total	Groups fed chow, high-chol, high-chol + L-arginine or high-chol + L-NAME x 12 weeks.	Vascular reactivity and superoxide release. Carotid artery plaque area.	+	Restoration of endothelial function.
	[38]	Rabbits-32 total	Cholesterol fed rabbits treated with L-arginine in water.	Aortic atherosclerosis, urinary nitrate and oxidative stress.	+	Reduced lesion formation, improved endothelial function, and decreased oxidative stress.
	[36]	Rabbits – 28 total	Cholesterol fed rabbits with L-arginine or L-NAME x 12 weeks	Myointimal cell proliferation. Aortic intima/media ratio.	+	Decreased cell proliferation and vascular monocyte accumulation.
	[362]	Rabbits – 12 total	Cholesterol fed rabbits treated with L-arginine x 10 weeks	Platelet aggregation. Platelet cGMP levels	+	Inhibition of platelet aggregation.
	[137]	Rabbits – 49 total	Cholesterol fed rabbits treated with L-arginine (2.5% in drinking water) x 12 weeks	NO metabolites, cGMP levels, vascular reactivity, tissue blood flow, superoxide levels, histological evaluation of atherosclerosis.	+	Reduced oxidative stress and reversed the progression of atherosclerosis.
	[391]	Rabbits	Cholesterol fed rabbits received 2.25% L-arginine in drinking water.	Plasma xanthine oxidase activity, vessel reactivity, LDL oxidation	+	Improved vasodilator function by reducing circulating levels of XO,

Smoking	[156]	Rabbits-32 total	Hypercholesterolaemic rabbits exposed to environmental tobacco smoke; some supplemented with L-arginine x 10 weeks	Vasorelaxation in aortic rings	+	Diminished endothelial dysfunction. Diminished endothelial dysfunction.
	[155]	Rabbits-32 total	L-arginine supplementation (2.25% solution) and environmental tobacco smoke (smoking chambers for 10 weeks)	Vasorelaxation in aortic rings	+	Diminished endothelial dysfunction.
Diabetes	[241]	Normal and diabetic rats	L-arginine added to PRP obtained from normal and diabetic rats	Platelet aggregation stimulated by ADP, thrombin and epinephrine	+	Inhibition of platelet aggregation
ischaemia reperfusion injury	[153]	Healthy Rabbits split into groups: 1: L-arginine treatment, hind limb operation with I/R 2: No L-arginine treatment, hind limb with I/R	L-arginine (4mg/kg/min) was infused for 1 hour before ischaemia).	NO and superoxide levels.	+	Decreased superoxide generation by NOS while increasing NO accumulation.

	[389]	6-hour model of myocardial ischaemia and reperfusion in pentobarbital-anaesthetised cats	A bolus administration (30 mg/kg) of L-arginine followed by a continuous infusion of 10 mg/kg/min for 1 hour starting 10 minutes before reperfusion.	Myocardial necrosis, cardiac myeloperoxidase activity, endothelial function in coronary artery rings.	+	Reduced necrotic injury and attenuated neutrophil accumulation in ischaemic cardiac tissue.
Hyper-tension	[58]	Pregnant rats	Treatment with L-arginine (2%) in the drinking water was initiated at day 10 of gestation	Arterial pressure	+	Reduced arterial pressure
Miscellaneous	[148]	Encephalomyo carditis virus-infected BALB/c mice	Dietary L-arginine and L-arginine + L-NAME administered over 4 weeks	Cardiac damage Survival	+	Reduced cardiac damage and increased survival.
	[223]	32 Adult male rats subjected to exhaustive exercise	Rats fed a standard diet Or 2% L-arginine diet for 30 days	xanthine oxidase, myeloperoxidase (MPO), SOD, catalase, glutathione peroxidase activity	+	Decreased XO and MPO activity and favourably increase antioxidant defence systems in the lung after exhaustive exercise.



1.5.1 Arginine Paradox

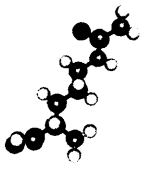
Evidence would suggest that L-arginine could be the key to future treatment of cardiovascular disease as it improves endothelium-dependent vasodilatation and abnormal interactions of vascular cells, platelets and monocytes. The question is therefore, how is L-arginine exerting these effects? The original hypothesis was that as the substrate of NOS, exogenous L-arginine increased NO production, however *in vitro* studies have shown the K_m of NOS to be in the micromolar range (1-5 μmol/l), whereas the intracellular concentration of L-arginine is thought to be in the milimolar range (0.8-2.0 mmol/l). L-arginine concentration should not therefore be rate limiting in the production of $NO^{\bullet}$. This discordance between *in vitro* studies estimating the K_m of NOS and *in vivo* experiments demonstrating an effect of L-arginine is known as the arginine Paradox. Several mechanisms have been postulated to explain the arginine paradox including:

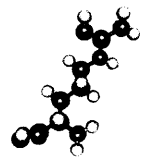
1.5.1.1 Intracellular compartmentalisation of L-arginine:

McDonald *et al* [236] demonstrated that a complex exists between eNOS and the y^+ L-arginine transporter in endothelial cells and that both molecules are located within the plasma membrane calveolae. This would suggest that eNOS is in fact partitioned from the intracellular L-arginine supply and is therefore dependent upon direct transfer of L-arginine into the subcellular compartment by the y^+ transporter. This would explain why exogenous L-arginine may increase $NO^{\bullet}$ production even though total intracellular levels of L-arginine may not appear to be rate limiting.

1.5.1.2 The presence of elevated levels of ADMA:

ADMA is an endogenous competitive inhibitor of NOS. It is derived from the hydrolysis of proteins that contain methylated arginine residues and has been shown to inhibit vascular $NO^{\bullet}$ production at concentrations found in pathophysiological conditions (3-15 μmol/L) [364]. ADMA competes for the L-





arginine transporter found in endothelial cell membranes and so can uncouple NOS by limiting L-arginine supply to the enzyme. Uncoupled NOS, as a product of limited L-arginine, produces superoxide in place of NO causing activation of redox-regulated transcription factors and concomitant up-regulation of endothelial adhesion molecules and monocyte adhesion [39]. Exogenous L-arginine would increase the concentration of L-arginine to a level sufficient to successfully compete with ADMA. This would result in the re-coupling of NOS thus increasing NO• production and decreasing superoxide production.

1.5.1.3 Increased arginase activity:

Arginase catalyses the breakdown of L-arginine into ornithine and urea and has been shown to be up regulated in plasma of individuals with cardiovascular disease. Up-regulation results in decreased L-arginine bioavailability and therefore exogenous administration of L-arginine may go some way to restore this balance.

1.5.1.4 Modulation of vascular tone

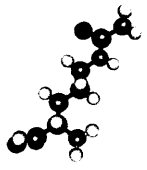
L-arginine may stimulated the release of several vasodilator modulators including insulin [109], histamine [108], glucagons and growth hormone [110,276,321]. L-arginine is also capable of inhibiting sympathetic tone [245].

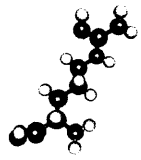
1.5.1.5 pH dependent affect

L-arginine can be administered as a hydrochloric salt that induces extracellular acidosis that in turn can alter intracellular pH. This affects pH dependent cell signaling pathways such as calcium transit, that modulate eNOS activity and NO synthesis. Acidic micorodomains also provide an appropriately acidic environment for the non-enzymatic reduction of nitrite into NO [423].

1.5.1.6 Up regulation of eNOS

L-arginine has been shown to up regulate eNOS protein expression, thus resulting in a long-term increase in NO production [79,255].





1.5.1.7 Antioxidant properties

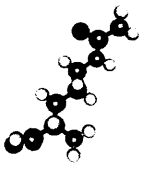
The final postulated mechanism for the beneficial effects on endothelial function seen with L-arginine is the ability of this molecule to act as an antioxidant. This would explain the success of this molecule on endothelial dysfunction in such a wide range of disease states (Table 9). As discussed at length in introduction, oxidative stress is the common link between nearly all cardiovascular risk factors and so the ability of a molecule to reduce levels of ROS offers a viable mechanism for improvement of endothelial function. The following section aims to give an overview of all the information to date concerning the antioxidant properties of L-arginine.

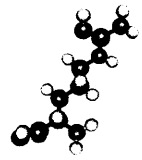
1.5.2 Antioxidant properties of L-arginine

The ability of L-arginine to scavenge ROS has been studied in a number of models with mixed results. Oxidation of lipoproteins is associated with the initiation of athrosclerosis and so a number of studies have looked at the ability of L-arginine to prevent this. The ability of L-arginine to inhibit copper mediated lipoprotein oxidation varies between studies with complete [379,383,411], moderate [38] and no inhibition [6] being seen.

L-arginine has been shown to directly inhibit oxidative stress by reducing superoxide release from endothelial cells [383] and hypercholesterolaemic rabbit aortic rings [37]. Reducing superoxide levels reduces ROS mediated degradation of nitric oxide, as demonstrated in a model of ischaemia-reperfusion injury in rabbits, where a reduction in superoxide levels was associated with increased levels of NO [153].

On a slightly larger scale, L-arginine has been shown to protect rat hearts from ROS damage. Incubation with L-arginine prevented myocardial injury and protected coronary function [199,347] from superoxide mediated damage.
L-arginine has also been shown to protect against oxidative stress via a less direct action. This includes decreasing xanthine oxidase activity in rats after

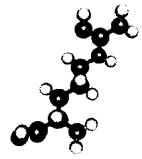




exhaustive exercise and improving antioxidant defence systems by increasing glutathione levels [223] and SOD and catalase activity [89]. A clinical trial in 2005 looked at the effect of L-arginine on endothelial function and LDL oxidation in patients with stable coronary artery disease [411]. In this study L-arginine was shown to improve endothelial function and inhibit LDL oxidation. L-arginine has also demonstrated an ability to reduce the release of ROS from circulating human neutrophils [392].

On a more negative note, a number of studies claim that L-arginine has no antioxidant effect. In 2005, Hayashi *et al* [137] looked at the ability of L-arginine to inhibit the progression of atherosclerosis in hypercholesterolaemic rabbits. Although L-arginine was able to reduce superoxide production and oxidative sensitive gene expression in combination with L-citrulline and/or ascorbic acid and α-tocopherol, no antioxidative effect was seen when L-arginine was administered on its own. Possibly the most damning study on the role of L-arginine as an antioxidant was a study by Adams *et al* who claim that at concentrations found in the plasma (~200μM), L-arginine lacked any antioxidant activity [6]. They demonstrated that L-arginine had no effect on lipid peroxidation, was unable to synergise with alpha-tocopherol. This shows that unlike ascorbic acid, L-arginine is incapable of recycling the alpha tocopherol radical to the antioxidant alpha tocopherol form. This study also looked at the ability of L-arginine metabolites to exert an antioxidant effect, but again no effect was seen. The study is limited however as only one concentration of L-arginine was investigated, 200μM, the reason being this is the concentration of L-arginine found in human plasma. The concentration of L-arginine in endothelial cells is much higher, 0.1-2mM, and at these concentrations several studies have shown L-arginine to be an effective antioxidant [199,379,383]. This study also only studied lipid oxidation, which although important in the development of cardiovascular disease, is not the only factor involved.

The role of L-arginine as an antioxidant therefore remains unclear as studies directly contradict each other. Even within studies that agree L-arginine has an antioxidant effect, there exists discord concerning the mechanism of action

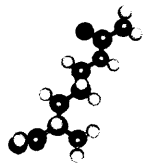


through which L-arginine scavenges free radicals, with some groups claiming the α-amino group mediates the antioxidant action [379] and others claiming it is the guanidinium group [199]. It is therefore clear that a comprehensive evaluation of the antioxidant properties of L-arginine is needed.

1.6 The role of L-citrulline in the vasculature

L-citrulline has received significantly less interest in comparison to L-arginine, mainly because until relatively recently it was thought to be merely the inactive bi-product of NO synthesis. One of the first groups to look at a potential physiological role of L-citrulline were Ruiz & Tejerina [308]. They *hypothesised* that L-citrulline was capable of controlling vascular tone and demonstrated that L-citrulline was able to relax pre-constricted aorta from healthy rabbit through activation of K_{ca} channels leading to hyperpolarisation [308]. These results have since been brought into question in a subsequent study by Marx *et al* [233] who upon repeating Ruiz & Tejerina's study showed no effect of L-citrulline on vascular tone. The reasons for such conflicting results were unclear, and explained as differences in protocol, such as difference in partial oxygen pressure, buffer and pH. The role of L-citrulline in the control of vascular tone remains unclear from experiments carried out, since two demonstrated that L-citrulline stimulated vasorelaxation [137,296] and one again showing no effect on aortic ring relaxation [157].

Other studies investigating the effect of L-citrulline include a study by Ikeda *et al* [157] who looked at the effect of L-citrulline on rat hearts exposed to ischaemia-reperfusion injury. Ischaemic hearts reperfused in the presence of L-citrulline exhibited a marked preservation of left ventricular developed pressure, decreased polymorphonuclear leukocyte accumulation in post-reperfused myocardium and decreased p-selectin expression on coronary endothelium. In another study the phenomena of post-operative hypertension development after congenital heart surgery in children was studied. Oral administration of L-citrulline was shown to be effective in reducing post-operative pulmonary hypertension. Probably the most informative study to date concerning L-citrulline was carried out in 2005 by Hayashi *et al* [137], who investigated the ability of a variety of antioxidant

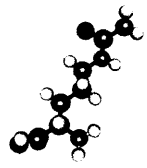


compounds to inhibit the progression of high-cholesterol induced atherosclerosis. Although the main aim of this study was to investigate the effects of L-arginine, L-citrulline was also tested based on the hypothesis that L-citrulline is converted into L-arginine and is thought to be one of the main ways of sustaining local L-arginine concentration for NO production [100,113,340]. L-citrulline was able to reduce atheromatous lesion size, markedly improve endothelial function, upregulate eNOS expression and to decrease superoxide levels.

1.6.1 Antioxidant properties of L-citrulline

The mechanism by which L-citrulline is working remains unclear. Hypotheses include NO independent effects, such as those demonstrated by & Tejerina [308] and NO dependent effects mediated through the conversion of L-citrulline into L-arginine. An alternative hypothesis, and one we are interested in, is the possibility that L-citrulline is working by decreasing ROS, thus preventing ROS mediated vascular damage and also increasing NO bioavailability. Indeed, some of the effects of L-citrulline do mimic those exhibited by NO, such as the inhibition of smooth muscle cell proliferation [306]. The potential for L-citrulline to act as an antioxidant is supported by a phenomenon seen in wild watermelon. Wild watermelon are found in the Kalahari desert where through the summer periods they have to survive conditions of drought, which in terms of plant physiology means they have to protect themselves against increased ROS. Botanists have discovered that these plants have evolved a survival mechanism that involves accumulation of L-citrulline via an enzyme called DRIP-1 [179,412]. L-citrulline has been shown to be a potent hydroxyl radical scavenger capable of reacting with hydroxyl radicals at a rate of 3.9×10^9 M^{-1} s^{-1} and therefore by increasing the levels of L-citrulline within the plant, ROS damage is limited.

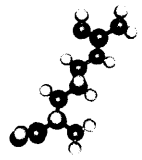
Although these studies only look at the hydroxyl radical, it is plausible that L-citrulline is capable of scavenging superoxide, as it is structurally similar to L-arginine, another potential superoxide scavenger. The positive effects of L-citrulline seen in the studies discussed above may therefore in part be mediated via an antioxidative action. A second aim of this thesis was to therefore



investigate whether L-citrulline does indeed exert an antioxidant effect.

1.7 Thesis aims

As oxidative stress plays such a prominent role in the development of endothelial dysfunction it is vital to characterise potential antioxidant molecules capable of preventing the development of cardiovascular disease. L-citrulline is being shown to be more than simply an inactive bi-product of NO synthesis with studies highlighting that L-citrulline is cardio-protective in a number of disease states. L-arginine, the precursor of NO, has shown in countless studies that it is capable of improving endothelial function. With evidence to suggest L-citrulline may have antioxidant properties in plants and the current contradictions that exist concerning the antioxidant properties of L-arginine, a study aimed at fully characterising the antioxidant profile of both molecules was needed. I feel that the contradictions that exist concerning the antioxidant nature of L-arginine may rest with the methodologies used to investigate its function. This thesis overcomes this by utilising the highly sensitive and specific technique of EPR which allows direct characterisation of free radicals. In a series of *ex vivo* studies, the significance of these antioxidant actions in both physiological and pathophysiological conditions is elucidated. Together, this provides an in depth profile of the antioxidant properties of L-arginine and L-citrulline and evaluates there potential in the prevention and treatment of endothelial dysfunction.

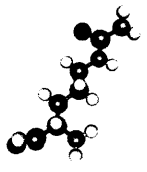


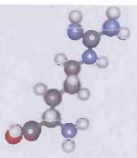
1.8 Specific aims

1. To characterise the antioxidant properties of L-arginine and L-citrulline focussing on radical specificity and the effect of the free radical:antioxidant ratio on function.

2. To characterise the mechanism by which L-arginine and L-citrulline exert their antioxidant actions.

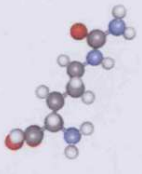
3. To investigate the effect of both molecules in physiological and pathophysiological processes including their effect on platelet function, lipid peroxidation and in a system of ROS mediated endothelial dysfunction.

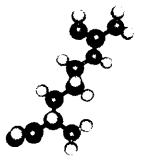




General Methods

Chapter Two





2.0 Formation of superoxide and hydroxyl radicals

2.01 Hydroxyl radical formation

A Fenton type reaction was chosen as the mechanism of hydroxyl radical synthesis as it is a relatively cheap and simple technique that could be adapted to the assay systems chosen for this thesis. Also, the importance of Fenton chemistry in biological systems has been long recognised [127,225].

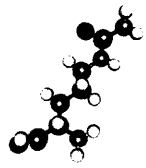
In 1894 [93] Fenton reported that mixtures of ferrous salts and hydrogen peroxide resulted in the formation of a species capable of oxidising dihydroxy maleic acid. This species was later suggested to be the hydroxyl radical by Haber and Weiss [125] which was confirmed by Baxendale *et al* [22] (Equation 14).

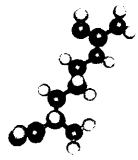
$$Fe^{2+} + H_2O_2 + H^+ \rightarrow Fe^{3+} + H_2O + {}^{\bullet}OH \qquad \textbf{Equation 14}$$

Numerous other transition metal complexes (in their lower valency state) have also been demonstrated to have the oxidative features of the Fe(II) including Cu(I), Ti(III), Cr(II) & Co(II). From these transition metals copper was chosen as it is a very physiologically relevant molecule that has been suggested to play a role in the oxidation of DNA damage associated with ageing and cancer [10,13,84,221,303]. As mentioned previously, it is lower valency transition metals that act as Fenton type reagents and so it is thought that hydroxyl radical production from Cu(II) most likely involves initial reduction to Cu(I). Note that Cu(I) was not used in these studies due to the rapid oxidation of Cu(I) to Cu(II) in aqueous solutions.

The mechanism of Cu(II) reduction and subsequent hydroxyl radical production is illustrated in equations 15 & 16.

$$Cu(II) + H_2O_2 \rightarrow CuOOH + H^+ \rightarrow Cu(I) + O_2^{\bullet-} + 2H^+ \qquad \textbf{Equation 15}$$





$$Cu(I) + H_2O_2 \rightarrow Cu(II) + {}^{\bullet}OH + OH^{-} \qquad \textbf{Equation 16}$$

The superoxide formed in reaction 16 can also go on to reduce Cu(II) as shown in equation 17 and directly reduce hydrogen peroxide to hydroxyl radicals via the Haber Weiss reaction as shown in Equation 18.

$$Cu(II) + O_2^{\bullet-} \rightarrow Cu(I) + O_2 \qquad \textbf{Equation 17}$$

$$O_2^{\bullet-} + H_2O_2 \rightarrow O_2 + {}^{\bullet}OH + OH^{-} \qquad \textbf{Equation 18}$$

As the aim was to produce a pure hydroxyl radical generating system, it was important to rule out the problem of superoxide contamination (equation 15). EPR studies, described in chapter three, demonstrated that no free superoxide was present in this system.

2.02 Superoxide formation

Throughout this thesis, superoxide was synthesised using a xanthine oxidase system. A general overview of xanthine oxidase is provided in the introduction and so the aim here is to briefly discuss superoxide production.

Xanthine oxidase is an oxidoreductase that exists as a homodimer of approximately 300kDa: each subunit contains four redox centres including a molybdenum cofactor (MoCo), one FAD and two Fe_2S_2 sites [146]. At the molybdenum site hypoxanthine is converted into xanthine, which then undergoes oxidation (specifically hydroxylation) to form urate. The electron introduced is rapidly transferred to the other linearly aligned redox centres and then passed on at the FAD centre to molecular oxygen (Figure). Univalent reduction of oxygen yields superoxide, whereas two and three electron reduction yields hydrogen peroxide and hydroxyl radicals respectively [26,191]. In this thesis a pure

superoxide generating system was required. EPR studies demonstrated that no hydroxyl radicals were being produced as no signal was detected using DEPMPO (a spin trap known to trap both superoxide and hydroxyl radicals) and the signal was completely inhibited by superoxide dismutase but not by catalase.

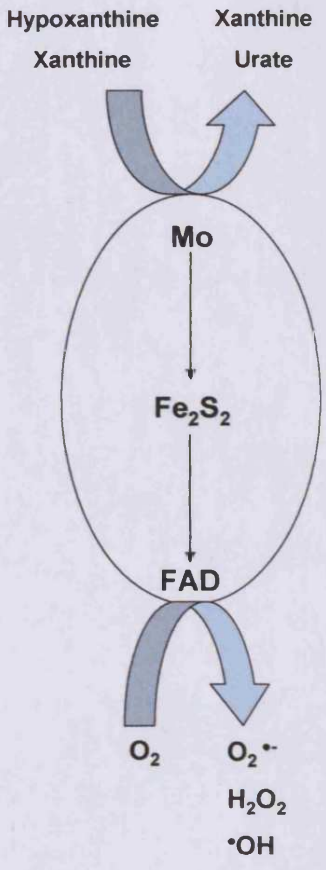


Figure 15 Schematic diagram illustrating xanthine oxidase oxidation of xanthine and hypoxanthine and reduction of molecular oxygen

2.1 Electron Paramagnetic Resonance spectroscopy

2.1.1 Introduction

Electron paramagnetic resonance spectroscopy is the gold standard in relation to the detection of ROS and has been used extensively in this thesis. To this end, the following provides a brief introduction to the complex field of EPR.

Spectroscopy generally utilises the fact that molecules interact with electromagnetic radiation. This results in the absorption or emission of energy by the molecule causing an energy transition from the ground state to an excited state (Figure).

16

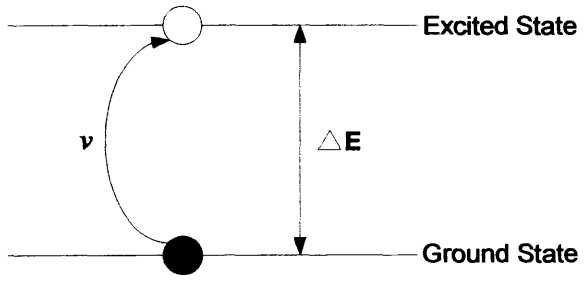
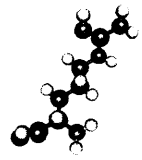


Figure 16 Spectroscopic transition involved in absorption spectroscopy.

Spectrometry refers to the measurement of radiation that is absorbed or emitted by the molecule involved. The difference, ΔE, between the two energy levels must be equal to the energy of the quantum radiation, i.e.

$$\Delta E = h\nu \qquad \textbf{Equation 19}$$

E is the energy of the quanta, v is the frequency of the radiation, and h is the Planck constant.



EPR is specific in that it only detects energy transitions within paramagnetic molecules i.e. those with an unpaired electron or nucleus with an odd mass number. This includes free radicals, transition metals and also spin traps, which are molecules that can be attached to free radicals to produce stable, detectable free radicals. EPR utilises the fact that free electrons and certain nuclei have a magnetic moment. All unpaired electrons spin about their axis and according to classical physics, any spinning charge will produce a magnetic field, and therefore the spinning electron (S) produces a magnetic moment (μ_S). The magnetic moment of an electron or a nucleus is related to its spin by:

$\mu = -g\beta s$ **for an electron** **Equation 20**

$\mu = g_n\beta_n I$ **for a nucleus** **Equation 21**

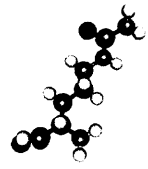
Where μ is the magnetic moment, g and g_n are proportionality constants called the electron and nuclear g-values, β and β_n are the Bohr and nuclear magnetons respectively (natural Units of electron and nuclear moments), s and I are the electron and nuclear spins respectively.

The electron and nuclear spin (S) are quantised, meaning there are only certain orientations allowed in relation to an applied external magnetic field, each corresponding to a distinct energy. The energy of the magnetic moment depends upon its projection along the magnetic field given by:

$E = -\mu.H$ **Equation 22**

In the case of a free electron or single nuclei, there are only two orientations allowed. The spins must either align with the magnetic field (parallel) in which case it has a lower energy state ($-^1/_2$) or against the external magnetic field (anti-parallel) in which case it has a higher energy state ($+^1/_2$) (Figure).

17



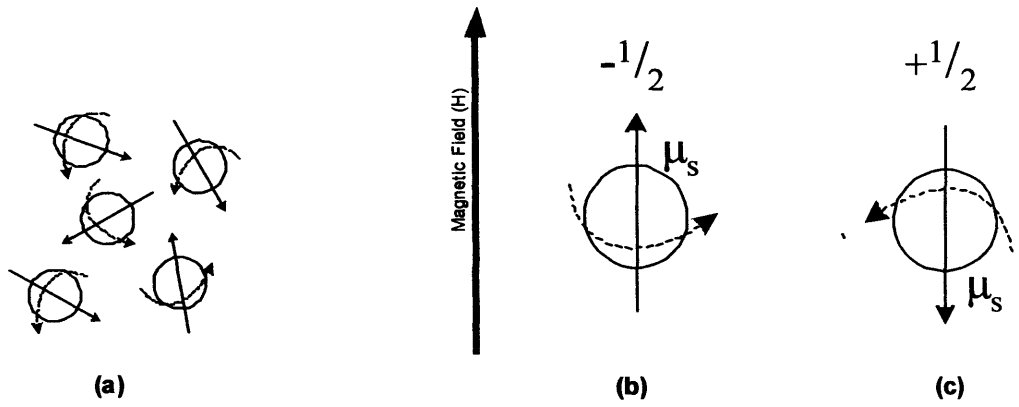


Figure 17 a) random magnetic moments of electrons in absence of magnetic field (H) (b) + (c) magnetic moment, μ_s, in presence of external magnetic field. (b) parallel, low-energy state, (c) anti-parallel, high energy state.

The energy states in a magnetic field are given by

$$\mathbf{E = \pm {}^1/_2\, g\beta H} \qquad \textbf{Equation 23}$$

Where H is the magnetic field.

Application of electromagnetic radiation can induce a transition from the low-energy (parallel) spin-state to the high-energy (anti-parallel) spin state. The conditions for this transition are given by:

$$\boldsymbol{h\nu = g\beta H} \qquad \textbf{Equation 24}$$

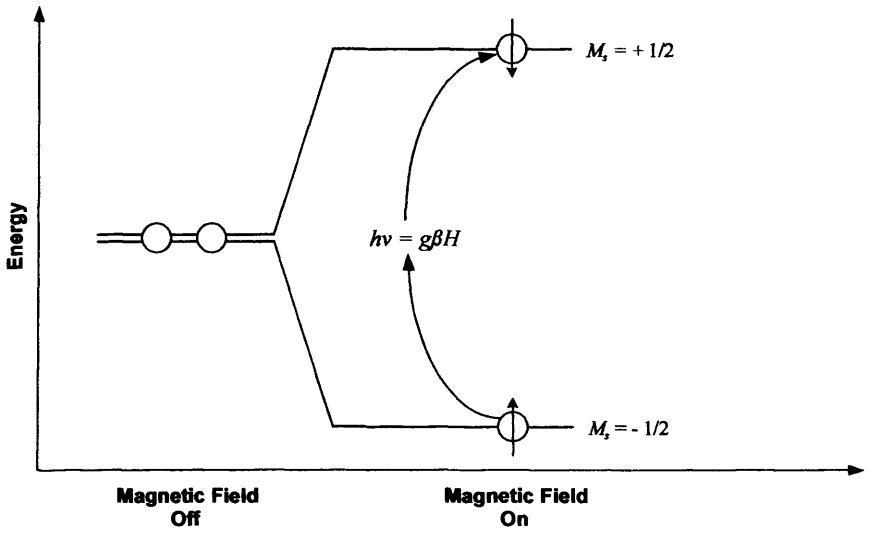


Figure 18 Electron spin levels in a magnetic field

$\Delta M_S = \pm 1$ **Equation 25**

$\Delta M_I = 0$ **Equation 26**

Where M_S and M_I is the spin quantum numbers of an electron and nucleus respectively.

According to these rules, only one transition is permitted; from $-^1/_2$ to $+^1/_2$ or vice versa. As a result only a single peak is observed in the spectrum. Magnetic resonance absorption is normally displayed as its first derivative, i.e. the amplitude representing the slope of the absorption peaks (Figure 18).

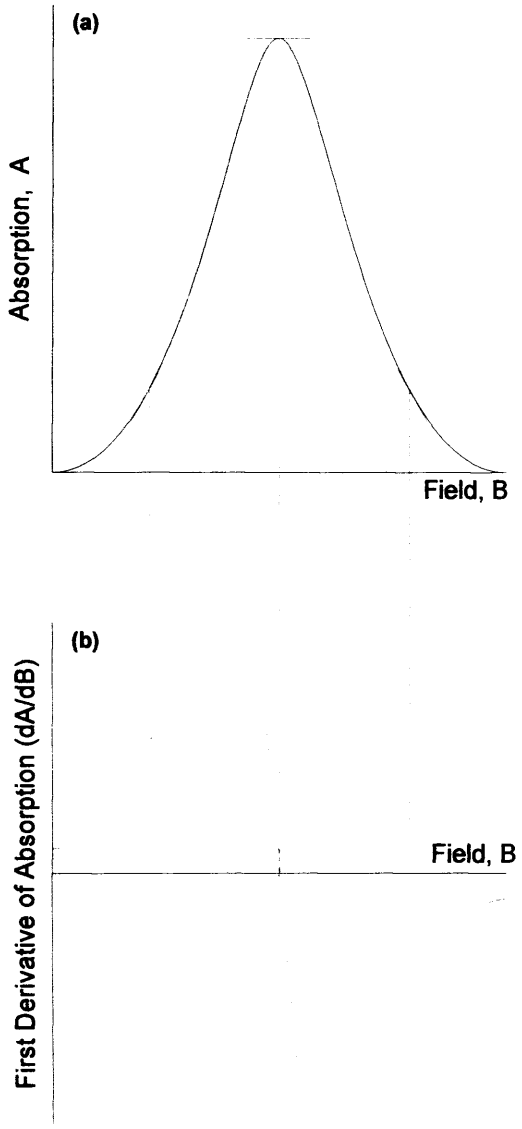


Figure 19 First derivative of the absorption intensity (a) the absorption (b) the first derivative signal, the slope of the absorption signal at each point.

The most important features of an EPR spectrum are the g-values – the points about which the spectrum is centred, and the splittings of these lines (hyperfine splittings). Hyperfine splittings arise from the interaction of the central magnetic moment (usually from the electron) and the magnetic moment of surrounding nuclei. Not all nuclei have a nuclear moment and Table 13 lists nuclei likely to give rise to EPR hyperfine splittings.

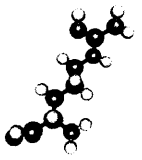


Table 13 Typical hyperfine splitting for magnetic nuclei.

Nucleus	Spin (I)	No. of lines
^{1}H	½	2
^{14}N	1	3
^{19}F	½	2
^{63}Cu	$^{3}/_{2}$	4
^{55}Mn	$^{5}/_{2}$	6

In the presence of a nuclear magnetic moment, the electron spin experiences both the external magnetic field (H) and the nuclear magnetic field (H_I). The number of hyperfine lines from a particular nucleus depends on the nuclear spin (I) and can be calculated from:

I= (2I +1) **Equation 27**

For I=$^{1}/_{2}$ e.g. ^{1}H, two hyperfine lines are seen. This is because EPR selection rules (Equation 25 & 26) states that only two possible transitions are permitted (Figure). The fact that all energy transitions are identical results in two peaks of equal intensity. One would expect both peaks to fall in the same position but this does not occur as the peaks are the product of both the electron and nuclear spin and are therefore displaced because they have slightly different energies i.e. different positions in the magnetic field.

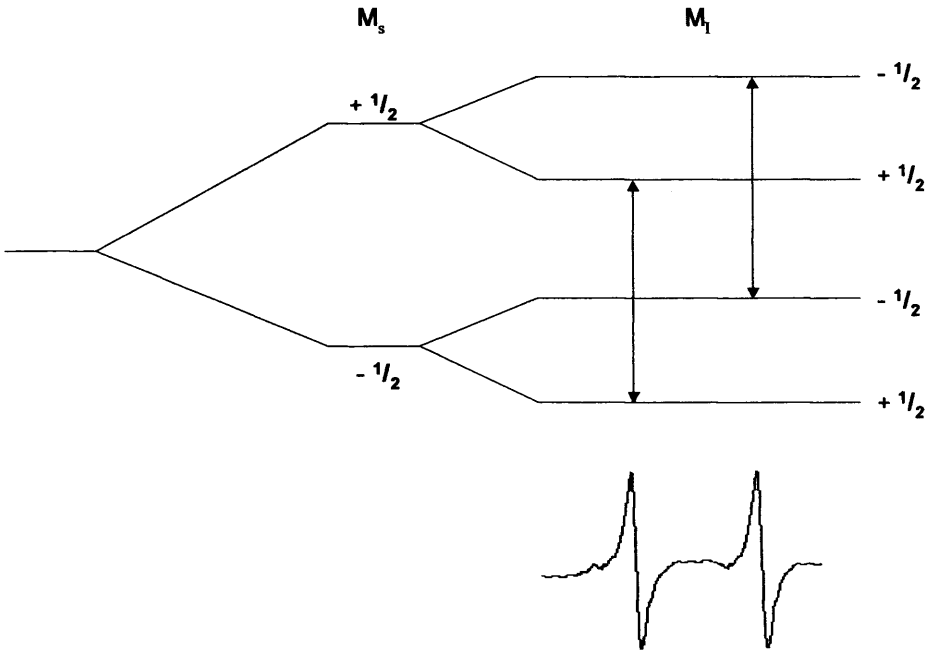


Figure 20 Hyperfine energy level splittings for nuclear spin $I={}^1/_2$.

Knowledge of a molecule's g-value and hyperfine splitting pattern means that EPR allows the quantification of the molecule in question, but to also unequivocally identify it.

2.1.2 Spin trapping

It is not always possible to observe paramagnetic species such as ROS for several reasons. The most obvious is that the concentration of ROS falls below the limit of detection of the EPR spectrometer. However some radicals, even at concentrations greater than the detection limit, are not observable at room/physiological temperature due to their short relaxation times. This results in line broadening so that the radical is effectively undetectable. This can be visualised using the Heisenberg Uncertainty principle. The uncertainty principle implies that if a system exists in an energy state for only a short time duration, then the energy of the state is not well defined causing a corresponding broadening in the spectral transition, the line width being given by Plancks constant:

$$\Delta v \approx 1/T \qquad \textbf{Equation 28}$$

where v=frequency of radiation, T= relaxation time

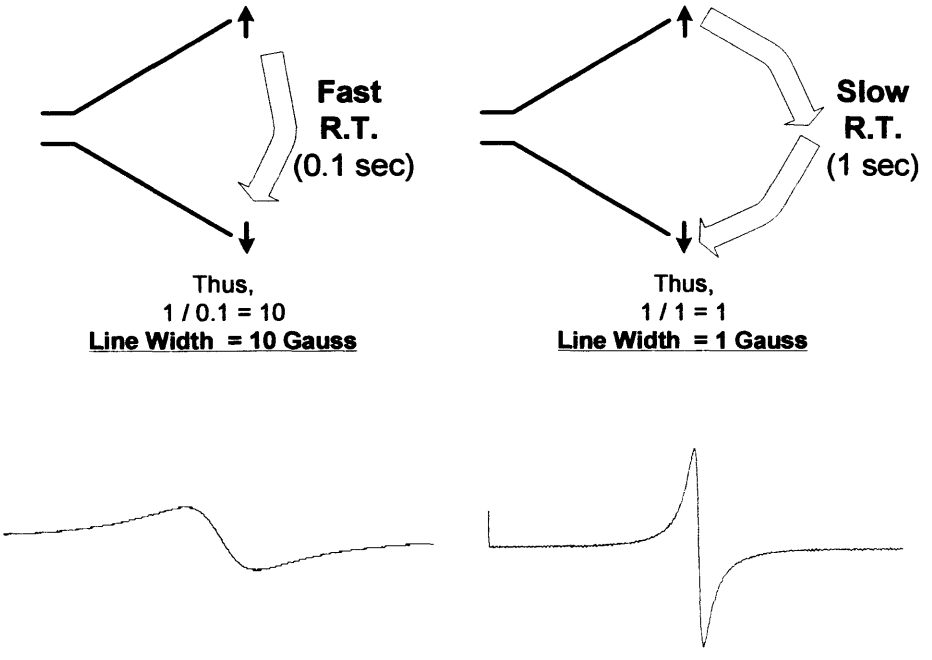
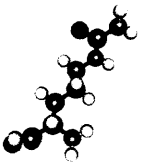


Figure 21 Schematic diagram illustrating how relaxation times affect the line width of a signal. Note that the area under the curve will be the same because the number of spins in the sample are the same.



Spin trapping provides a method to overcome the problem of unstable radicals or detection problems due to line broadening via the complexity of free radicals to nitroso or nitrone compounds (or spin-adduct). This results in the formation of a detectable nitroxide product. Simply put, a free radical ($R^{\bullet}$) reacts with a double bond of a diamagnetic compound, the spin trap (S=T), to form a less reactive radical, the spin adduct $(S\text{-}T\text{-}R)^{\bullet}$.

$$R^{\bullet} + S{=}T \rightarrow (S\text{-}T\text{-}R)^{\bullet} \qquad \textbf{Equation 29}$$

There are many types of spin trap and within this thesis the nitroso spin trap TEMPONE-H, and the nitrone spin traps DEPMPO and PBN have been focused on.

2.1.2.1 Nitroso and nitrone derivatives

Both nitroso and nitrone derivatives react with free radicals to from a stable nitroxide product (Figure 22). Nitroxides are relatively stable because the unpaired electron is resonance stabilised and sterically protected.

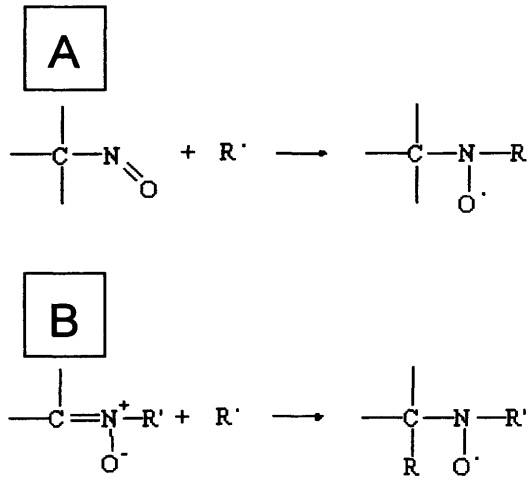


Figure 22 A) Nitroso molecule B) Nitrone molecule reacting with a free radical to give a nitroxide

The EPR spectrum of a nitroxide is a characteristic triplet resulting from the effect of the nitrogen nuclear spin (I=1) on the free electron. As discussed previously, in the presence of a nuclear magnetic moment, the electron spin experiences both the external magnetic field (H) and the nuclear magnetic field (H_I). The number of hyperfine lines from a particular nucleus depends on the nuclear spin (I) which in the case of nitrogen is 1. Following the equation I= (2I +1), nitrogen will therefore give rise to three peaks. The spectrum shows a 1:1:1 intensity ratio (Figure).

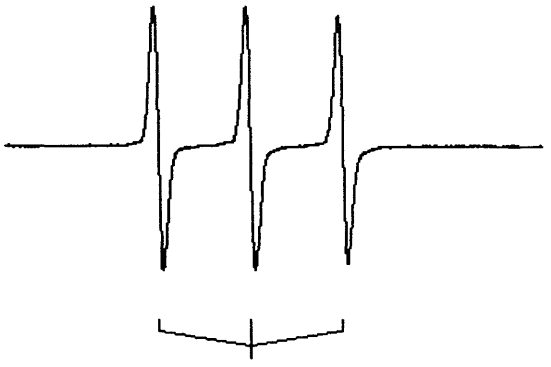


Figure 23 Representation of 1:1:1 spectrum resulting from the nuclear spin of Nitrogen

The primary triplet may be split into secondary lines, and with all spin traps used in this thesis this is due to the interaction of the β-proton (illustrated on all spin tap diagrams), which results in the formation of a doublet for each resonance of the primary nitrogen (See Table 13 & Figure 23). The magnitude of the hyperfine splitting for the β-proton provides information on the position of the hydrogen in relation to the nitrogen, which varies upon binding of different ROS. Thus the hyperfine splittings for the β-proton are used together with the primary triplet coupling for identification of the reactive free radical.

2.1.2.2 N-t-Butyl-α-phenylnitrone (PBN)

Almost all radicals form spin adducts with PBN, including carbon, oxygen and nitrogen centred radicals.

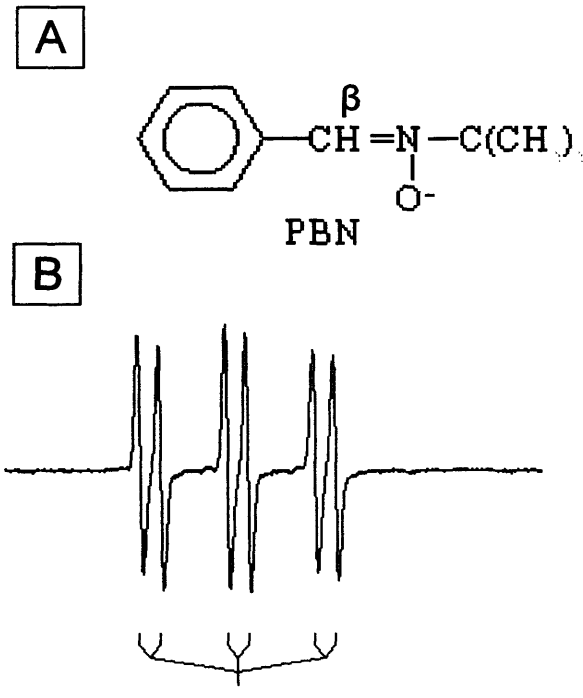
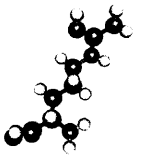


Figure 24 A) Structure of PBN B) example of PBN-adduct spectrum illustrating the splittings arising from the hydrogen and nitrogen nuclear moments

Although ROS are the primary radical generated *in vivo*, PBN reacts more rapidly with carbon-centred radicals. However as ROS react with cellular biomolecules such as lipids leading to the formation of secondary carbon-centred radicals, it is possible to use PBN *ex vivo* to demonstrate ROS formation as evidenced by trapping of the secondary carbon radicals [357]. Specifically, DMSO can be added as a source of carbon groups [43]. Any hydroxyl radicals formed react with DMSO to form carbon based radicals which can then be detected using PBN (Figure).

24



A

$$(CH_3)_2SO + \cdot OH \rightarrow CH_3SO_2H + \cdot CH_3$$

B

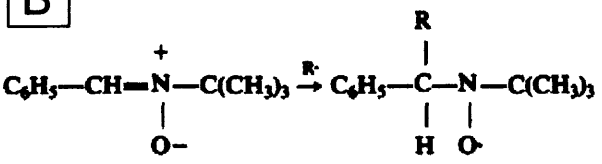


Figure 25 A) Formation of carbon centred radicals through reaction of DMSO and hydroxyl radicals. B) Formation of PBN adduct via addition of R group. In this experiment, R represents a carbon centred radical

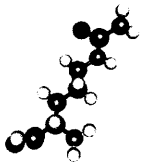
The differences in hyperfine splitting caused by the binding of different free radicals are small and therefore PBN is ill suited in the identification of specific ROS. PBN is more hydrophobic than traps such as DEPMPO however, meaning it readily crosses membranes and so can be used to measure free radical production within cells.

2.1.2.3 5-(Diethoxyphosphoryl)-5-methyl-1-pyrroline-N-oxide (DEPMPO)

Due to the shortcomings of PBN in terms of characterisation of specific ROS, the spin trap DMPO was developed as this allowed differentiation between ROS within a given system. DMPO was replaced later on with a phosphorylated derivative DEPMPO. DEPMPO-OOH is 15 times more stable than DMPO-OOH and does not spontaneously decay to $^{\bullet}OH$ or DEPMPO-OH [369]. Therefore unlike DMPO, DEPMPO can be used to detect between superoxide and hydroxyl radicals, a quality that is utilised throughout this thesis.

DEPMPO forms characteristic superoxide and hydroxyl radical adduct spectra as the position of the β-proton varies between adducts (Figure).

26



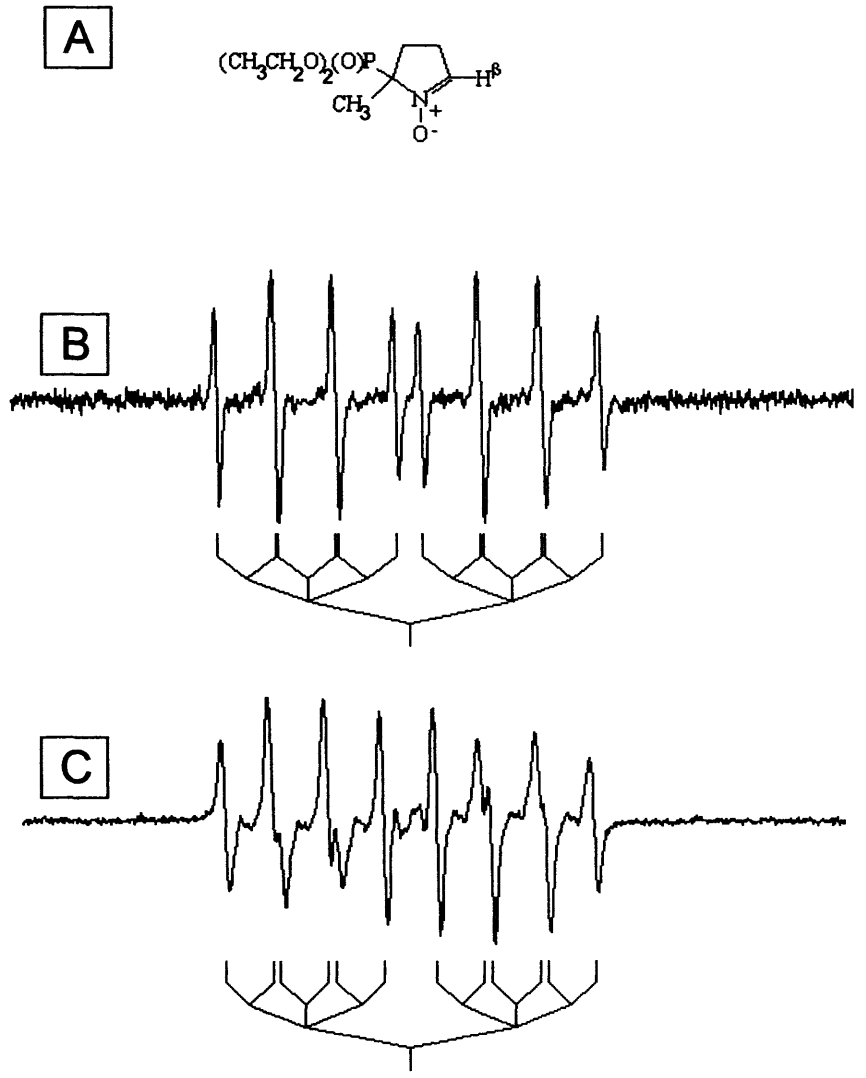


Figure 26 A) Structure of DEPMPO B) example of DEPMPO-OH spectrum C) Example of DEPMPO-OOH spectrum. Both B&C illustrates the splittings arising from the phosphate, hydrogen and nitrogen nuclear moments

2.1.2.4 TEMPONE-H

Unlike the spin traps discussed so far, TEMPONE-H does not allow characterisation of free radicals *per se*, rather quantification of oxidising potential. This is because unlike PBN and DEPMPO, the ROS do not bind to the nitrogen, rather they oxidise the diamagnetic TEMPONE-H into the paramagnetic TEMPONE. Formation of TEMPONE is therefore proportional to the concentration of ROS and so allows semi-quantification of the ROS (Figure) 27

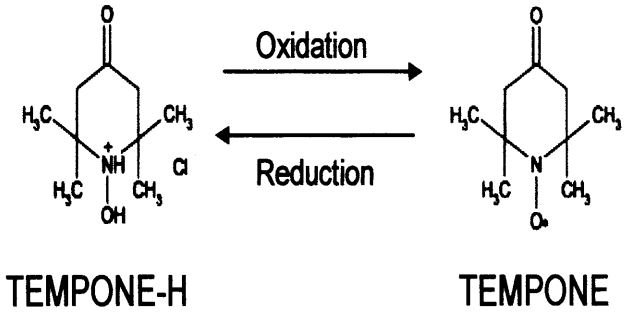


Figure 27 Inter-conversion between TEMPONE-H and TEMPONE

2.1.3 Detection of transition metals

In order to test whether L-arginine and L-citrulline were able to reduce hydroxyl radical generation through chelation of copper sulphate, the ability of EPR to quantify copper sulphate levels was utilised. EPR spectra for transition metals are often more complicated than those derived from spin traps and therefore a brief discussion of the spectrum observed with Cu(II) shall be undertaken.

The transition metal Cu(II) has one unpaired electron in the outer shell ($3d^9$) (Figure) making it paramagnetic and therefore detectable by EPR.

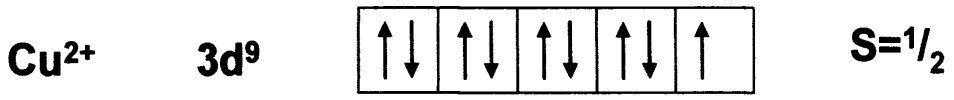


Figure 28 Electron configuration of copper(II)

However, as with ROS discussed above, copper has a short relaxation time resulting in signal broadening. Therefore, in order to detect copper, it must be bound to a metal chelator, in the case of these studies MGD, which has the effect of increasing relaxation time.

In the case of Cu(II), the EPR spectrum is complicated by the fact that the copper nucleus has a nuclear spin (I) of $^3/_2$. In simple terms, while a proton (I = ½) can only spin on its axis in two opposite directions (+½ or –½, often called 'clockwise' and 'anticlockwise' spin) the copper nucleus can actually spin in four opposite directions (labelled $+^3/_2$, $+^1/_2$, $-^1/_2$, $-^3/_2$). By applying the same set of EPR selection rules, one observes that four permitted energy transitions now become feasible, as illustrated in Figure . Notice, that no change occurs in the nuclear spins during the transition (i.e., one goes from the $M_I = -^3/_2$ level in the lower $M_S = -^1/_2$ spin state to $M_I = -^3/_2$ level in the upper $M_S = +^1/_2$ spin state; the electron spin changes, but the nuclear spin does not change during the transition).

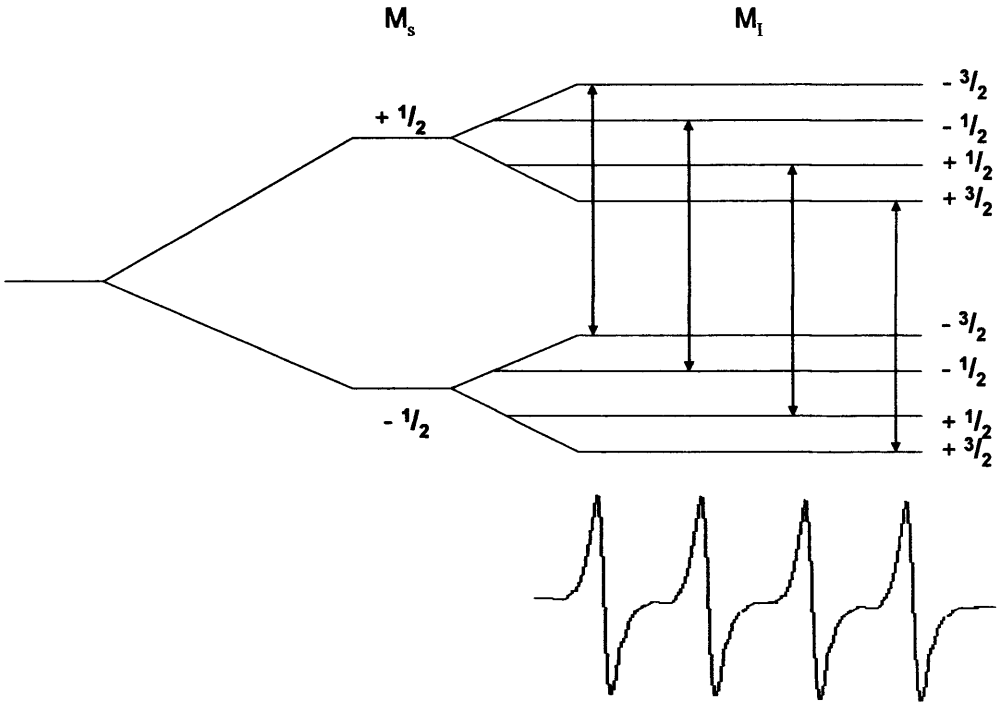
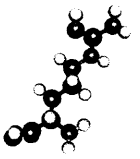


Figure 29 Hyperfine energy level splittings for nuclear spin $I={}^3/_2$. The EPR transitions for a single electron, $S={}^1/_2$ are shown. The energy level splittings mirror the hyperfine splittings are characteristic of the nuclear spin $I={}^3/_2$.

The spectra observed for Cu-MGD complex is not as straightforward as that illustrated in Figure due to the following factors:

1. Rotational correlation time
2. Spectral anisotropy
3. The presence of a nitrogen nucleus on the MGD molecule



2.1.3.1 Rotational correlation time

A dissolved copper ion in a solvent of low viscosity presents very rapid rotational motion whose correlation time (τ_c) is given by the Debye relation;

$$\tau_c = 4\pi\eta R^3/3kT \qquad \textbf{Equation 30}$$

Where R is the radius of the molecule and η is the viscosity of the solvent. One can simply see from this equation that the radius (i.e. size of the molecule) and the viscosity will affect the motion of the ion. For very fast motion (i.e., $\tau_c < 10^{-11}$ s), the EPR spectrum is insensitive to the rate of molecular motion and will consists of four lines of equal intensity. For relatively fast motion, $10^{-11} < \tau_c < 10^{-9}$ s, the effective rotational correlation time changes and this affects the shape of the spectrum, with all four peaks having slightly different intensities.

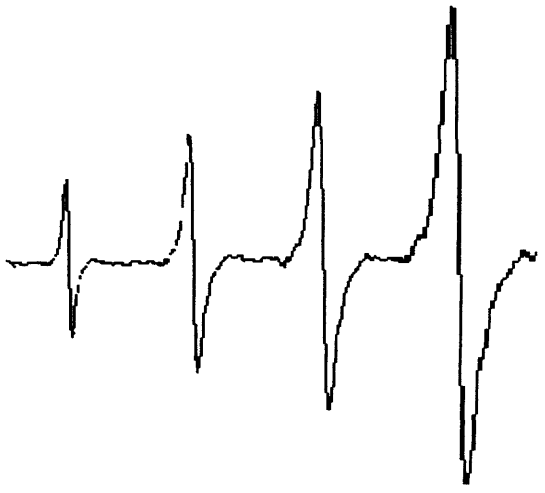
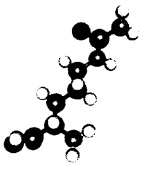


Figure 30 Schematic representation of the effect of rotational correlation time on copper(II) spectrum



2.1.3.2 Spectral anisotropy

The position and splittings of the lines (specified by the g-values and the hyperfine constants) seen in an EPR spectrum is dependent upon the direction of the magnetic field in relation to the molecular axes. A simple example to demonstrate this effect can be seen for a fully hydrated Cu(II) ion (Figure).

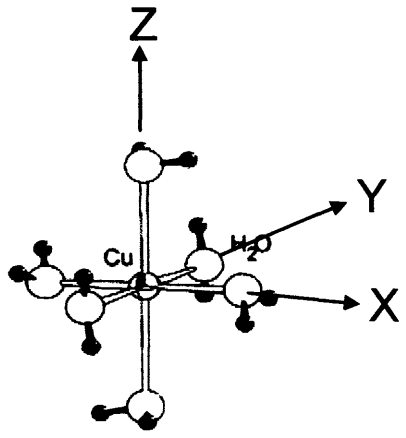


Figure 31 Schematic illustration showing the distorted octahedral shape of a fully hydrated Cu(II)

As can be seen, the Cu(II) ion binds six water molecules along the x, y and z-axis. According to the classic Jahn-Teller theory, all d9 ions will undergo a distortion along the z axis. This means the symmetry of the ion is reduced from pure octahedral (in which the Cu-H_2O distance is identical in every direction) to a distorted octahedral (in which the Cu-H_2O distance in the z direction is longer compared to the Cu-H_2O distance in the x and y direction). As a result the energy experienced by the electron is different along the z axis compared to the x and y axes. Under these circumstances, two peaks will appear in the EPR spectrum (disregarding for the moment the nuclear spin of copper); one corresponding to resonance along the z axis and another peak (of twice the intensity) corresponding to resonance along the x and y direction (Figure).

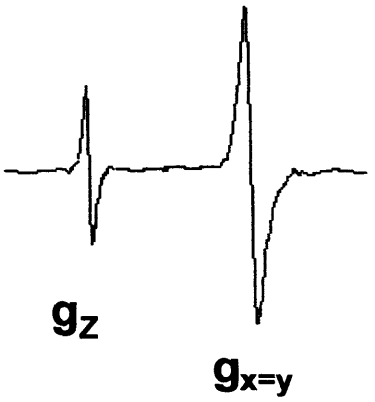


Figure 32 Schematic of spectrum obtained from fully hydrated copper ion experiencing a Jahn-Teller distortion

In frozen solution (e.g. 77K) two sharp, defined peaks will be seen as all molecules will be stationary and therefore exist in a random distribution (known as a polycrystalline glass) in which all orientations of the Cu ion are exposed to the external magnetic field. As the mobility of the molecule increases i.e. as temperature rises, rotation between the x-, y- and z-axis increases resulting in g-averaging in which the two peaks overlap to form one broad peak. As discussed before, this is slightly complicated due to the nuclear spin of copper ($I=^3/_2$). This results in a four-line hyperfine splitting in both the z and x=y directions as shown in the room temperature spectrum shown below (Figure 33).

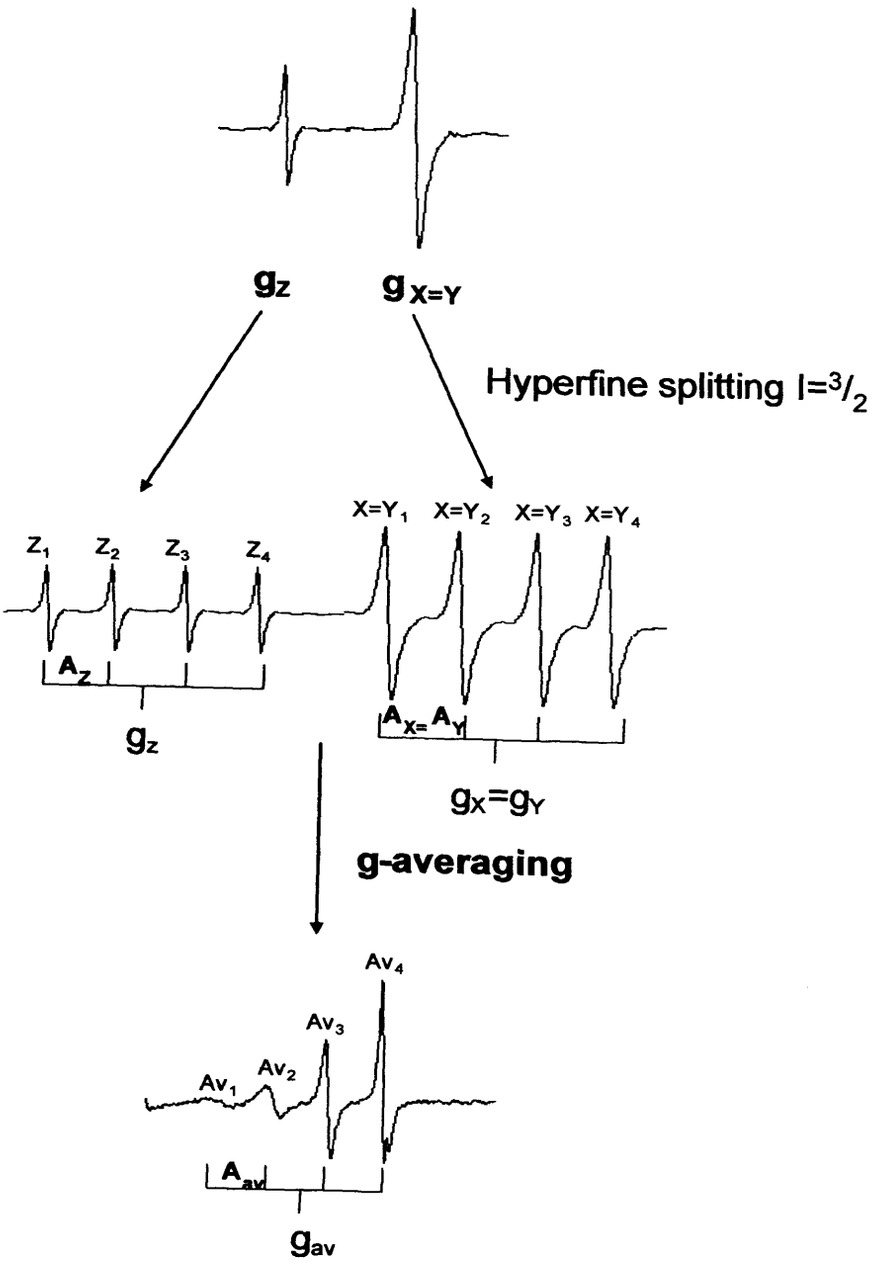
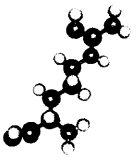


Figure 33 g-averaging of Cu(II). Av1=Z1 + X=Y1, Av=X=Y2.....etc



It can be seen from the above illustration that the g_z component will average with the g_x and g_y component.

2.1.3.3 Presence of a nitrogen nucleus on the MGD molecule

Finally the effect of the interacting nitrogen nucleus on the Cu(II) spectra was taken into account. This produces a superimposed *super*hyperfine structure (due to nitrogen) on the hyperfine structure of copper. Copper most likely binds to MGD *via* interaction with the nitrogen, and this is responsible for the effect of the nitrogen nucleus (I = 1) in the spectra.

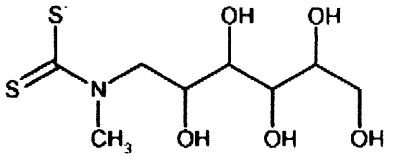


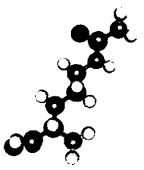
Figure 34 Chemical structure of MGD

The nitrogen nucleus results in a 3-way splitting of each peak. Due to the effects of decreased rotational correlation time and g-averaging, the three-way splitting can not be seen in detail due to signal broadening. The presence of a shoulder on peak 3 and peak 4 however can be observed and this is most likely due to the nitrogen splitting (A_N=14G).

2.1.4 Conclusion

The combined effects of the rotational correlation time, spectral anisotropy and binding to the chelator MGD, converts the simple four lined spectra derived from the electron spin ($S\pm^1/_2$) and nuclear spin ($I=^3/_2$) into the characteristic copper spectra see below at room temperature (Figure).

35



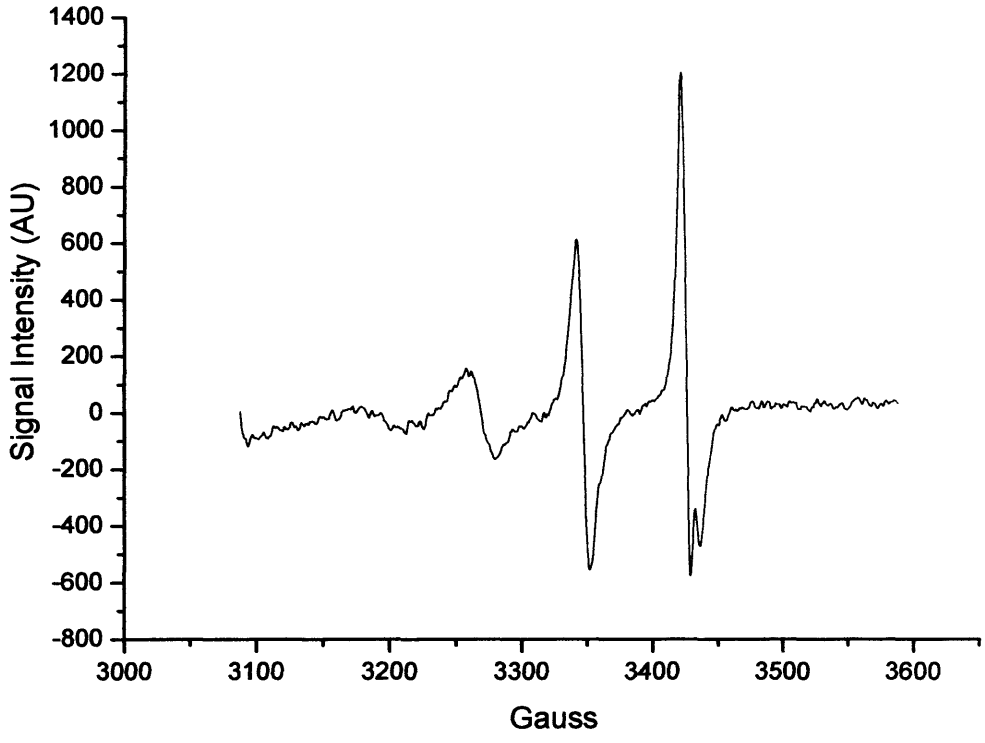


Figure 35 Example spectrum of 200μM copper (II) and 6.25mM MGD

2.1.5 EPR methodology

2.1.5.1 Generation of superoxide and hydroxyl radicals

The assay mixes used to generate EPR detectable superoxide and hydroxyl radicals are summarised in Table 14.

Table 14 Radical generation mix

Hydroxyl radical	**Superoxide radical**	
	DEPMPO	**TEMPONE-H**
PBS 10mM DEPMPO 1μM $CuSO_4$ 4.41mM H_2O_2	PBS 10mM DEPMPO 0.1mM DTPA 5μM hypoxanthine 0.0025 Units xanthine oxidase	PBS 2μM TEMPONE-H 0.1mM DTPA 25μM hypoxanthine 0.0025 Units xanthine oxidase

2.1.5.2 Superoxide and hydroxyl radical scavenging by L-arginine and L-citrulline

Samples were placed in a quartz glass cell and EPR spectra recorded at room temperature, pH 7.4 using a Varian 104 EPR spectrometer. Instrument settings varied depending on the spin trap being used, of which details can be found in the appropriate chapters. Samples were run for 20 minutes (10 x 2 minute scans) after a 90 second incubation period. Signal intensity for each two minute scan was determined. Area under curve was calculated for the full 20 minute period from these intensities.

The antioxidant capacity of L-arginine and L-citrulline was assessed according to their ability to quench the signal detected from the above radical generating systems.

2.1.5.3 Detection of copper sulphate

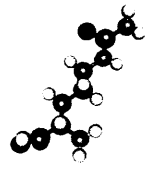
The dithiocarbamate, MGD, was added to Cupric sulphate (Cu(II)) forming a paramagnetic Cu-MGD complex with characteristic spectra. The effect of L-arginine and L-citrulline on Cu(II) levels was investigated via incubation of a range of test compound concentrations ($1x10^{-6}$M, $1x10^{-4}$M, $1x10^{-2}$M, $1x10^{-1}$M and 1M) with 50µM Cu(II) for five minutes. After the five-minute period, 6.25mM MGD was added and samples immediately placed in a quartz glass cell and EPR spectra recorded at room temperature using a Varian 104 EPR spectrometer. The instrument settings were as follows; Gain $5x10^4$, modulation 1.6Gauss, time constant 0.128sec, 500 Gauss scan width, power 5mW. Samples were run for 5 minutes (5 x 1 minute scans). The average of the five scans was taken and signal intensity determined.

2.1.5.4 Detection of ROS in Platelet Rich Plasma

Blood was taken by standard venepuncture from the antecubital vein. The anticoagulant trisodium citrate (3.8%), 1:9 v/v (Sodium Citrate : Blood)was used. Blood was then centrifuged at 150g and 4^0C for 10 minutes and platelet rich plasma (PRP) removed and stored at room temperature.

A round bottomed glass tube was then coated with $5x10^{-5}$moles of PBN and a total volume of 500µL added to the tube to give a final concentration of 100mM PBN. The composition of the 500µL varied as described in Table 15. All solutions were vortexed to ensure full mixing of PBN with solution. Samples were placed in a quartz glass cell and EPR spectra recorded at room temperature using a Varian 104 EPR spectrometer.

The instrument settings were as follows; Gain 8x10, modulation 1.6 Gauss, time constant 0.128sec, Gauss scan width 200 Gauss, power 5mW. Samples were run for 10 minutes (10 x 1 minute scans), with no initial incubation period.



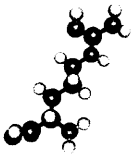
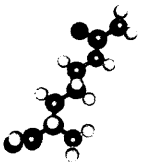
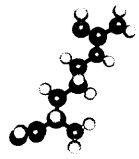


Table 15 Composition of EPR platelet assay mix

Sample Type	PBN Conc[a]	Collagen Conc[a]	PBS volume	PRP volume	DMSO volume
Control	100mM	0μg/ml	500μL	0	0
ROS level in unstimulated platelets	100mM	0μg/ml	250μL	250μL	0μL
ROS level in stimulated platelets	100mM	1μg/ml or 10μg/ml	250μL	250μL	0μL
Carbon centred radical levels in unstimulated platelets	100mM	0μg/ml	200μL	250μL	50μL
Carbon centred radical levels in stimulated platelets	100mM	1μg/ml or 10μg/ml	200μL	250μL	50μL





2.2 Luminol based chemiluminescence

2.2.1 Introduction

Luminol based chemiluminescence is a widely used technique in the detection and quantification of oxygen derived free radicals. The interaction of luminol and ROS leads to emission of light which is consequently detected using a range of luminometers. Luminol based chemiluminescence is an effective and reliable technique for quantifying hydroxyl radicals as shown by Cheng *et al* 2003 [59] who demonstrated the ability of luminol to not only detect and quantify hydroxyl radicals but to also provide a suitable assay for investigations into the antioxidant properties of different compounds. The data obtained from this assay provided complimentary data to the EPR study. EPR is specific to the radical of interest but is not as convenient as each sample takes twenty minutes to run and kinetic studies are more difficult to obtain. Luminol based experiments are the opposite, numerous samples can be run at once meaning kinetic studies over one hour are feasible.

2.2.2 Methodology

2.2.2.1 Generation of varying concentrations of hydroxyl radical

Hydroxyl radicals were generated using Fenton chemistry described previously. Briefly, varying concentrations of hydrogen peroxide and copper sulphate (ratio kept constant (Table 16)), were mixed to produce a range of hydroxyl radical concentrations.

Due to difficulties in quantifying exact concentrations of hydroxyl radical, all radical systems were expressed in terms of the concentration of copper sulphate and hydrogen peroxide, and for simplicity referred to as system 1-4 as explained in Table 16.

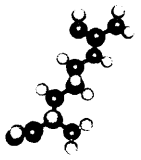
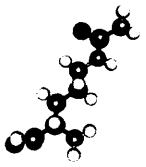


Table 16 Composition of hydroxyl radical formation assay mixes

System	Concentration of copper sulphate	Concentration of hydrogen peroxide
1	4.5μM	44.1mM
2	9μM	88.2mM
3	18μM	176.4mM
4	36μM	352.8mM

Hydroxyl radicals were measured via chemiluminescence utilising a luminol probe. Measurements were carried out at pH 7.4, 37°C using a MLX microtitre ® plate luminometer. Briefly, 100μL Luminol (final concentration 1mM dissolved in 10% DMSO and 90% PBS) was added to each well in a 96 well plate along with the appropriate concentration of hydrogen peroxide (see Table 16) and amino acid ($1x10^{-6}$M – 1M). As a control, PBS was added in place of the amino acid. Background chemiluminescence activity was measured for one minute and then the appropriate volume of copper sulphate (Table 16) was injected into each well using an automated injector system to initiate the reaction. Copper sulphate was auto injected with the plate already in the spectrometer as preliminary data showed that if copper sulphate and hydrogen peroxide were pre-mixed, a significant proportion of hydroxyl radicals were produced before recording started. Given the short half life of the hydroxyl radical ($<10^{-4}$s), this lead to large differences in signal between plates. This injection system therefore allowed continuous radical detection and real time capture of radical production. Photon emission was continuously recorded for sixty minutes. Total hydroxyl radical production was expressed as area under curve. The formation of superoxide was rules out in a preliminary study by the addition of 50-1000 Units of SOD which had no effect on total yield (Figure S3). The antioxidant capacity of L-arginine and L-citrulline was assessed as ability to reduce hydroxyl radical levels.



2.3 Platelet aggregation

2.3.1 Introduction

Platelet activation and aggregation plays a vital role in maintenance of the cardiovascular system. To investigate the ability of L-arginine and L-citrulline to act in an antioxidant capacity, a platelet model was chosen as platelets are known to synthesise ROS under normal conditions and it has been shown that ROS levels increase upon activation of the platelets by agonists such as collagen [46,186,284,376]. These endogenous ROS are thought to be involved in platelet activation and aggregation and so a platelet model provides an ideal system to investigate the antioxidant activity of both L-arginine and L-citrulline in a physiologically relevant model.

2.3.2 Methodology

Blood was taken by standard venepuncture from an antecubital vein. The anticoagulant trisodium citrate (3.8%), 1:9 v/v was used. Blood was then centrifuged at 150g and 4 ^{0}C for 10 minutes and platelet rich plasma (PRP) kept at 37 ^{0}C (i.e. body temperature – to return platelet metabolism to normal rates after cooling).

Platelet aggregation was measured on a dual channel aggregometer (Chrono-log, Model 560). The aggregometer measures change in electrical resistance which occurs as a result of aggregating platelets building up between two electrodes. Electrodes were calibrated before each measurement and aggregation consequently measured for 10 minutes at 37°C with continuous stirring at 1000rpm (Figure).
36

The effect of a range of compounds, L-arginine, L-citrulline, D-arginine, ascorbic acid, superoxide dismutase, catalase and Mannitol on collagen-induced platelet aggregation were analysed (see Table 17 for exact concentrations). All samples were made up to a total volume of 1ml, consisting of 500µl platelet rich plasma at all times and 500µl of the appropriate test compound or PBS (control).

All samples were incubated for ten minutes at 37°C prior to initiation of aggregation. 3 minutes of this 10 minute incubation period was spent in the aggregometer with stirring at 1000rpm.

Table 17 Concentration of test compounds in platelet aggregation assay

Test Compound	Final concentration
L-arginine	$1x10^{-4}M - 1M$
D-arginine	$1x10^{-4}M - 1M$
L-citrulline	$1x10^{-3}M - 1.6x10^{-1}M$
Ascorbic acid	$1x10^{-3}M - 1M$
Mannitol	$1x10^{-3}M - 5x10^{-1}M$
Superoxide dismutase	10, 100 & 500 Units/ml
catalase	100, 1000 & 2000 Units/ml

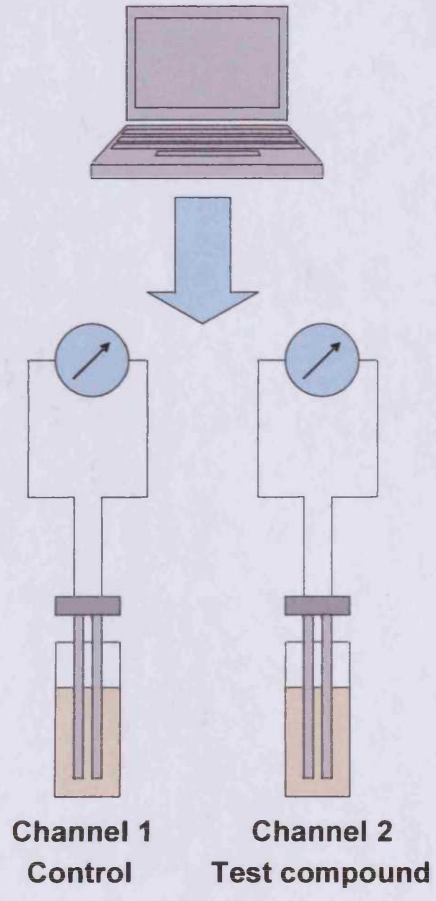


Figure 36 Diagrammatic representation of the platelet aggregometer

2.4 Nitric oxide Analyser (NOA)

2.4.1 Introduction

Ozone based chemiluminescence is one of the most sensitive nitric oxide detection systems available. It utilises the fact that the reaction between nitric oxide and ozone results in the emission of light that is proportional to nitric oxide concentration. This assay is specific as ozone is un-reactive with other gases such as NO_2, NH_3, CO, C_2H_4, SO_2, CO_2, or H_2O under the conditions used to detect nitric oxide.

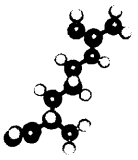
Ozone reacts selectively with NO to yield nitrogen dioxide (NO_2), a proportion of which is in an excited form (NO_2*).

$$4NO + 2O_3 \rightarrow 2NO_2^* + 2NO_2 + O_2 \qquad \textbf{Equation 31}$$

The excited form of nitrogen dioxide is unstable and so energy is dissipitated in the form of photons in order to regain the stable ground state (NO_2).

$$NO_2^* \rightarrow NO_2 + h\nu \qquad \textbf{Equation 32}$$

The light emitted from this reaction is in the red and infrared section of the spectrum (640-3000nm) with peak intensity occurring at ~1100nm.. Although the photomultiplier used to amplify the signal only detects wavelengths below 900nm, the amount of light emitted in the 640nm-900nm region is sufficiently high to be detected, making chemiluminescence one of the most sensitive NO assays available [130].



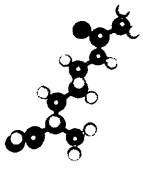
2.4.1.1 Chemiluminometer

The NO chemiluminescence (NOA) analyser in its simplest form consists of a reaction chamber in which NO reacts with ozone, a light detector and a recorder. A photomultiplier is used since this allows the relatively weak red and infrared light given off to be amplified. The photomultiplier also is the detection method of choice due to the stability of the dark current (background output) compared to other devices.

Because of the partition coefficient of nitric oxide (~20) even though nitric oxide is produced in an aqueous environment, it is 20 times more likely to exist in the gaseous phase. By bubbling nitrogen through the sample, the removal of NO from the sample in to the chemiluminometer is accelerated. Also, nitrogen removes oxygen from the system and therefore prevents interaction of nitric oxide and oxygen.

2.4.2 Methodology

Oxygen-free nitrogen gas was bubbled through the assay mix, which was heated to 37°C (± 1°C) in a water bath on a thermostatically controlled hotplate. A chemical trap containing 25ml of 1M sodium hydroxide (16g NaOH/400ml HPLC grade water) was placed between the reagent vessel and the NO analyser (Sievers NOA 280i, Analytix, UK) to prevent damage to the NOA and to remove N-oxides. The carrier gas (oxygen free nitrogen) was maintained at a constant flow rate by two adjacent flow meters (Figure). The assay mix was made up in PBS to a volume of 5ml which was bubbled with nitrogen for 5 minutes prior to the start of the experiment to remove all oxygen from the sample.



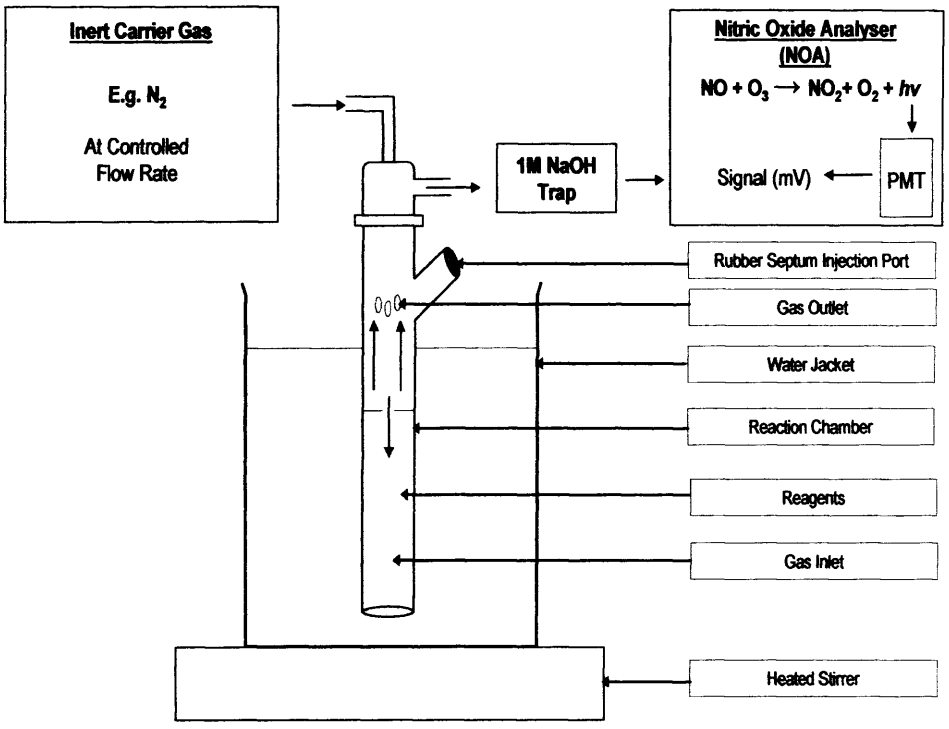
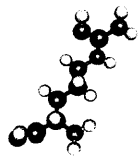


Figure 37 Experimental set-up for ozone based chemiluminescence determination of NO release from GSNO. Picture courtesy of Andrew Pinder.

The effects of a range of compounds on the release of NO from 10nmoles and 80nmoles GSNO were investigated (Table 22). Fresh vials of 10mM GSNO stock (25mg in 7.44ml 100μM DTPA; DTPA 15.7mg/400ml HPLC water; used to increase the stability of the compound) were stored in light protected vials in the freezer (-20°C). Once thawed, vials were stored on ice (4°C) and diluted to the appropriate concentrations in HPLC grade water.



2.5 Aortic ring preparation

2.5.1 Introduction

The aortic ring preparation is a type of bioassay that is classically used for determining the potencies of endothelium-dependent relaxants on vascular smooth muscle. *In this thesis the aortic ring preparation was chosen as it allowed* the quantitative measurement of the degree of relaxation to acetylcholine, an agent known to release NO from endothelial sources resulting in smooth muscle relaxation. Relaxation can therefore be used as a measure of NO bioavailability.

2.5.2 Methodology

2.5.2.1 Removal of aortae

Male, New Zealand White rabbits (approximately 2-2.5Kg) were terminally anaesthetised with an intravenous injection of sodium pentobarbitone (120mg/Kg). A transverse incision was made into the abdominal cavity using blunt-ended scissors. The diaphragm was then transected and two longitudinal cuts made into the rib cage to allow the thoracic aorta to be exposed. The aorta was excised and placed directly into fresh Krebs buffer (see Table 18 for composition). Care was taken not to stretch the tissue at any point.
The aorta was then cleaned of fat, blood and connective tissue before being cut into 2-3mm wide rings on a purpose built apparatus using equidistant upturned razor blades.

2.5.2.2 Isometric tension recording

Aortic rings were prepared and mounted in 5ml tissue baths containing Krebs buffer and gassed with 95% O_2 / 5% CO_2 at 37°C. A resting tension of 2.6g was set. Tissues were allowed to equilibrate for one hour, during which time the tension was reset to allow for stress-induced relaxation, and washed out at 30

minutes and 60 minutes. After one hour all rings were exposed to phenylephrine (PE, $1x10^{-6}$M) until a reproducible constriction was achieved. Upon achieving a steady plateau, rings were exposed to $1x10^{-5}$M acetylcholine to assess endothelial function followed by washing and re-equilibration. Various experiments were subsequently performed with differing protocols (Chapter 6).

Table 18 Composition of KREBS buffer

REAGENT	CONCENTRATION
NaCl	109 mM
KCl	2.68 mM
KH_2PO_4	1.2 mM
$MgSO_4.7H_2O$	1.2 mM
$NaHCO_3$	25 mM
Glucose	11 mM
$CaCl_2.7H_2O$	1.5 mM

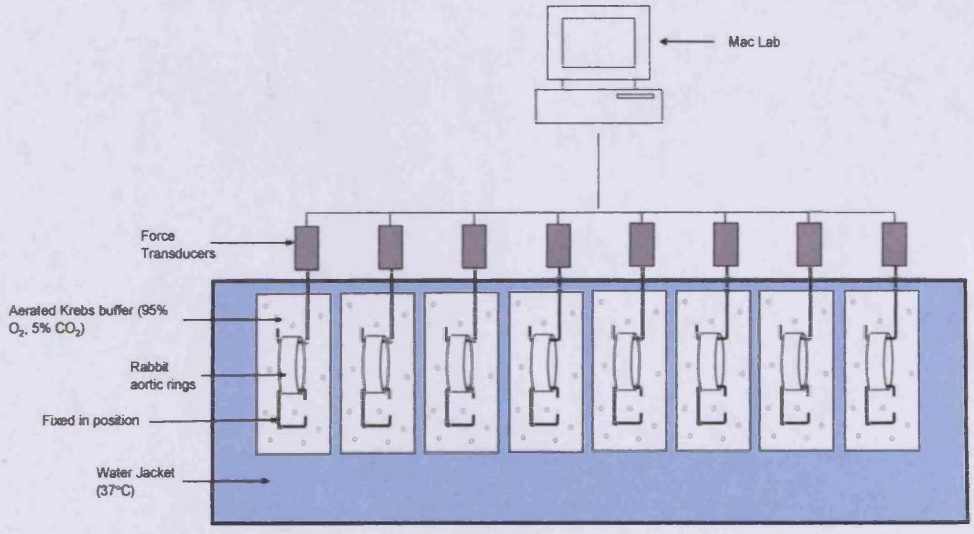


Figure 38 Diagrammatic representation of aortic ring apparatus

2.6 TBARS assay

2.6.1 Introduction

The thiobarbituric acid reactive substances test (TBARS) is one of the oldest and most frequently used tests for the measurement of oxidation of fatty acids, membranes and food products. It is a very simple technique in which the reagent, thiobarbituric acid reacts with the products of lipid oxidation under acidic conditions producing a detectable product. Phosphatidylcholine (Figure 39) was chosen as it is a phospholipid that is a major constituent of cell membranes.

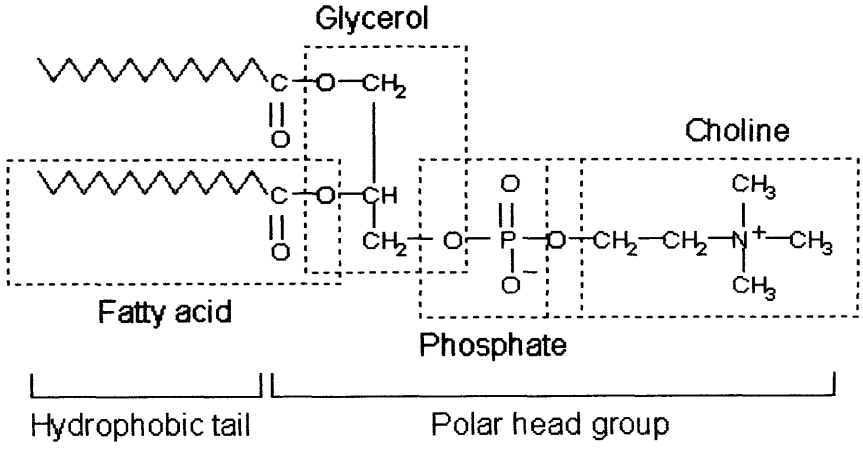


Figure 39 Structure of phosphatidylcholine

Lipid oxidation occurs in three stages, initiation, in which the lipid is attacked by a free radical, in this case the hydroxyl radical; propagation in which the resulting lipid radical abstracts hydrogen from an adjacent lipid resulting in the formation of new lipid radicals, and termination in which reactions between lipid radicals results in the formation of a stable non-radical product (Figure 40).

Monodialdehyde (MDA), one of the products of lipid oxidation, allows lipid oxidation to be quantified as MDA reacts with TBA to form TBA-MDA, also

known as TBARS (ThioBarbituric Acid Reactive Substances). TBA-MDA is a coloured product that absorbs light at 525nm and emits at 547nm and therefore fluorescence can be used to effectively quantify lipid oxidation.

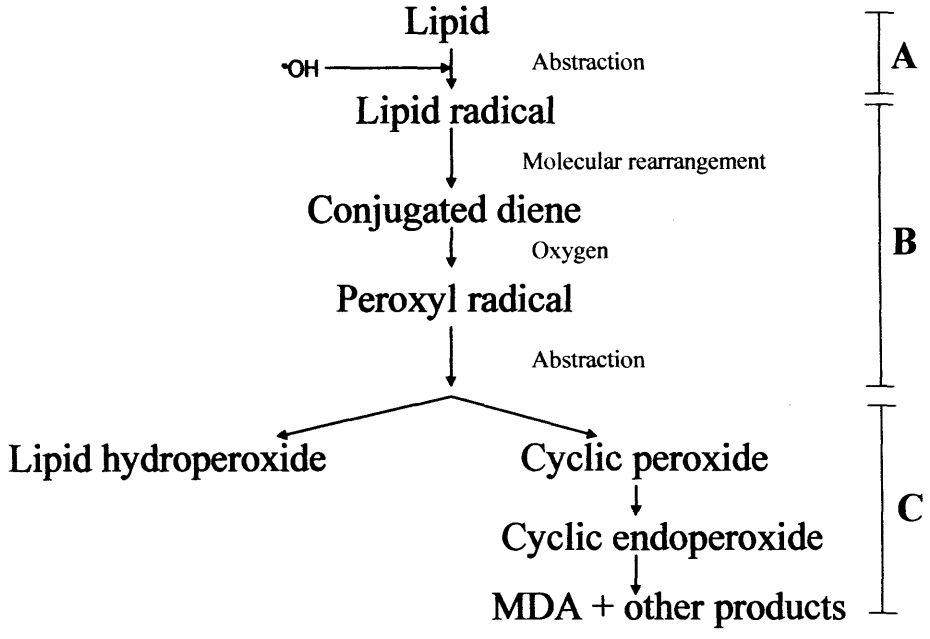
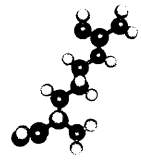


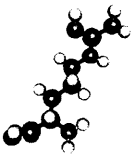
Figure 40 Summary of lipid oxidation A) Initiation B) Propagation C) Termination



2.6.2 Methodology

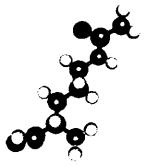
Thiobarbituric acid solution (TBA) was prepared by dissolving TBA in 50% v/v Acetic acid to give a final concentration of 29mM. The standard solution of malondialdehyde (MDA) was prepared by dissolving 328µl (2mmol) Tetramethoxypropane (TMP) in 100ml absolute ethanol to give a final concentration of (20mM). MDA is an unstable molecule and was therefore generated by hydrolysis of TMP. Phosphatidylcholine (PC) was dissolved in absolute ethanol, concentrated under liquid nitrogen and then re-suspended in PBS (pH 7.4) giving a final suspension of 4mg/ml. To investigate the effects of the amino acids, 50% of the PBS volume was replaced with the appropriate concentration of amino acid dissolved in PBS. Hydrogen peroxide (final concentration 44.1mM) and copper sulphate (final concentration 4.5µM) were added to the phosphatidylcholine solution to initiate oxidation. All samples were incubated at 37°C, pH 7.4 for 150 minutes. After the incubation period, samples were placed on ice and 250µM EDTA was added to terminate the oxidation reaction (adapted from Wasowicz *et al* [385]. 100µl of sample or the appropriate volume of MDA standard were added to glass tubes containing 1ml of HPLC grade water. 1ml of the TBA solution was then added and all samples heated at 100°C for 1 hour. Samples were cooled on ice and the reaction mixture was extracted using 3.5ml n-butanol and mixing for 5minutes. Centrifugation at 1500g for 10minutes allowed separation of the butanol layer and fluorescence was measured at 525nm for excitation and 547nm for emission using a Perkin Elmer Luminescence spectrometer (LS50B). The calibration curve was prepared using 0-0.3nmol/ml MDA.

The effect of a range of L-arginine and L-citrulline concentrations on lipid oxidation was measured ($1x10^{-7}M$ – $2x10^{-1}M$ & $5x10^{-1}M$ for L-citrulline and L-arginine respectively).

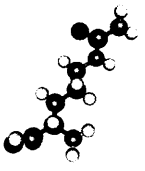


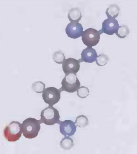
2.7 Chemicals and other agents

Product	Source
Spin Traps	
DEPMPO	Alexis Bio-chemicals UK
TEMPONE-H	Alexis Bio-chemicals UK
N-t-Butyl-α-phenylnitrone (PBN)	Alexis Bio-chemicals UK
Reagent Chemicals	
HPLC Grade Water	Fisher Scientific UK
Glacial Acetic Acid	Fisher Scientific UK
Sodium Hydroxide (NaOH)	Sigma Aldrich UK
Diethylenetriaminepentaacetate (DTPA)	Sigma Aldrich UK
Phenylephrine (PE)	Sigma Aldrich UK
Acetylcholine (Ach)	Sigma Aldrich UK
Dimethyl Sulfoxide (DMSO)	Sigma Aldrich UK
catalase	Sigma Aldrich UK
Luminol	Sigma Aldrich UK
Phosphobuffered Saline tablets	Sigma Aldrich UK
Hydrogen peroxide	Sigma Aldrich UK
Hypoxanthine	Sigma Aldrich UK
Xanthine oxidase	Sigma Aldrich UK
L-arginine	Sigma Aldrich UK
L-citrulline	Sigma Aldrich UK
D-arginine	Sigma Aldrich UK
Mannitol	Sigma Aldrich UK
Ascorbic acid	Sigma Aldrich UK
Ethylenediaminetetra-acetic acid (EDTA)	Sigma Aldrich UK
Thiobarbituric Acid (TBA)	Sigma Aldrich UK
Aminoguanidine	Sigma Aldrich UK
glycine	Sigma Aldrich UK
N-α-acetyl-arginine	Sigma Aldrich UK
Phosphatidylcholine	Sigma Aldrich UK
S-nitrosoglutathione (GSNO)	Sigma Aldrich UK
N-(Dithiocarbamoyl)-N-methyl-D-glucamine (MGD)	Alexis Bio-chemicals UK



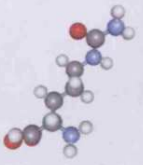
Superoxide dismutase (SOD)	Alexis Bio-chemicals UK
Tetramethoxypropane (TMP)	Alexis Bio-chemicals UK
n-Butanol	Aldrich
Copper sulphate	Aldrich
Collagen	Chronolog
Krebs	
Sodium Chloride (NaCl)	Fisher Scientific UK
Potassium Chloride (KCl)	Fisher Scientific UK
Potassium Dihydrogen Orthophosphate (KH_2PO_4)	Fisher Scientific UK
Magnesium sulphate ($MgSO_47H_2O$)	Fisher Scientific UK
Sodium hydrogen Carbonate ($NaHCO_3$)	Fisher Scientific UK
Glucose	Fisher Scientific UK
Calcium Chloride ($CaCl_2$)	Fisher Scientific UK





In Vitro studies

Chapter Three

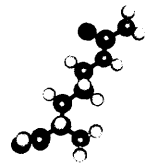


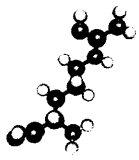
3.0 Introduction

Both L-arginine and L-citrulline have been shown to have beneficial effects on the vasculature, potentially via an antioxidative action. Although a significant amount of work has been carried out investigating the antioxidant effects of L-arginine, controversy exists as to its ability to act as a free radical scavenger [6,199,379,383]. To date nearly all studies have focused on superoxide with only a handful investigating its effect on hydroxyl radicals [290,298]. Conversely, the antioxidant properties of L-citrulline have focused mainly on the hydroxyl radical (as demonstrated in plants) where L-citrulline has been shown to scavenge hydroxyl radicals with second-order rate constant of 3.9x109 M^{-1} s^{-1}[8], with its effect on superoxide remaining uncharacterised. This study was aimed at fully characterising the antioxidant profile of both amino acids on both radicals.

This investigation into both the superoxide and hydroxyl radical is unique in providing a direct comparison between radical types, something which is currently lacking in the literature. Although superoxide is more commonly associated with endothelial dysfunction, wherever superoxide is produced hydroxyl radicals are also generated via Haber-Weiss and Fenton Chemistry. The hydroxyl radical is one of the most aggressive reactive oxygen species in the vasculature with targets including proteins, lipids, carbohydrates and DNA [27]. Its action results in various levels of cell damage leading to impaired cell function and cell death. The hydroxyl radical has also been shown to react directly with nitric oxide forming nitrite at a rate of 1-2 × 10^{10} L mol^{-1} s^{-1}[327]. This results in decreased NO bioavailability and may lead to increased endothelial dysfunction. It is therefore vital that any molecule considered as having a cardioprotective effect via an antioxidant action must be defined in terms of both superoxide and hydroxyl radical scavenging properties.

In summary this body of work is the first study to fully characterise the antioxidant properties of L-arginine and L-citrulline not only in terms of free radical type, but also the effect of free radical concentration on antioxidant capacity. Thus two radical generating systems were developed for detection of free radical concentrations and comparisons were made with an *in vitro* model

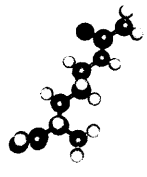




system to test antioxidant capacity.

3.1 Aims

- To characterise the superoxide generating capacity of a xanthine oxidase based system utilising EPR.
- To assess the antioxidant properties of L-arginine and L-citrulline against superoxide radicals.
- To characterise the hydroxyl radical generation capacity of an adapted Fenton system utilising EPR.
- To assess the antioxidant properties of L-arginine and L-citrulline against hydroxyl radicals.
- To assess the antioxidant capacity of L-arginine and L-citrulline against varying concentrations of hydroxyl radical utilising luminol based chemiluminescence.



3.2 Materials and Methods

3.2.1 Detection of free radicals by EPR

3.2.1.1 Generation of superoxide

Superoxide is a product of the catalysis of hypoxanthine into uric acid by xanthine oxidase as discussed in detail in Chapter 2. Superoxide formed can be measured using EPR (Table 19).

3.2.1.2 Generation of hydroxyl radicals

Hydroxyl radicals were generated via an adapted Fenton chemistry system, discussed in detail in Chapter two. Briefly, hydrogen peroxide and copper sulphate react to form hydroxyl radicals which can be measured by EPR and luminol based chemiluminescence. Table 19 lists the components of the reaction mix required to generate a detectable hydroxyl radical yield.

Table 19 Radical generation mix

Hydroxyl radical	**Superoxide radical**	
	DEPMPO	**TEMPONE-H**
PBS 10mM DEPMPO 1mM $CuSO_4$ 4.41mM H_2O_2	PBS 10mM DEPMPO 0.1mM DTPA 5μM hypoxanthine 0.0025 Units xanthine oxidase	PBS 2μM TEMPONE-H 0.1mM DTPA 25μM hypoxanthine 0.0025 Units xanthine oxidase

3.2.1.3 Detection of superoxide and hydroxyl radicals

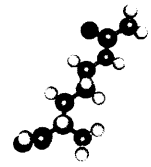
Samples were placed in a quartz glass cell and EPR spectra recorded at room temperature, pH 7.4 using a Varian 104 EPR spectrometer. Typical instrument

settings were as follows; Gain 8x10, modulation 1.6 Gauss, time constant 0.128 seconds, Gauss (3378Mt), scan width 200 Gauss for DEPMPO, 100 Gauss for TEMPONE-H, power 5mW. Samples were run for 20 minutes (10 x 2 minute scans) after a 90 second incubation period. The intensity of the first peak of the DEPMPO-OH and TEMPONE spectra and the fourth peak of the DEPMPO-OH spectra for each two minute scan was determined (Figure). Area under curve was calculated for the full 20 minute period from these intensities.

3.2.1.4 Scavenging effects of L-arginine and L-citrulline

The antioxidant capacity of L-arginine and L-citrulline was assessed according to their ability to quench the signal detected from the above radical generating systems. Both amino acids were added to the assay mix and as with control samples, samples were run for 20 minutes (10 x 2 minute scans) after an initial 90 seconds incubation period. Area under curve was recorded in the presence (AUC_{AA}) and absence ($AUC_{Control}$) of the selected amino acids, and inhibition of control (I) was calculated as:

$$\mathbf{I=((AUC_{control} - AUC_{AA}) / AUC_{control}) * 100}$$



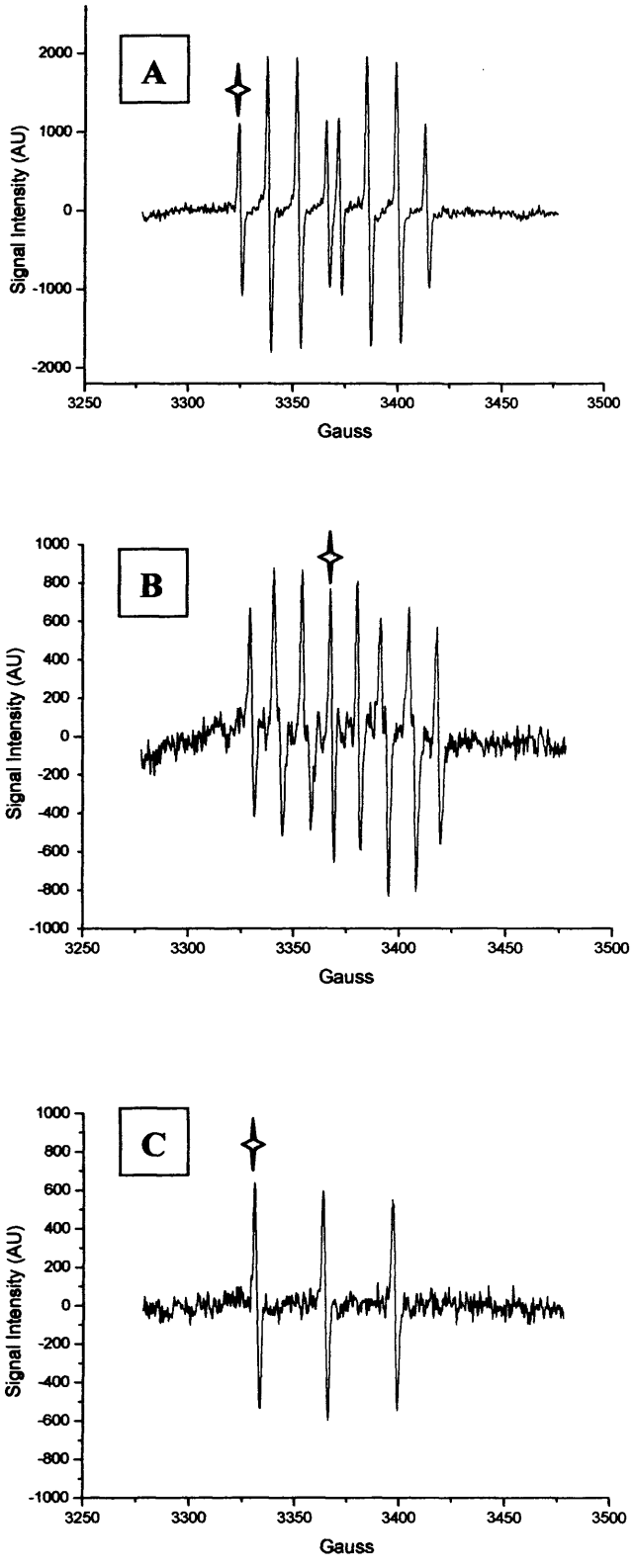
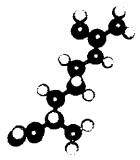


Figure 41 Examples of A) DEPMPO-OH B) DEPMPO-OOH and C) TEMPONE spectra. Star illustrates the peak chosen for signal intensity measurements



3.2.2 Detection of free radicals by luminol based chemiluminescence

3.2.2.1 Generation of varying concentrations of hydroxyl radical

Hydroxyl radicals were generated using Fenton chemistry [277]. Briefly, varying concentrations of hydrogen peroxide and copper sulphate (ratio kept constant (Table 16), were mixed to produce a range of hydroxyl radical concentrations.

Due to difficulties in quantifying exact concentrations of hydroxyl radical, all radical systems were expressed in terms of the concentration of copper sulphate and hydrogen peroxide, and for simplicity referred to as system 1-4 as explained in Table 20.

Table 20 Fenton Chemistry mix composition

System	Concentration of copper sulphate	Concentration of hydrogen peroxide
1	4.5μM	44.1mM
2	9μM	88.2mM
3	18μM	176.4mM
4	36μM	352.8mM

Hydroxyl radicals were measured as described in section 2.2.2.1 via chemiluminescence utilising a luminol probe. Photon emission was continuously recorded for sixty minutes. Total hydroxyl radical production was expressed as Area under curve. The formation of superoxide was ruled out in a preliminary study by the addition of 50-1000 Units of SOD, which had no effect on total yield.

3.2.2.2 Assessment of antioxidant capacity of L-arginine and L-citrulline

The antioxidant capacity of L-arginine and L-citrulline was assessed according to their ability to quench the signal detected from the above Fenton reaction. Area under curve was recorded in the presence (AUC_{AA}) and absence ($AUC_{Control}$) of the selected amino acids, and inhibition of control (I) was calculated as:

$$\mathbf{I=((AUC_{control} - AUC_{AA}) / AUC_{control}) * 100.}$$

3.2.3 Statistical Analysis

Data expressed as mean ± SEM. Student t-tests and one or two way analysis of variance with a Bonferroni *post hoc* test were used to calculate significance which was set at $p<0.05$ unless otherwise stated.

3.3 Results

3.3.1 Superoxide radical scavenging activity of L-arginine and L-citrulline determined by EPR spectroscopy

3.3.1.1 Characterisation of superoxide generation from xanthine oxidase by DEPMPO

As discussed in chapter two, DEPMPO is the spin trap of choice for superoxide and hydroxyl radical detection. 10mM DEPMPO was chosen as this has previously been shown to be the most efficient at trapping superoxide with roughly 60% of the superoxide present being detected at concentrations of DEPMPO greater than 10mM [304]. Upon analysis of results obtained, a DEPMPO-OOH spectra (Figure 42) was identified using the following parameters

- A_N of 13.4g which is consistent with published results for DEPMPO-OOH splittings [173]
- Signal was inhibited by 10 Units of SOD .
- 100 Units of catalase had no effect on signal intensity.

Superoxide production from 0.0025 Units xanthine oxidase was proportional to the concentration of substrate (hypoxanthine) (Figure 43). A concentration slightly higher than the EC_{50} value of 1.66μM was chosen in order to minimise the signal to noise ratio.

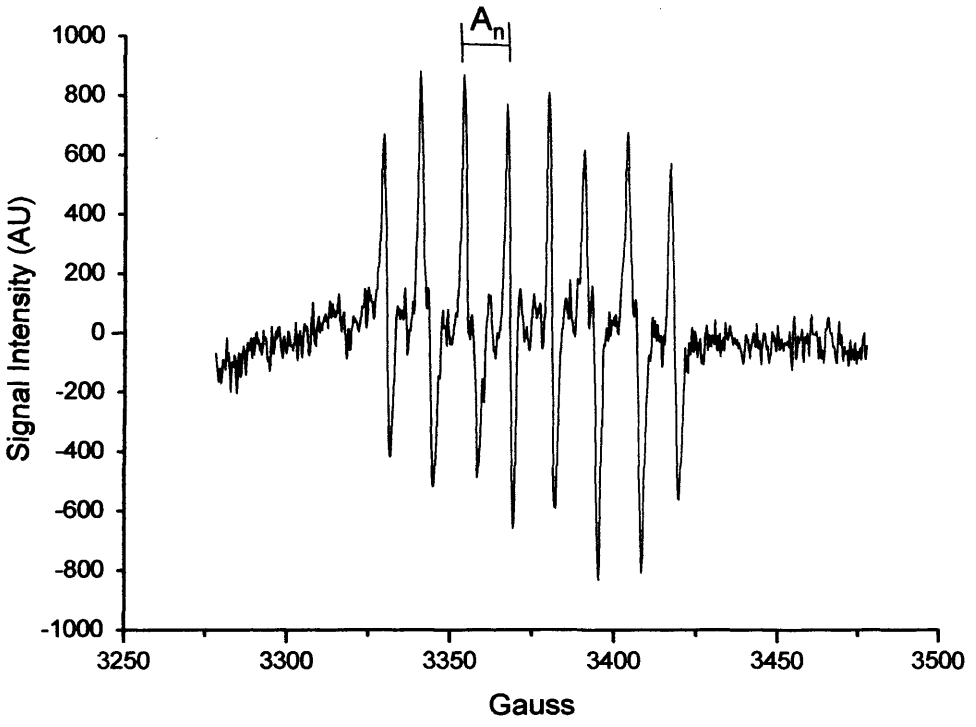


Figure 42 Example Spectrum of DEPMPO-OOH. Scan taken over two minutes, eighteen minutes after the reaction was initiated.

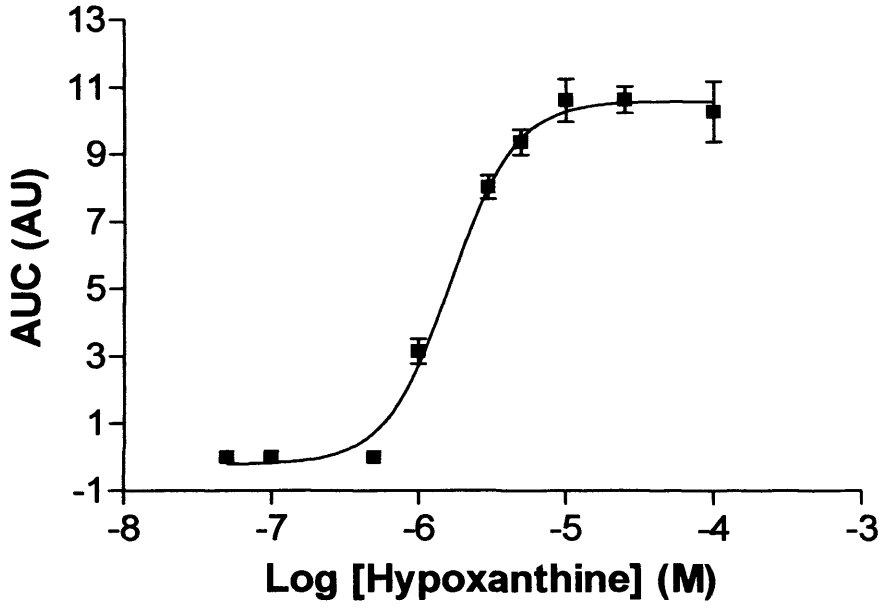


Figure 43 Hypoxanthine concentration effect curve. Varying hypoxanthine concentrations incubated with 0.025U Xanthine oxidase for 20 minutes. n=5 except for $5x10^{-6}$M where n=11. Data expressed as mean±SEM. EC_{50}=1.66μM

3.3.1.2 Effect of L-arginine and L-citrulline on superoxide levels

L-arginine and L-citrulline had negligible superoxide scavenging capacity with antioxidant activity only seen at concentrations greater than $1x10^{-1}$M (Figure 44). L-citrulline exerted stronger superoxide scavenging activity than L-arginine, which at no point was capable of inhibiting 100% of superoxide detected. At all concentrations which an antioxidant capacity was observed, L-citrulline more efficiently scavenged superoxide in comparison to L-arginine.

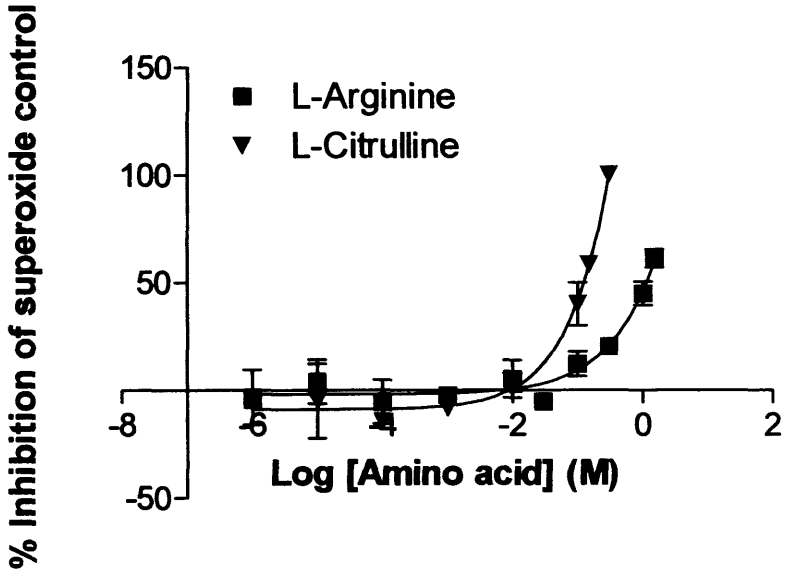


Figure 44 Effect of L-arginine and L-citrulline on DEPMPO-OOH levels.

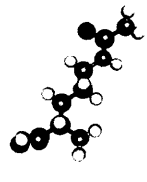
Data expressed as mean ± SEM. n=4.

3.3.1.3 Characterisation of superoxide generation from xanthine oxidase by TEMPONE-H

Superoxide can oxidise the EPR silent TEMPONE-H into the paramagnetic TEMPONE (Figure 45). Although it is acknowledged that TEMPONE-H is not specific for superoxide, in a pure chemical system, the formation of TEMPONE is a measure of superoxide concentration. The concentration of xanthine oxidase was maintained at 0.0025 Units as used in the DEPMPO assay, however the concentration of hypoxanthine was increased to 25μM as little signal could be seen with 5μM (Figure 46). As with DEPMPO, superoxide generation was proportional to hypoxanthine concentration.

3.3.1.4 Effect of L-arginine and L-citrulline on superoxide radical levels

L-arginine had no effect on TEMPONE formation indicating that L-arginine was unable to reduce superoxide levels (Figure 47). L-citrulline was only able to significantly reduce superoxide levels at a concentration of $1x10^{-1}$M (Figure 47) ($p<0.001$) confirming experiments using DEPMPO.



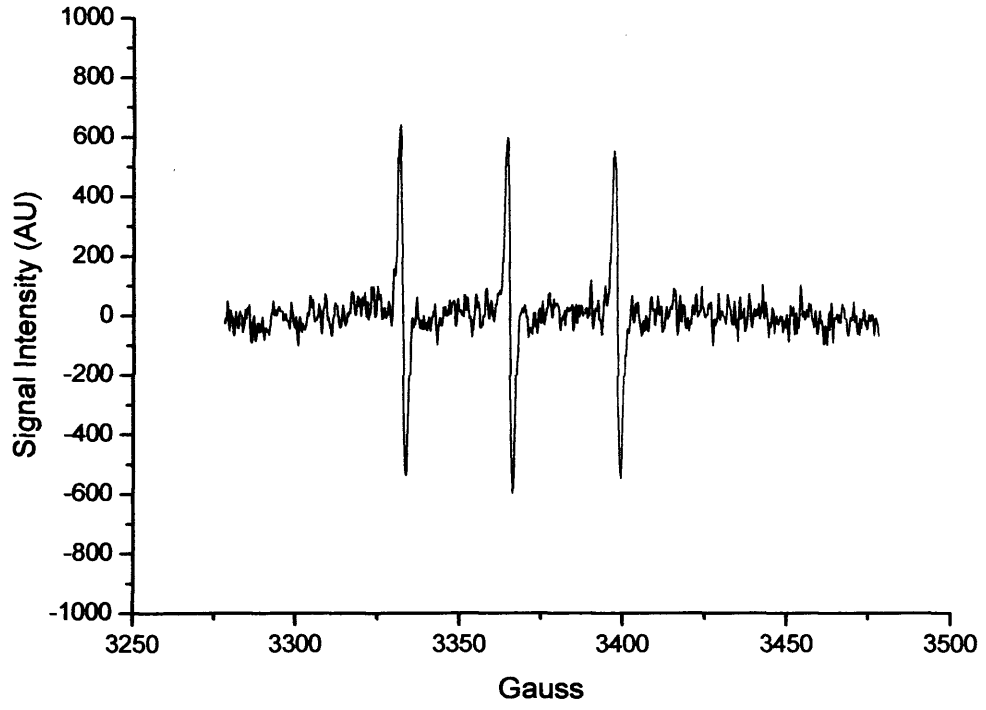


Figure 45 Example Spectrum of TEMPONE. Scan taken over two minutes, eighteen minutes after the reaction was initiated.

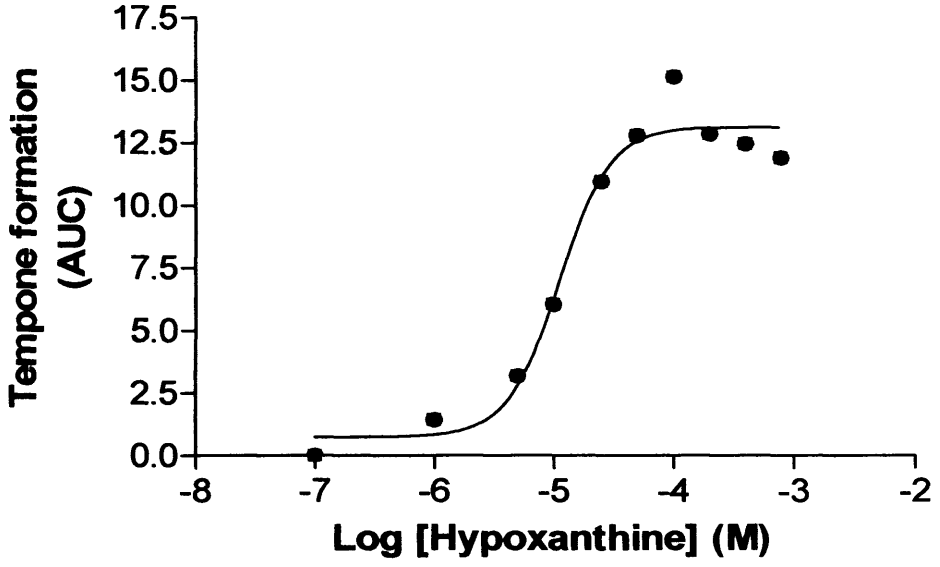


Figure 46 Hypoxanthine concentration effect curve. n=4. Data expressed as mean±SEM. EC_{50}=11.14μM

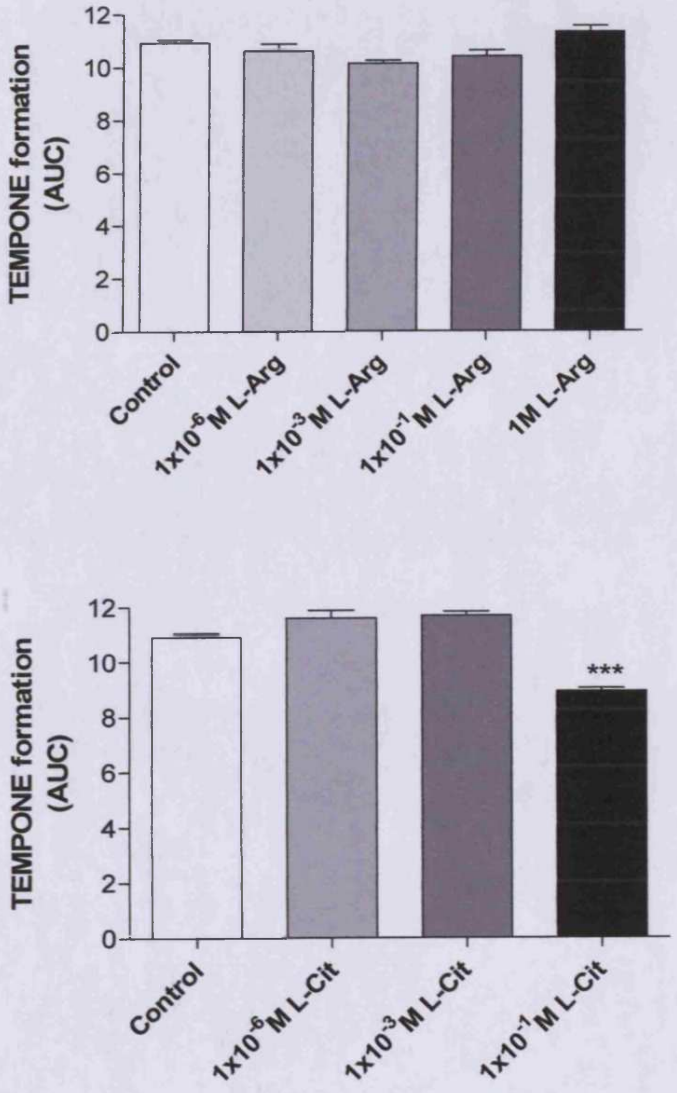


Figure 47 Effect of L-arginine and L-citrulline on TEMPONE formation. Data expressed as mean ± SEM. n=4 * represents $p<0.001$ compared with control.**

3.3.2 Hydroxyl radical scavenging activity of L-arginine and L-citrulline determined by EPR spectroscopy

3.3.2.1 Characterisation of hydroxyl radical generation

Hydroxyl radicals were generated through the reaction of copper sulphate with hydrogen peroxide (adapted Fenton chemistry system). A range of hydrogen peroxide concentrations were incubated with 1μM copper sulphate in order to evaluate the most efficient concentrations for hydroxyl radical production. Based on these results (Figure 48), a final concentration of 1μM copper sulphate and 4.41mM hydrogen peroxide was chosen so as to allow both increases or decreases in radical levels to be detected whilst maintaining acceptable signal to noise ratio. Trapping of hydroxyl radicals by DEPMPO resulted in the formation of a characteristic DEPMPO-OH spectrum (A_N=13.4) (Figure 49). Production of DEPMPO-OH increased steadily over the twenty minute experimental period (Figure 50).

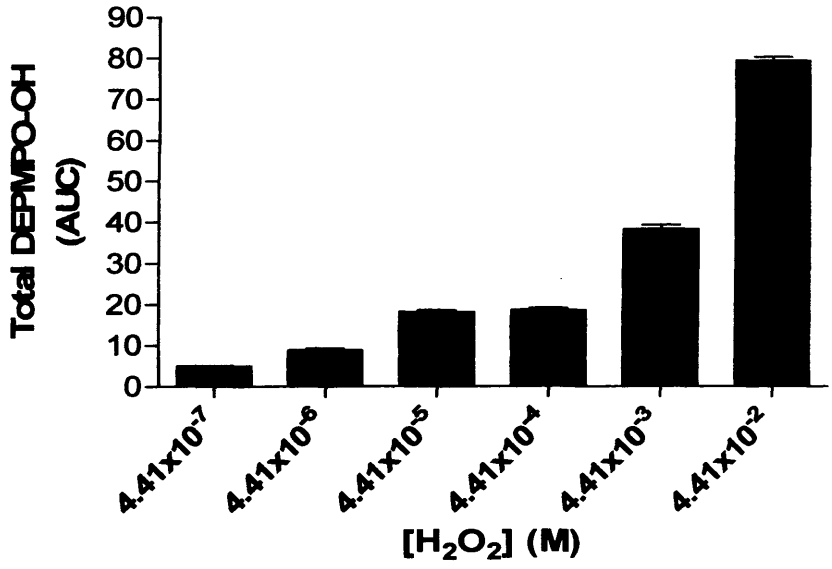


Figure 48 Effect of hydrogen peroxide concentration on hydroxyl radical production in the presence of 1μM copper sulphate. Data expressed as mean±SEM. n=4.

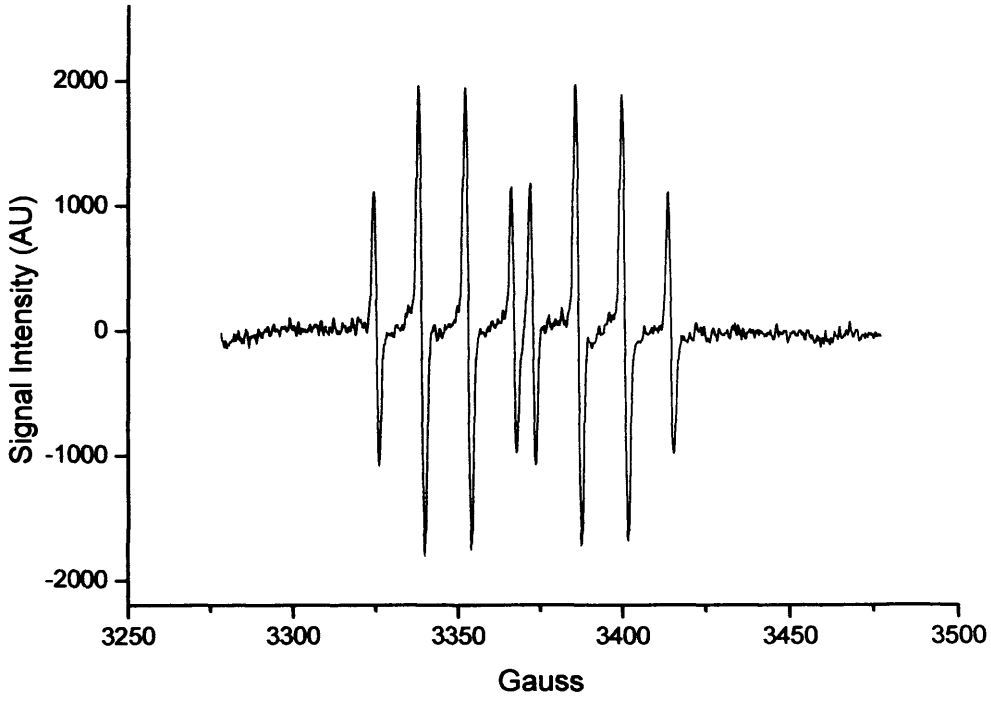


Figure 49 DEPMPO-OH spectrum. Readings taken over two minutes, eighteen minutes after the reaction was initiated.

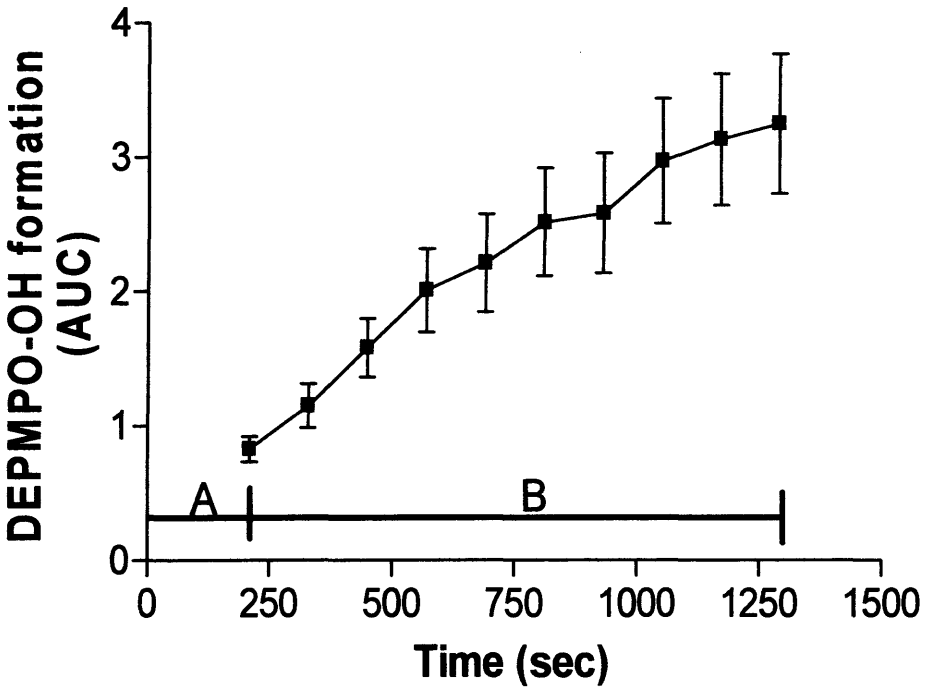


Figure 50 Production of DEPMPO-OH from 1μM copper sulphate and 4.41mM hydrogen peroxide. (A) Hydroxyl radical was produced using Fenton chemistry and incubated with 10mM DEPMPO, pH 7.4 for ninety seconds at room temperature. (B) DEPMPO-OH formation was then recorded for twenty minutes (10 x 2min scans). Data expressed as mean ± SEM. n=9.

3.3.2.2 Effect of L-arginine and L-citrulline on hydroxyl radical levels

Both L-arginine and L-citrulline were able to significantly inhibit DEPMPO-OH formation at concentrations $>1x10^{-6}$M ($p<0.01$) in the case of L-arginine and $1x10^{-6}$M ($p<0.05$) and $>1x10^{-4}$M ($p<0.01$) in the case of L-citrulline. EC_{50} values for L-arginine and L-citrulline were 16.19μM and 44.06μM respectively. There was no significant difference between the antioxidant activity of L-arginine and L-citrulline as assessed by a two-way ANOVA ($p>0.05$) (**Figure** 51).

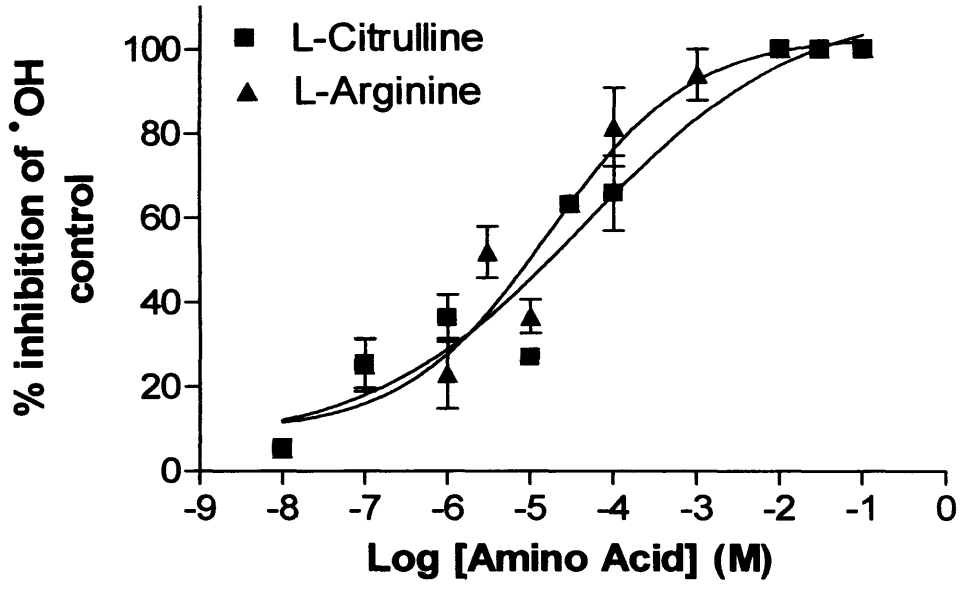
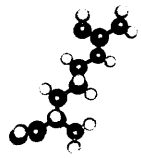


Figure 51 Effect of L-arginine and L-citrulline on DEPMPO-OH levels. Data expressed as mean ± SEM. n=3-5 except for $1x10^{-6}$M where n=6.



3.3.3 Effect of hydroxyl radical concentration on antioxidant capacity

The effect of hydroxyl radical concentration on the redox activity of L-arginine and L-citrulline was tested using luminol-based chemiluminescence. Redox activity was tested against four concentrations of hydroxyl radicals produced from increasing concentrations of copper sulphate and hydrogen peroxide (System 1 - 4). Increasing the concentration of copper sulphate and hydrogen peroxide resulted in a significantly larger yield of hydroxyl radical as determined by chemiluminescence yield ($p<0.01$) (Figure 52). Radical production was rapid and peaked immediately after copper sulphate was auto injected into the system, with signal gradually returning to baseline over one hour (Figure 52). Both amino acids tested exhibited antioxidant activity at all concentrations of hydroxyl radicals (Figure 54) with antioxidant activity shown to be dependent upon both amino acid and hydroxyl radical concentration (Table 21).

Table 21 EC_{50} values for L-arginine and L-citrulline against a range of hydroxyl radical concentrations. Details for systems 1-4 can be found in Table 20.

Amino Acid	EC_{50} system 1	EC_{50} system 2	EC_{50} system 3	EC_{50} system 4
L-arginine	375.6μM	578.7μM	856.1μM	1363μM
L-citrulline	226.3μM	441.9μM	746.1μM	1038μM

Also of interest is the fact that at amino acid concentrations of $<1x10^{-3}$M, and with the three highest concentrations of free radical (system 2, 3 & 4), a pro-oxidative effect was seen, defined as the ability to increase the signal to that above the control value. The concentration of $1x10^{-4}$M was chosen to investigate this pro-oxidant activity as this concentration produced consistent pro-oxidative effects with both amino acids. When radical concentration increased from system 2 to system 3, redox activity changed from antioxidative to pro-oxidative (Figure 55). In the case of L-arginine, pro-oxidant activity was proportional to the

concentration of hydroxyl radical present (r^2=0.9431).

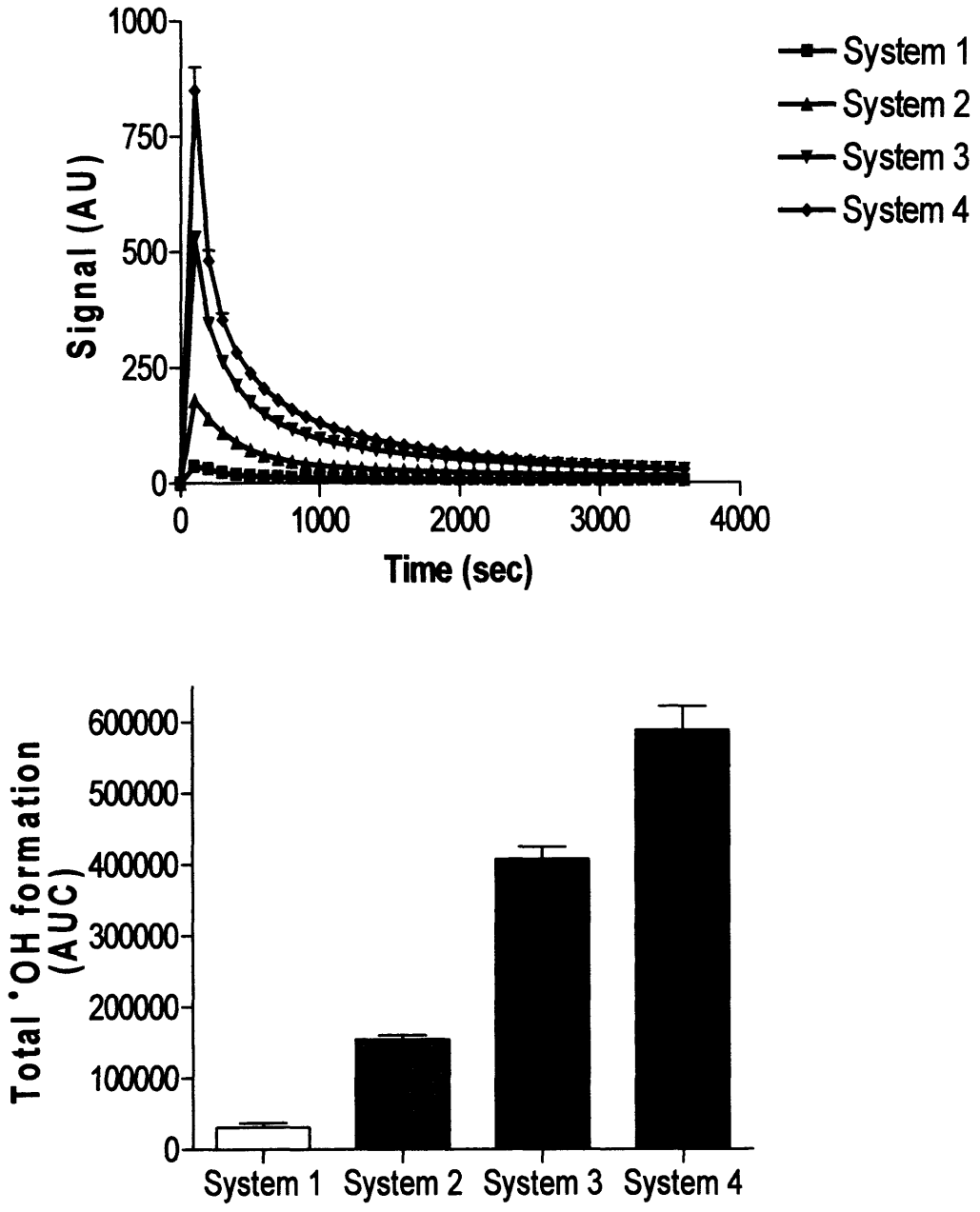


Figure 52 Hydroxyl radical production and quantification from varying concentrations of copper sulphate and hydrogen peroxide. For explanation of concentrations of copper sulphate and hydrogen peroxide in systems 1-4 refer to Table 20. Data expressed as mean ± SEM. n=16.

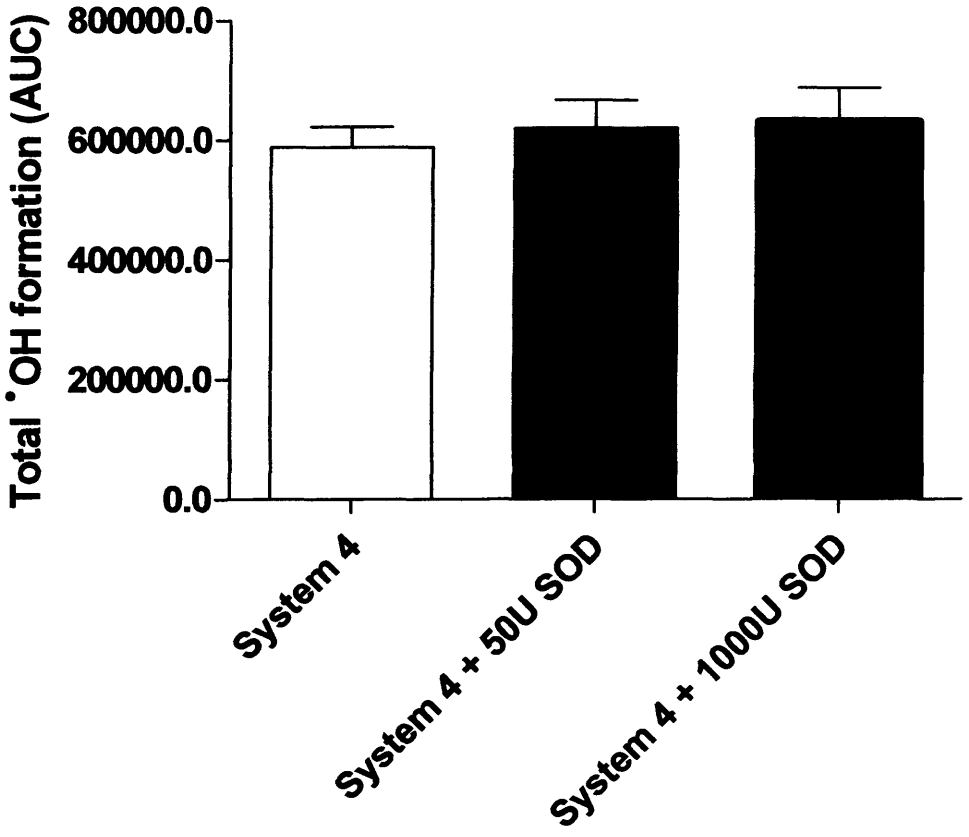


Figure 53 Effect of SOD on total hydroxyl radical yield from 36μM copper sulphate and 352.8mM hydrogen peroxide (system 4). No significant difference between groups ($p>0.05$). Data expressed as mean ± SEM. n=9-11.

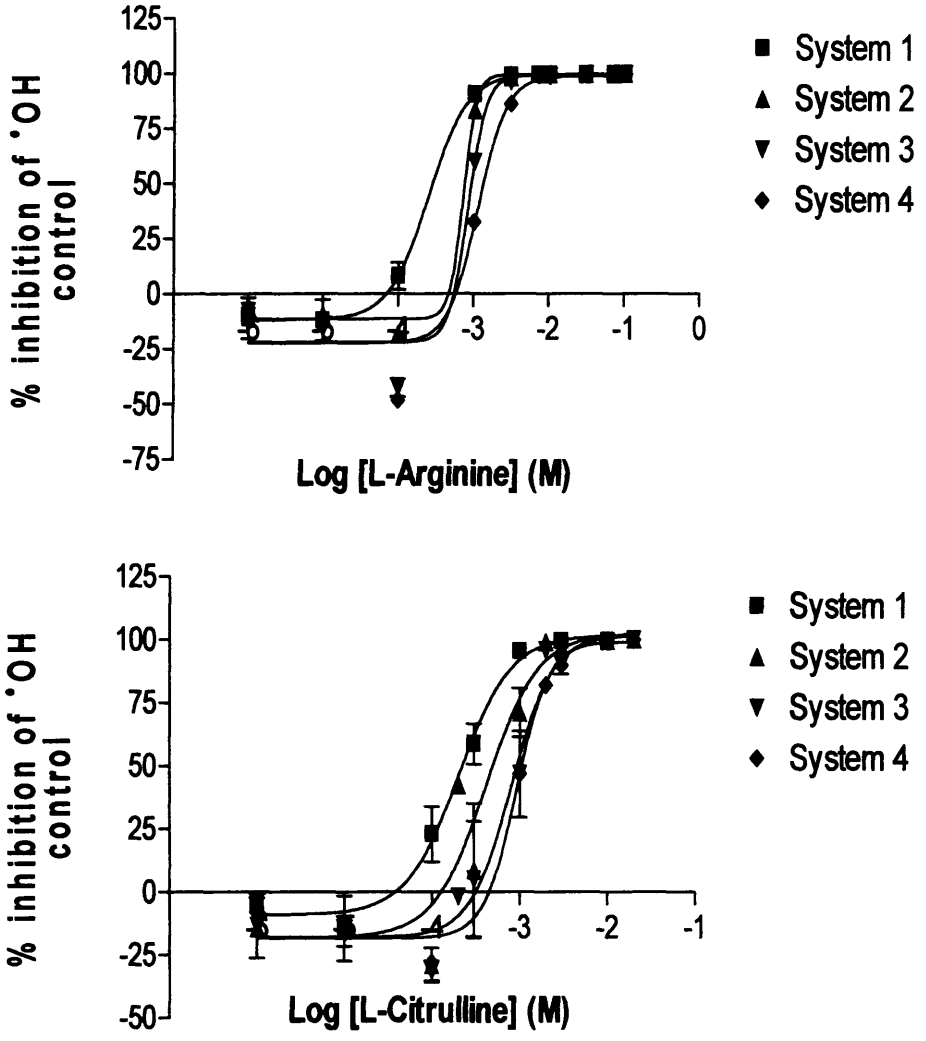


Figure 54 Effect of a range of L-Arginine and L-Citrulline concentrations ($1x10^{-6}$M – $1x10^{-1}$M) on hydroxyl radical levels. Moving from left to right indicates an increase in hydroxyl radical concentration. Positive values indicate a decrease in hydroxyl radical production (antioxidant activity); negative values indicate an increase in signal (pro-oxidative activity). For explanation of concentrations of copper sulphate and hydrogen peroxide in systems 1-4 refer to Table 20. Data expressed mean ± SEM. n=16.

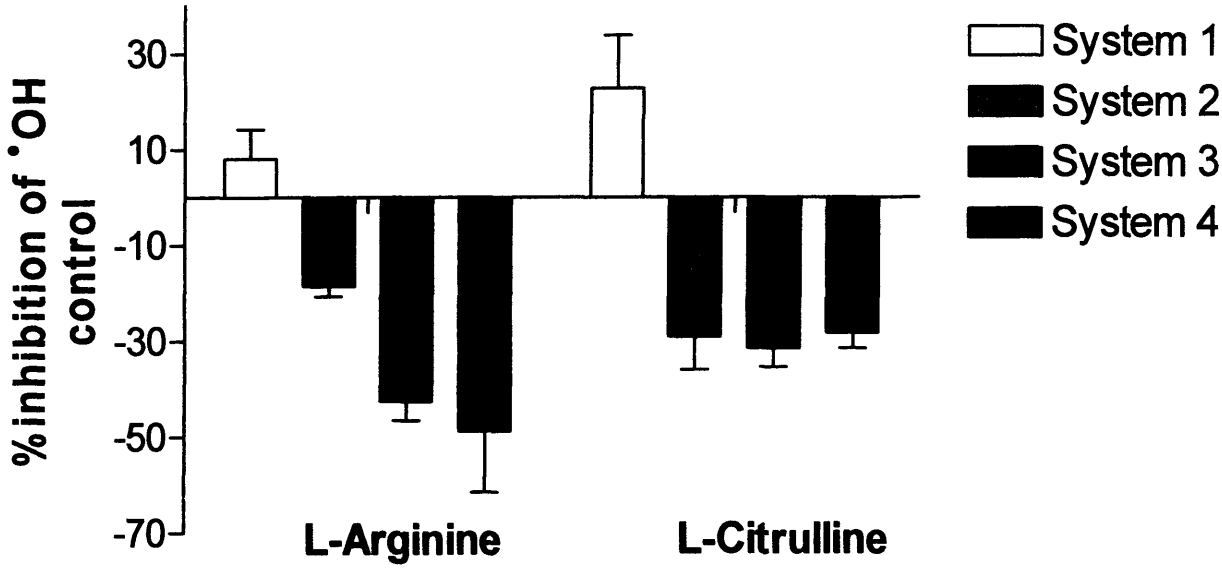
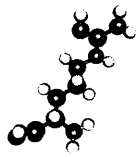


Figure 55 Effect of hydroxyl radical concentration on the antioxidant / pro-oxidant activity of $1x10^{-4}$M L-arginine and L-citrulline. Positive values indicate a decrease in hydroxyl radial concentration (antioxidant activity); negative values indicate an increase in hydroxyl radical concentration (Pro-oxidant activity). Data expressed mean ± SEM. n=4-6 except for L-Citrulline Systems 1 and 3 where n=9.



3.4 Discussion

3.4.1 Antioxidant properties in relation to superoxide

Two spin traps were chosen to investigate the antioxidant properties of L-arginine and L-citrulline against superoxide. This was based on the initial finding that L-arginine and L-citrulline had little effect on superoxide levels when measured by DEPMPO, with no activity seen below concentrations of $1x10^{-1}M$ (Figure). Based on this finding, a second spin trap (TEMPONE-H) was chosen to rule out the possibility that this result was due to the efficiency of radical binding by DEPMPO i.e. the DEPMPO-OOH adduct was being formed before the amino acid could scavenge the superoxide. Unlike DEPMPO, TEMPONE-H could not be used to characterise the scavenged radical, however results with DEPMPO showed conclusively that the ROS generating system used was only producing superoxide (spectra demonstrated A_N=13.4, inhibited by SOD but not catalase) and therefore radical contamination was not a problem in this system. Results with TEMPONE-H also demonstrated a lack of antioxidant activity against superoxide, with L-arginine having no effect on superoxide levels and L-citrulline only significantly reducing levels at 1x10-1M ($p<0.001$). As plasma concentrations are 70-150μM and 30-100μM for L-arginine and L-citrulline respectively [204,402] and exogenous i.v. administration of L-arginine leading to levels between 0.8mM to 8mM in plasma it can be concluded that neither amino acids would be likely to have any relevant antioxidant activity in a biological setting.

The fact that the spin traps may be more efficient at reacting with superoxide than the amino acids demonstrates the limited antioxidant properties of L-arginine and L-citrulline in regard to superoxide. When applied to an *in vivo* setting it is likely that superoxide would react more favourably with other molecules, meaning that the amino acids would afford little protection to molecules such as NO, especially considering NO and superoxide react at almost diffusion controlled rates. This work supports studies carried out by Adams *et al* [6] and Hayashi *et al* [137] who demonstrated that L-arginine and L-citrulline has

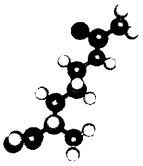
no antioxidant properties *in vitro* or *in vivo* respectively. However, this conclusion is based on chemical interactions alone (a factor returned to below). One must consider however the number of studies that point towards an effect of L-arginine on superoxide levels. Such studies have demonstrated that L-arginine can directly inhibit oxidative stress by reducing superoxide release from hypercholesterolaemic rabbit aortic rings [37], decrease ROS mediated NO degradation [153] and protect rat hearts from ROS damage. The following offers some explanation for the differences seen in these contradictory observations.

3.4.1.1 Incorrect identification of ROS

Several studies including a study by Lass *et al* [199], claim that L-arginine is able to scavenge superoxide generated from xanthine oxidase and hypoxanthine. Any results with xanthine oxidase must be carefully interpreted however. It is commonly assumed that the main product of xanthine oxidase is superoxide, however this radical generation system has also been shown to produce hydrogen peroxide and the hydroxyl radical through two and three electron reductions of molecular oxygen [191] [26]. Hydroxyl radical generation also occurs when hydrogen peroxide and superoxide are present through Haber Weiss chemistry. As not all studies use catalase, and even fewer directly identify the ROS in question, one cannot be sure that superoxide and not hydrogen peroxide or hydroxyl radicals were being produced. It is therefore possible that the antioxidant effect of L-arginine observed in earlier studies was actually mediated via scavenging of hydroxyl radicals, a finding supported by this data (Figure S1).

3.4.1.2 Reduction of superoxide production

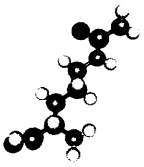
L-arginine has also been shown to protect against oxidative stress through indirect actions such as decreasing xanthine oxidase activity [223] and improving antioxidant defence systems by increasing glutathione levels [223] and SOD and catalase activity [89]. L-arginine could also reduce superoxide levels via re-coupling of NOS, thus converting it from a superoxide generator back to NO



synthesis [153].

Care must also be taken when interpreting results, as a change in superoxide levels may not correlate to antioxidant activity. For example, a study carried out by Boger *et al* [37] demonstrated that L-arginine was able to reduce superoxide release from the vasculature of cholesterol fed rabbits. Superoxide release was stimulated by PMA, a chemical that stimulates a respiratory burst in leukocytes, which are known to infiltrate the vessel wall in hypercholesterolaemia [133,261,310,378]. L-arginine may therefore not be scavenging superoxide release *per se*, but may be increasing the concentration of NO, thus inhibiting leukocyte adhesion to vascular endothelium. If this was the case, superoxide levels would decline, not because of the antioxidant effect of L-arginine, but because there are fewer leukocytes present in the endothelium generating superoxide upon PMA stimulation.

By directly investigating the interaction of amino acids and superoxide, this work overcame many of the complicating factors discussed above. Although *in vivo*, the chemistry between these two molecules would not be as straight forward as that witnessed in an *in vitro* system, in order to answer the question of whether L-arginine can directly scavenge superoxide, a simple *in vitro* system was needed. By using EPR it is indisputable that the xanthine oxidase system was only synthesising superoxide as only this radical could have provided the hyperfine splittings seen in the spectrum (Figure 42). This was most likely due to the low concentration of hypoxanthine used (25μM), as low substrate concentrations favour superoxide generation [191], whilst previous studies have used concentrations up to 1mM [199] which would push the enzyme towards generating hydrogen peroxide or even hydroxyl radicals. Also, through the use of a simple chemical system, complications arising *in vivo* and *ex vivo* such as the ability of L-arginine to increase antioxidant defences e.g. SOD and catalase were avoided. This allowed us to categorically state that there was direct interaction between L-arginine / L-citrulline and superoxide and suggests that antioxidant effects seen in other studies are most likely due to confounding factors such as incorrect radical identification and/or alternative physiological effects of the amino acid.



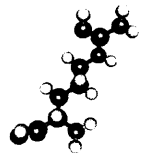
Although the results seem to directly contradict several studies, it is not that mistakes have been made; rather that different end points or indices of superoxide scavenging have been used. Although there can be no doubt that L-arginine is capable of reducing superoxide levels both *in vivo* and ex vivo, it is unlikely that this is via a direct scavenging effect and is most likely due to the reasons discussed above such as decreased production and increased degradation by other antioxidant systems. In terms of L-citrulline, little work has been carried out investigating the effects on superoxide. and so there is no work to compare this study to. This work would suggest that in terms of direct superoxide scavenging potential, L-citrulline is similar and would appear to have no role in antioxidant defence.

3.4.2 Antioxidant properties in relation to the hydroxyl radical

To the best of our knowledge this is the first study demonstrating the ability of L-arginine and L-citrulline to scavenge hydroxyl radicals at physiologically relevant concentrations. It is also the first to highlight potential pro-oxidant activity of both amino acids under certain oxidative conditions i.e. at specific concentrations of hydroxyl radial. An EPR based assay was chosen to investigate antioxidant activity as this allowed the antioxidant capacity against relatively low levels of hydroxyl radical to be quantified. Both L-arginine and L-citrulline scavenged hydroxyl radicals with an EC_{50} of 17.09μM and 44.06μM respectively (Figure). These are concentrations well within the range found in human plasma, reported to be 70-150μM and 30-100μM for L-arginine and L-citrulline respectively [205,403]. It has been shown that L-citrulline is released with nitric oxide [307] from endothelial cells and could therefore play a protective role in regards to preventing radical mediated breakdown of nitric oxide. The results within this study demonstrated that L-citrulline and L-arginine were able to scavenge hydroxyl radicals, however the difference in reaction rates between hydroxyl radicals with nitric oxide (1-2 x 1010 L mol–1 s–1) [327] and L-citrulline and L-arginine (3.9 x 109 L mol–1 s–1 and 3.5 x 109 L mol–1 s–1 respectively) [9,289], would suggest that neither would be very effective in

protecting nitric oxide from hydroxyl radical mediated breakdown. This study was concluded with an investigation into the effect of free radical concentration on antioxidant capacity. This is important to consider because the term 'oxidative stress' covers a wide range of increased hydroxyl radical concentrations. This study highlights how increased levels of hydroxyl radical not only reduce antioxidant capacity, but that the ratio between hydroxyl radical and amino acid governs whether an antioxidant or pro-oxidant effect could be seen (Figure SS). Between concentrations of 10μM-1mM both L-arginine and L-citrulline were converted from antioxidants to pro-oxidants and increased the concentration of hydroxyl radicals produced (Figure SS). This leads to the question of whether this could occur under physiological conditions and also whether exogenously administered amino acids have the potential to act in a pro-oxidative manner. This is of great relevance as numerous clinical trials have investigated the effects of administering supra-physiological concentrations of L-arginine and L-citrulline on endothelial function [8,53,164,212,279]. Little is currently known about administration of exogenous L-citrulline and the plasma levels achieved, but exogenous i.v. administration of L-arginine has been shown to lead to levels between 0.8mM to 8mM in plasma, depending on the dose administered (between 6g and 30g) [50,163,211,281]. At these concentrations, a direct pro-oxidative manner across concentrations of hydroxyl radicals was observed. The problems associated with quantification of hydroxyl radicals in plasma however make it difficult to predict the behaviour of L-arginine and L-citrulline *in vivo*. Results from this study suggest that as long as hydroxyl radical levels are relatively low, only an antioxidant effect would be observed. If hydroxyl radical levels increase however, both amino acids would have the potential to act in a pro-oxidant fashion, further increasing harmful oxidant levels.

In summary, this study demonstrates that at physiological levels, L-arginine and L-citrulline have no effect on superoxide but are effective hydroxyl radical scavengers. Under certain oxidative conditions however, both have the capacity to act in a pro-oxidative fashion. The potential for pro-oxidative activity is of great relevance as a growing number of studies are investigating the effect of exogenous L-arginine and L-citrulline in treatment of disease states associated



with oxidative stress. This study highlights the importance of achieving the correct balance between amino acid and hydroxyl radical if a protective antioxidant effect is to be attained.

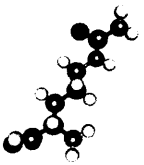
3.5 Summary

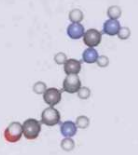
- L-arginine and L-citrulline are ineffective at scavenging superoxide with neither exerting any antioxidant activity at physiological concentrations or at concentrations achieved after exogenous administration

- The ability of L-arginine to reduce superoxide level *in vivo* and *ex vivo*, as demonstrated in published studies, is unlikely to be via direct scavenging.

- This is the first study highlighting the potential pro-oxidant activity of both amino acids under certain oxidative conditions concerning the hydroxyl radical. It also demonstrates the ability of L-arginine and L-citrulline to scavenge hydroxyl radicals at physiologically relevant concentrations.

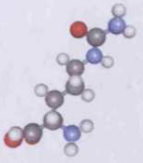
- The potential for pro-oxidative activity is of great relevance as a growing number of studies are investigating the effect of exogenous L-arginine and L-citrulline in treatment of disease states associated with oxidative stress. This study highlights the importance of achieving the correct balance between amino acid and hydroxyl radical if a protective antioxidant effect is to be attained.





Mechanism of antioxidant action

Chapter Four



4.0 Introduction

Chapter three focused on characterising the antioxidant properties of L-arginine and L-citrulline, demonstrating a propensity to scavenge hydroxyl but not superoxide radicals at physiologically relevant concentrations. Based on these findings, the aim of this chapter was to characterise the mechanism by which hydroxyl radical scavenging was occurring.

There are two main mechanisms by which an antioxidant may reduce hydroxyl radical formation and in relation to the adapted Fenton system these are:

1. Interaction with the substrate involved in hydroxyl radical production e.g. copper sulphate / hydrogen peroxide
2. Direct scavenging of hydroxyl radicals

4.0.1 Indirect reduction of hydroxyl radical levels

The majority of published work has focused on the ability of L-arginine and L-citrulline to interact directly with hydroxyl radicals. Within the literature however, there is evidence to suggest that amino acids have the ability to bind copper [258]. Amino acids are known to form salts with d-block transition metals through dative bonding between the α-amino and carboxylate groups with the metal ion (Figure).

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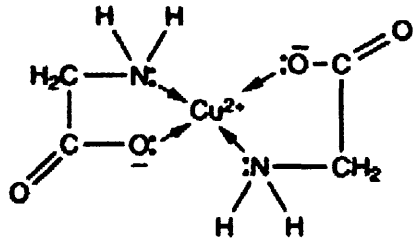
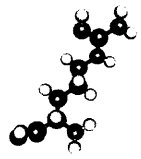


Figure 56 Product of amino acid – copper interactions



The vast majority of serum copper is transported bound to ceruloplasmin; the rest is bound to albumin, transcuprein, and copper–amino acid complexes. L-arginine, but not L-citrulline, is capable of binding copper within human plasma [258] and all evidence would suggest that although L-arginine is capable of interacting with copper however the effect this has on copper catalysed reactions remains uncharacterised. The effect of L-citrulline is also unknown and so this chapter aims to provide more information on this topic.

4.0.2 Direct scavenging of hydroxyl radicals

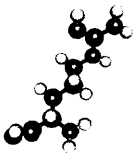
To date, no work investigating the mechanism by which L-arginine scavenges hydroxyl radicals has been undertaken. All work has focused on the superoxide anion with contradictory results ensuing. Lass *et al* 2002 [199] suggested that the anti-oxidative activity of L-arginine was based on the guanidinium group which is in direct contrast to a study by Wallner *et al* 2001 [379] who reported that the α-amino group is responsible for the antioxidant properties of this amino acid.
Significantly less work has been carried out investigating the mechanism of action of L-citrulline [8]. In this study two techniques, HPLC and LCMS, were used and two potential mechanisms of action proposed: 1) abstraction of a hydrogen ion from the α-carbon forming a carbon centred radical as an intermediate and 2) the hydroxyl radical attacks the side chain of L-citrulline, resulting in decomposition to several radical derivatives and eventual formation of unknown secondary products of various molecular weights through polymerisation and/or condensation reaction.

The aim of this chapter was therefore to characterise the mechanism of action of both L-arginine and L-citrulline against the hydroxyl radical. First, the ability of both amino acids to reduce hydroxyl radicals via an indirect mechanism, i.e. through the reduction of substrate, was investigated utilising EPR and an ozone based chemiluminescence assay to measure the ability of L-arginine and L-citrulline to reduce Cu(II) levels. Next, commercially available compounds, that structurally represent various chemical groups found within L-arginine and L-

citrulline, were used to try and pin point the moiety that controls antioxidant and pro-oxidant activity. Finally, the mechanism by which both amino acids directly interact with hydroxyl radicals was investigated via the manipulation of pH to control the protonation state.

4.1 Aims

- To clarify whether the reduction in hydroxyl radical levels seen upon administration of L-arginine and L-citrulline is via a direct or indirect mechanism
- To investigate the structural moiety that controls the redox activity (antioxidant vs pro-oxidant) of L-arginine and L-citrulline
- To elucidate the mechanism by which this moiety confers redox activity.



4.2 Materials and Methods

4.2.1 Investigation into indirect reduction of hydroxyl radical levels

4.2.1.1 Detection of NO release from s-nitrosothiols

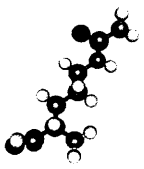
Ozone based chemiluminescence was used to detect NO release from S-nitrosoglutathione as described in chapter two. The assay mix was made up in PBS to a volume of 5ml which was bubbled with nitrogen for 5 minutes prior to the start of the experiment to remove all oxygen from the sample.
The effects of a range of compounds on the release of NO from 10nmoles and 80nmoles GSNO were investigated (Table 22). EDTA, a well known metal chelator, was used to demonstrate the transition metal dependence of NO release.

Table 22 Concentration of compounds used in the nitric oxide analyser (NOA) assay

Compound	Concentration Range
L-arginine	100μM, 5mM & 100mM
L-citrulline	100μM, 5mM & 100mM
Ascorbic acid	50μM, 100μM, 200μM, 5mM & 100mM
EDTA	5mM, 20mM, 100mM

4.2.1.2 Detection of copper (II) by EPR

The dithiocarbamate, MGD, was added to Cupric sulphate (Cu(II)) forming a paramagnetic Cu-MGD complex with characteristic spectra (Figure & Figure).
The effect of L-arginine and L-citrulline on Cu(II) levels was investigated



through the incubation of a range of test compound concentrations ($1x10^{-6}$M, $1x10^{-4}$M, $1x10^{-2}$M, $1x10^{-1}$M and 1M) with 50μM Cu(II) for five minutes. After the five-minute period, 6.25mM MGD was added and samples immediately placed in a quartz glass cell and EPR spectra recorded at room temperature using a Varian 104 EPR spectrometer. The instrument settings were as described in chapter 2. Samples were run for 5 minutes (5 x 1 minute scans). The average of the five scans was taken and the intensity of peak 4 (Figure 57) was determined.

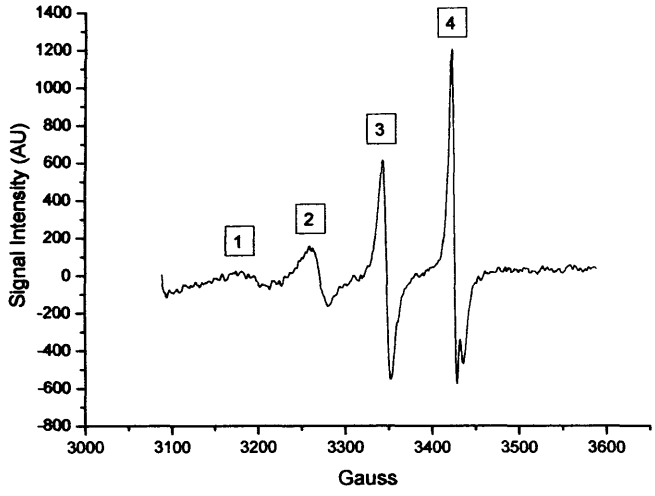
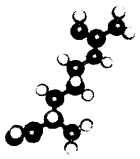


Figure 57 Example of Cu-MGD spectrum. The Spectrum was taken between 4-5 minutes after initiation of the reaction.

4.2.2 Investigation into the direct reduction of hydroxyl radical levels

4.2.2.1 Formation of hydroxyl radicals

Hydroxyl radicals were generated using Fenton chemistry as described in 2.01. Briefly, 36μM copper sulphate and 352.8mM hydrogen peroxide were mixed to produce hydroxyl radicals. Hydroxyl radical was measured using chemiluminescence with a luminol probe. Measurements were all made at pH 7.0, 37°C using a MLX microtitre ® plate luminometer. Briefly, 100μL Luminol



(final concentration 1mM dissolved in 10% DMSO and 90% PBS) was added to each well in a 96 well plate along with 352.8mM hydrogen peroxide and 10µl of either PBS (control) or the appropriate concentration of test compound ($1x10^{-1}$M – $1x10^{-6}$M L-arginine, N-α-Acetyl-L-arginine, glycine, Aminoguanidine). Background chemiluminescence activity was measured for one minute and then 36µM copper sulphate injected into each well using an automated injector system to initiate the reaction. Photon emission was continuously recorded for sixty minutes. Redox activity was assessed according to the ability to quench or increase the signal detected from the above Fenton reaction. Area under curve was recorded in the presence (AUC_{AA}) and absence ($AUC_{Control}$) of the test compounds, and change from control (%change) was calculated as:

% change=$((AUC_{control} - AUC_{AA}) / AUC_{control}) * 100$

4.2.2.2 Effect of various structural moieties on redox activity

In order to investigate the moiety that controls the redox activity of L-arginine and L-citrulline, a number of commercially available molecules with similar structures to both amino acids were used. Aminoguanidine was chosen as it is structurally similar to the guanidinium group contained within L-arginine. glycine was chosen because as the most structurally simple amino acid, it allows the effect of the α-amino group contained within both amino acids to be studied. Finally the effects of N-α-acetyl-L-arginine were investigated as within this molecule the α-amino group is replaced with an acetyl group, and so allows the effect of removing the α-amino group to be investigated (Figure).

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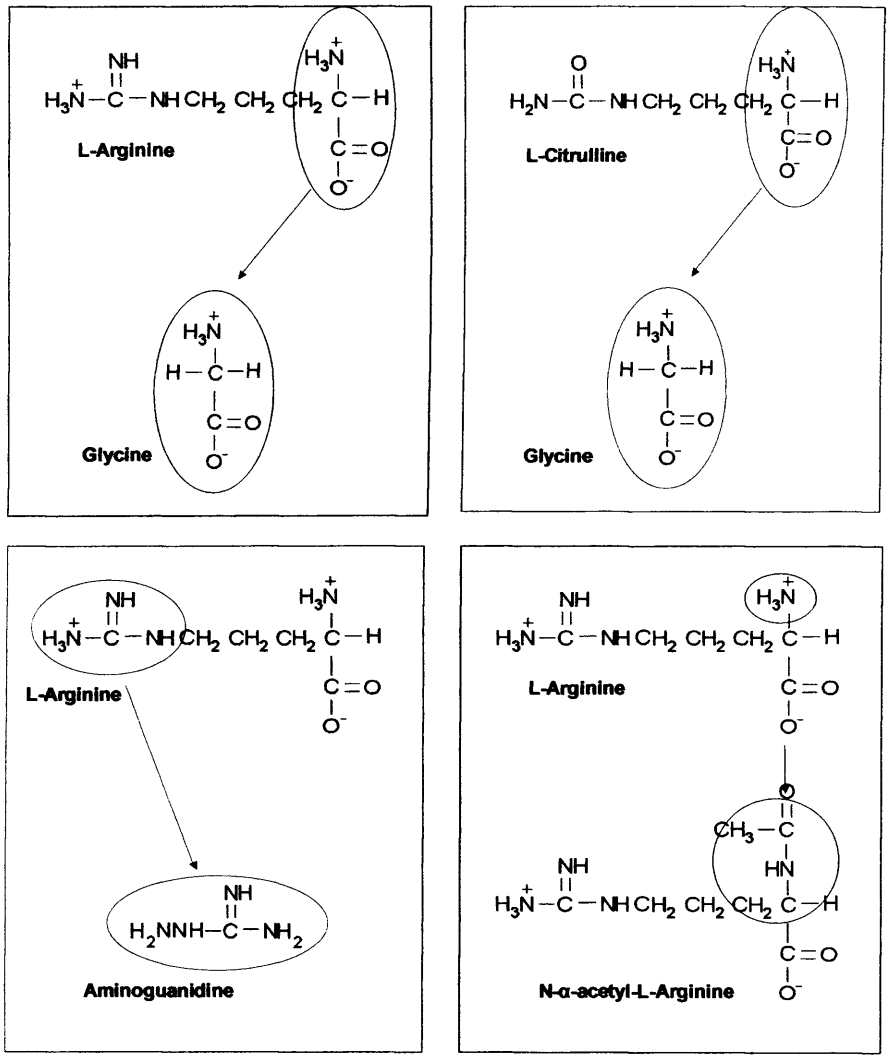
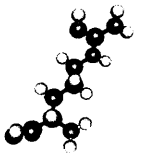


Figure 58 Illustration of the structural similarities between L-arginine, Glycine, L-citrulline, Aminoguanidine & N-α-acetyl-L-Arginine.



4.2.2.3 Mechanism by which the alpha amino group confers redox activity

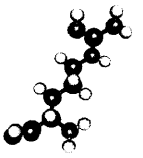
In order to investigate the effect of the protonation state of the α-amino group on redox activity, pH systems were chosen based on the pKa values of L-arginine and L-citrulline.
These are summarised in Table 23.

Table 23 pH values for each luminol based chemiluminescence system. Details on systems 1-4 can be found in table Table 20.

	L-arginine (pH)		L-citrulline (pH)	
	NH_3	NH_2	NH_3	NH_2
System 1 Fenton chemistry	7.2±0.19	9.6±0.58	7.2±0.04	10.7±0.015
System 2 Fenton chemistry + amino acid	7.0±0.16	9.9±0.08	7.1±0.025	10.8±0.05
System 3 Hydrogen peroxide alone	7.2±0.19	9.0±0.33	7.2±0.045	10.7±0.01
System 4 Hydrogen peroxide + amino acid	7.2±0.17	9.8±0.05		

System one and System three - to test the effect of pH on Fenton chemistry and hydrogen peroxide, pH was increased to the above values via addition of 1.2µl and 4µl of 2.5M NaOH respectively.

System two and System four - to test the effect of pH on the redox activity of L-arginine and L-citrulline, pH was increased to the above values via addition of 5µl 2.5M NaOH.



4.2.3 Statistical analysis

Data expressed as mean ± SEM. Unpaired Student t-test and one way ANOVA followed by Bonferroni *post hoc* test was used to calculate significance which was set at $p<0.05$ unless otherwise stated.

4.3 Results

Indirect hydroxyl radical scavenging

4.3.1 Effect of L-arginine and L-citrulline on Cu(II) levels - Ozone based chemiluminescence study

4.3.1.1 Effect of EDTA on basal NO release

In order to investigate the effect of L-arginine and L-citrulline on copper levels, the technique of ozone based chemiluminescence was used based on the fact that NO release from S-nitrosothiols is copper dependent [115,338,394]. In PBS alone, a small amount of NO was seen to be released from GSNO with an area under curve of 320.9AU and 929.7AU for 10nmoles and 80nmoles GSNO respectively (Figure 59). In order to prove that this NO release was copper dependent, varying concentrations of the metal chelator EDTA were added to the PBS. Figure 59 & Table 24 illustrate the the decrease in NO release from 10nmoles and 80nmoles GSNO upon the addition of EDTA ($p<0.05$).

4.3.1.2 Effect of L-arginine and L-citrulline on basal NO release

Both L-arginine and L-citrulline significantly reduced basal NO release from 10nmoles and 80nmoles of GSNO (for individual p values see Figure & Figure) suggesting that these amino acids were able to reduce unbound copper levels, thus inhibiting NO release. In order to further prove that the release of NO within this system was dependent on copper, the effects of ascorbic acid on NO release was tested based on studies demonstrating that ascorbic acid catalyses the release of NO from GSNO through reduction of Cu(II) to Cu(I) [174,326,409]. Upon addition of 10nmoles and 80nmoles GSNO to a range of ascorbic acid concentrations, a significant increased in NO release (~3000% ($p<0.05$ & $p<0.001$)) was seen providing further evidence that copper is involved in NO release within this system (Figure , Figure & Table 26).

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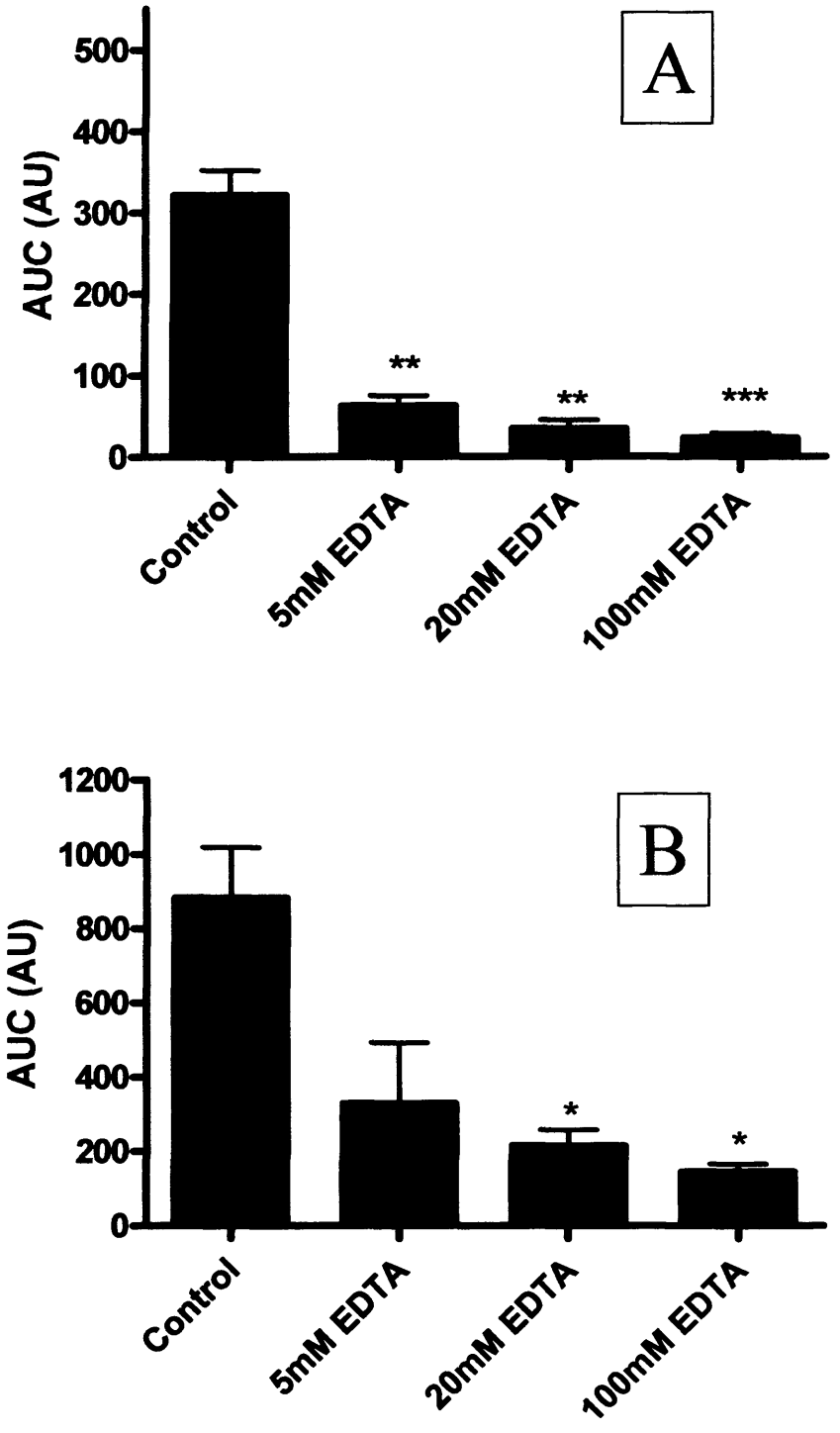


Figure 59 Effect of EDTA on NO release from (A) 10nmoles GSNO and (B) 80nmoles GSNO. Data expressed as mean±SEM. For control n=23. For all other treatment groups n=4. * represents p<0.05, ** represents p<0.01, * represents p<0.001 cf control.**

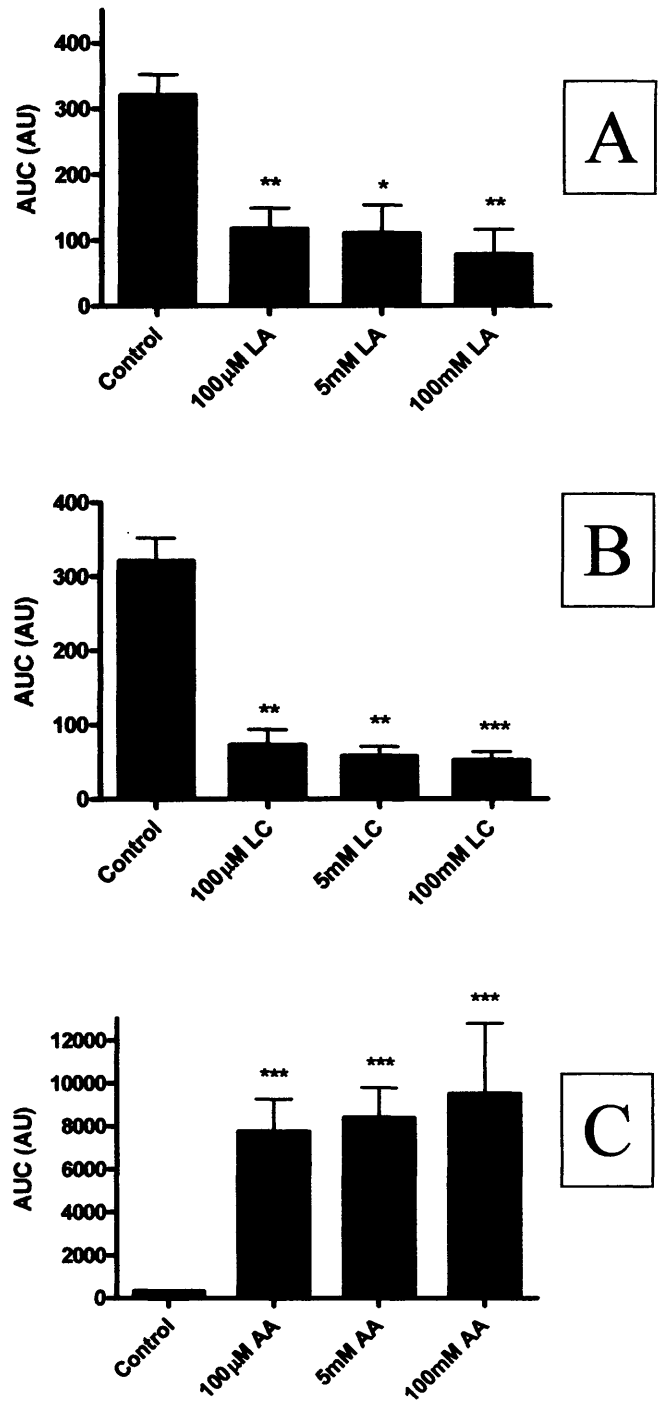


Figure 60 Effect of (A) L-arginine, (B) L-citrulline, (C) Ascorbic acid on NO release from 10nmoles GSNO. Data expressed as mean±SEM. For control n=23. For all other treatment groups n=4-5. * represents $p<0.05$, ** represents $p<0.01$, * represents $p<0.001$ cf control.**

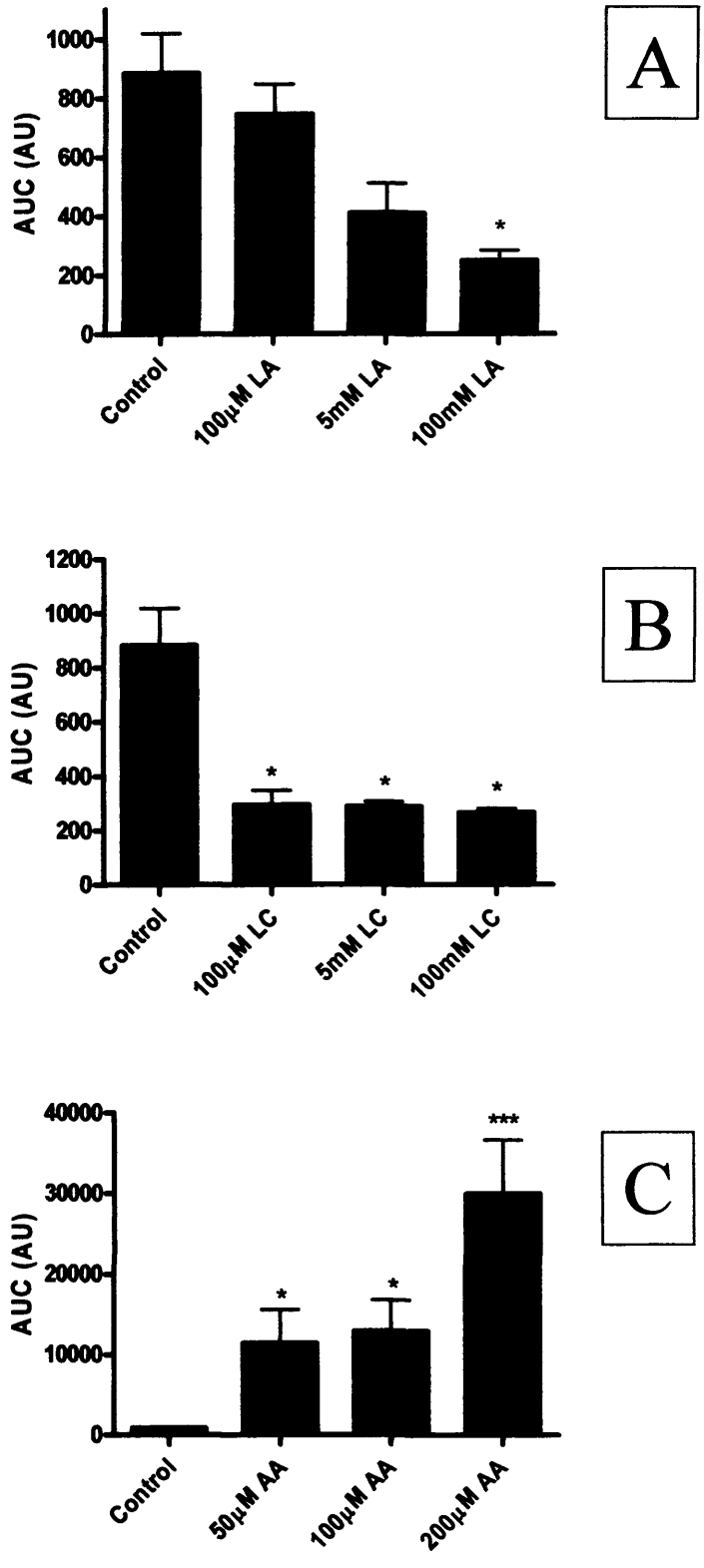


Figure 61 Effect of (A) L-arginine, (B) L-citrulline,

(C) Ascorbic acid on NO release from 80nmoles GSNO. Data expressed as mean±SEM. For control n=23. For all other treatment groups n=4-5. * represents p<0.05, * represents p<0.001 cf control.**

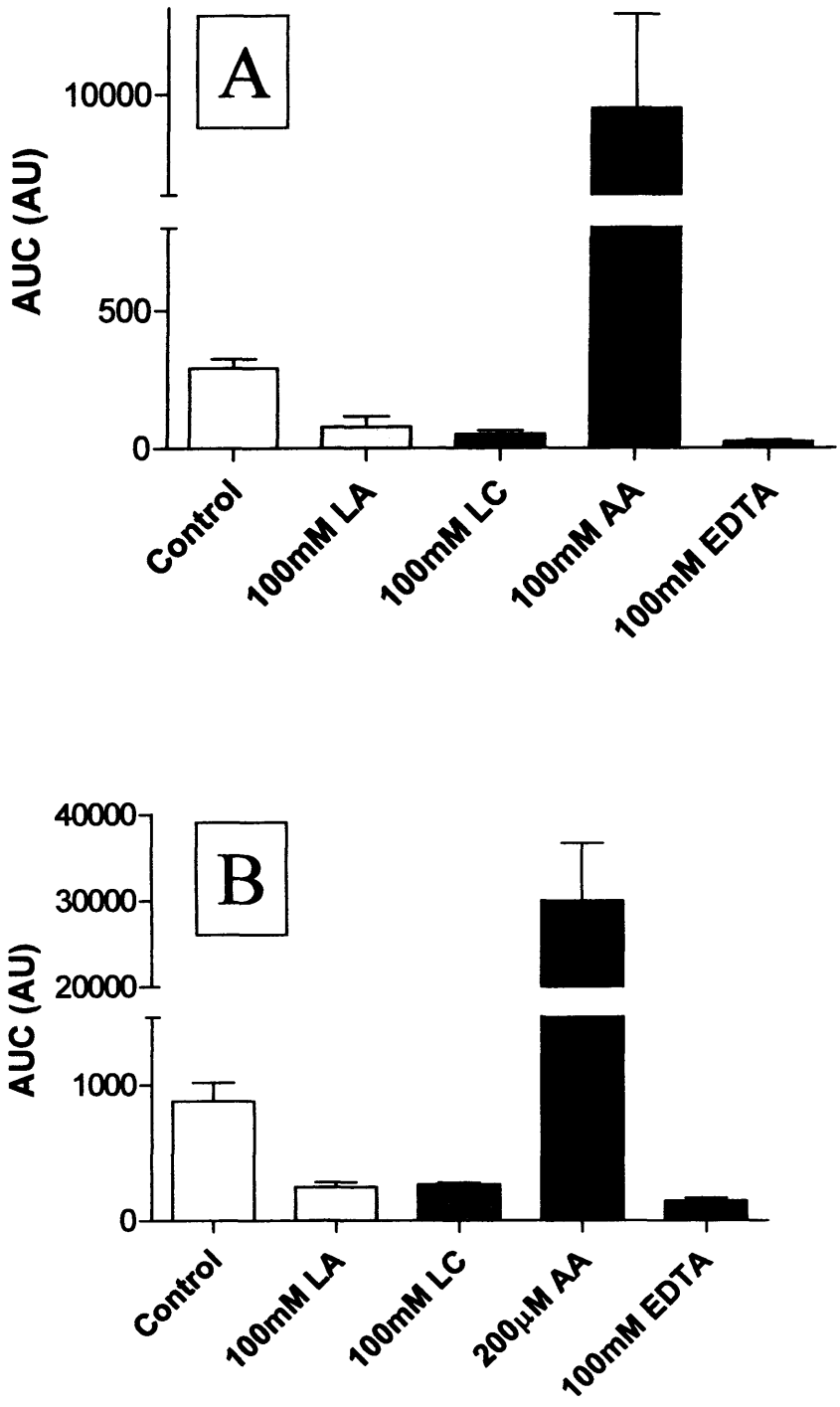


Figure 62 Effect of all tested compounds on NO release from A) 10nmoles GSNO and B) 80nmoles GSNO. Data expressed as mean±SEM. For control n=23. For all other treatment groups n=4.

Table 24 Effect of EDTA on NO release from GSNO. Values correspond to **Figure** . Percentage values represent % decrease from control

	10nmoles GSNO			**80nmoles GSNO**		
[Test compound]	***5mM***	***20mM***	***100mM***	***5mM***	***20mM***	***100mM***
EDTA	-80%	-89%	-93%	-64%	-77%	-84%

Table 25 Effect of amino acids on NO release from GSNO. Values correspond to **Figure** and **Figure** . Percentage values represent % decrease from control

	10nmoles GSNO			**80nmoles GSNO**		
[Test compound]	***100μM***	***5mM***	***100mM***	***100μM***	***5mM***	***100mM***
L-arginine	-64%	-66%	-76%	-20%	-56%	-73%
L-citrulline	-77%	-82%	-84%	-68%	-67%	-71%

Table 26 Effect of ascorbic acid on NO release from GSNO. Values correspond to **Figure** and **Figure** . Percentage values represent % increase from control

	10nmoles GSNO			**80nmoles GSNO**		
[Test compound]	***100μM***	***5mM***	***100mM***	***50μM***	***100μM***	***200μM***
Ascorbic Acid	2316%	2510%	2859%	1129%	1291%	3126%

4.3.2 Effect of L-arginine and L-citrulline on Cu(II) levels – an EPR based study

In order to investigate the ability of L-arginine and L-citrulline to interact with Cu(II), the ability of EPR to detect paramagnetic species was used. As discussed previously (chapter three), Cu(II) is paramagnetic due to the presence of a free electron in its outer electron shell. Chelation of Cu(II) to MGD therefore allows quantification of this molecule (see chapter two for a more detailed discussion). A range of copper sulphate concentrations were tested in order to determine the optimum concentration of copper to bind to the spin trap MGD. A concentration of 50μM was chosen. Although slightly higher than the concentrations used previously (1μM, 4.5μM, 9μM, 18μM and 36μM; Chapter three), lower concentrations were prone to a low signal:noise ratio. Also, as demonstrated in Figure , MGD can detect both a linear increase and decrease from 50μM. This concentration was therefore optimum for detection of regardless of an increase or decreases in copper levels.

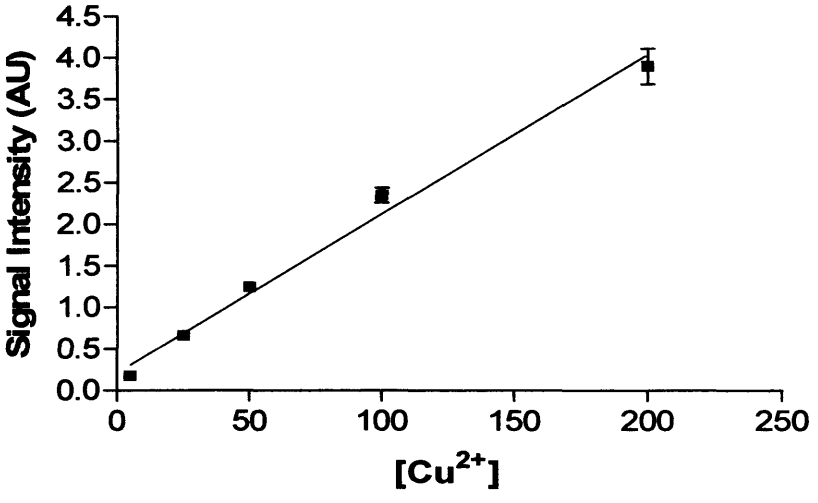
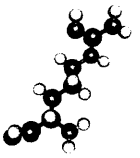
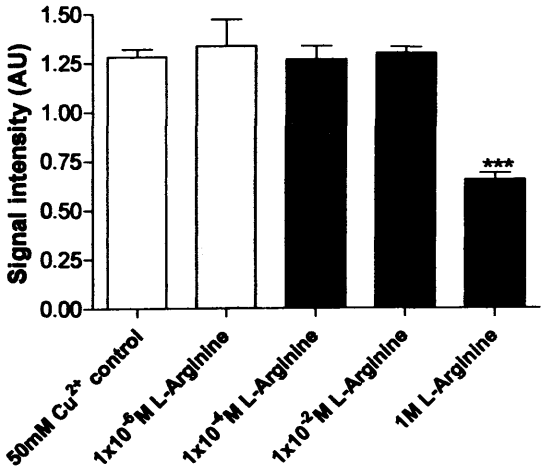


Figure 63 Effect of copper sulphate concentration on signal intensity of Cu^{2+}-MGD. r^2=0.9639. Data expressed as mean±SEM. n=3-5



L-arginine and L-citrulline had little effect on Cu(II) levels. L-arginine reduced Cu(II) levels but only at supraphysiological concentrations of 1M ($p<0.001$). L-citrulline had no significant effect on Cu(II) levels ($p>0.05$) (Figure)

64



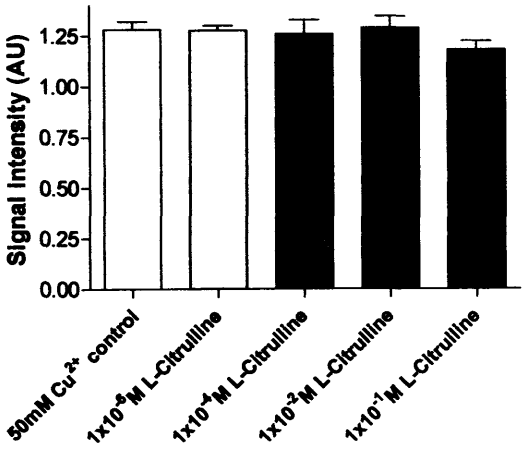
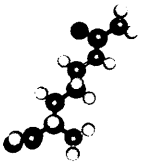


Figure 64 Effect of test compounds on Cu(II) levels as detected by 6.25mM MGD. * represents $p<0.01$ cf control. Data expressed as mean±SEM. For control n=9. For all other treatment groups n=4-5.**



4.3.3 The effect of various structural moieties on redox activity

4.3.3.1 Antioxidant effects

glycine, representative of the α-amino group exerted an almost identical redox (antioxidant/pro-oxidant) profile to that of *L*-arginine and *L*-citrulline (EC_{50} values: 1.047mM, 1.363mM and 1.062mM respectively) (Figure 65). Although aminoguanidine demonstrated a degree of antioxidant activity it was to a lesser extent than L-arginine (EC_{50} values 1.363mM and 3.02mM respectively). Finally N-α-acetyl-L-arginine structurally representing *L*-arginine minus the α-amino group, demonstrated no antioxidant or pro-oxidant activity at any concentration.

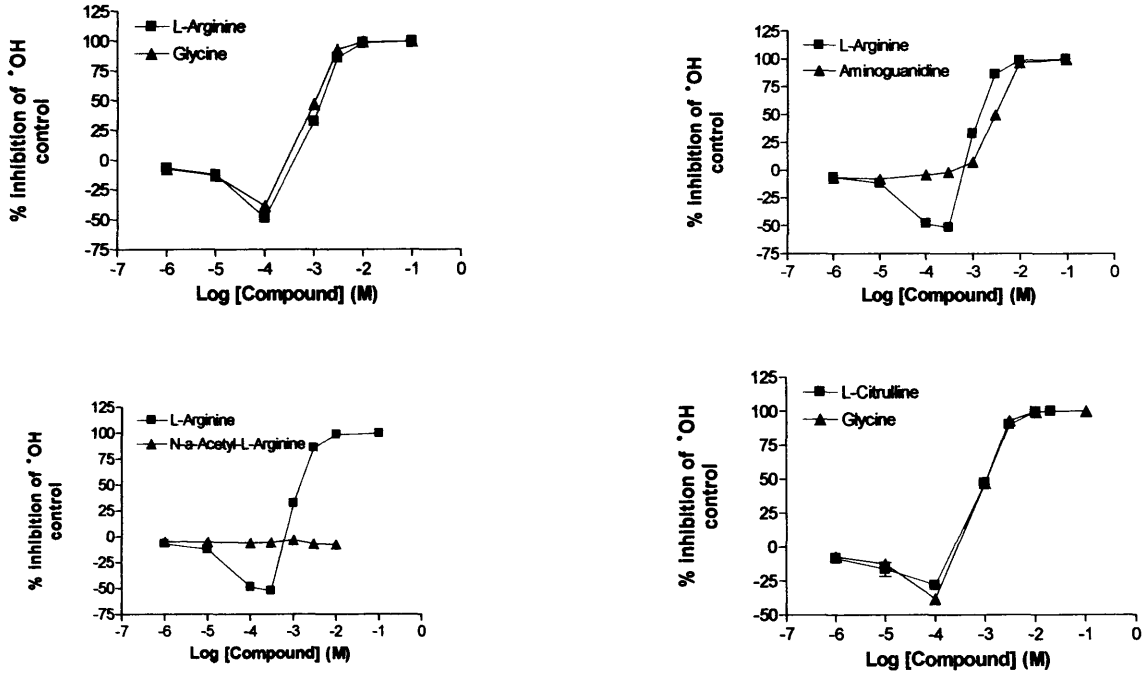


Figure 65 Effect of structural derivatives of L-Arginine and L-Citrulline on hydroxyl radical levels. Positive values indicate a decrease in hydroxyl radical production (antioxidant activity); negative values indicate an increase in signal (pro-oxidative activity). EC_{50} values for L-Arginine, L-Citrulline, glycine and aminoguanidine 1.363mM, 1.062mM, 1.047mM and 3.023mM respectively. Data expressed mean ± SEM. n=4-6.

4.3.3.2 Pro-oxidative effects

As demonstrated in chapter three, both L-arginine and L-citrulline demonstrated pro-oxidative activity at $1x10^{-4}$M. As with antioxidant action, the moiety responsible for this effect was investigated. Glycine demonstrates an identical pro-oxidative response at $1x10^{-4}$M to L-arginine and L-citrulline (Figure 66), all three resulting in a significant increase in chemiluminescence compared with the control ($p<0.001$ for both molecules). Neither aminoguanidine nor N-α-acetyl-L-arginine showed any pro-oxidant effects ($p>0.05$ for both molecules) (Figure 66)

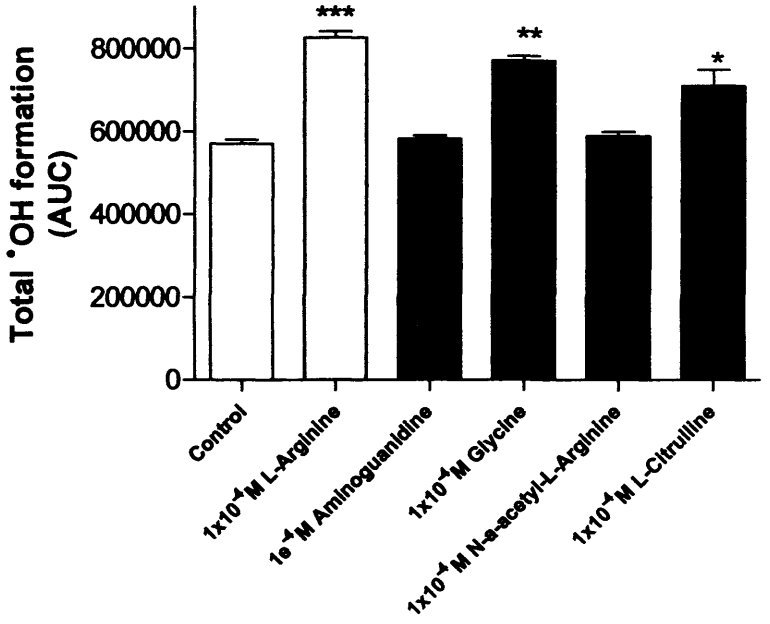


Figure 66 Pro-oxidative activity of L-arginine, L-citrulline and glycine at $1x10^{-4}$M. * represents $p<0.001$, ** $p<0.01$ and * $p<0.05$ compared with control. Data expressed as mean ± SEM. n=4-5**

4.3.4 Direct hydroxyl radical scavenging - Effect of protonation state of the α-amino group on antioxidant activity

Based on the above results, the mechanism by which the α-amino groups confers redox activity was investigated. The α-amino group exists in two different protonation states, NH_2 and NH_3, of which the NH_3 state is predominant at physiological pH. A potential antioxidant mechanism is that the hydroxyl radical abstracts hydrogen from the α-amino group to form water. To test this hypothesis, pH was used to manipulate the protonation state of the α-amino group, as a change in protonation state should not affect the chelation properties of the amino acids. This assay allowed us to investigate the direct hydroxyl radical scavenging properties of L-arginine and L-citrulline.

4.3.4.1 Determination of NH_3:NH_2 ratio in each system

The pKa value of a molecule gives us information on the percentage of each protonation state at any given pH using the Henderson-Hasselbach equation (Figure).

$$pH = pK_a + \log\frac{[A^-]}{[HA]}$$

Figure 67 Henderson-Hasselbach equation

Based on this, pH values were chosen that resulted in the amino acids being entirely in either the NH_2 or NH_3 form. These systems were then used to analyses the effect of NH_2:NH_3 ratio on redox activity.

Table 27 & Table 28 summarise the pH values chosen for both L-arginine and L-citrulline and the corresponding levels of NH_2 and NH_3 at each pH.

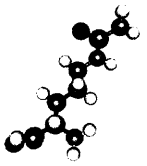


Table 27 pH values chosen for L-arginine and the corresponding levels of NH_2 and NH_3 at each pH.

L-arginine	**-NH_2**	**-NH_3**
pH 7.0	1%	99%
pH 9.9	89%	11%

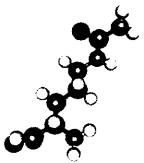
Table 28 pH values chosen for L-citrulline and the corresponding levels of NH_2 and NH_3 at each pH.

L-citrulline	**-NH_2**	**-NH_3**
pH 7.1	1%	99%
pH 10.8	98%	2%

4.3.4.2 Effect of pH on hydroxyl radical production and detection

Before the effect of protonation state on redox activity could be investigated, the effect of pH on hydroxyl radical production (i.e. the Fenton chemistry system) and detection was investigated. pH has been shown to affect both luminol luminescence and hydroxyl radical production through both an increase in radical production [346] (Equations 3-5). and an increase in the degree of chemiluminescence for a set concentration of free radical [178].

Indeed, a significant increase in hydroxyl radical production / detection was seen when pH increased from 7.2 (control) to 9.6 and 10.7 respectively ($p<0.05$ and 0.001 respectively). For this reason, control experiments (i.e. hydroxyl radical production in the absence of amino aids) were run at the same pH as the corresponding system containing the amino acid.



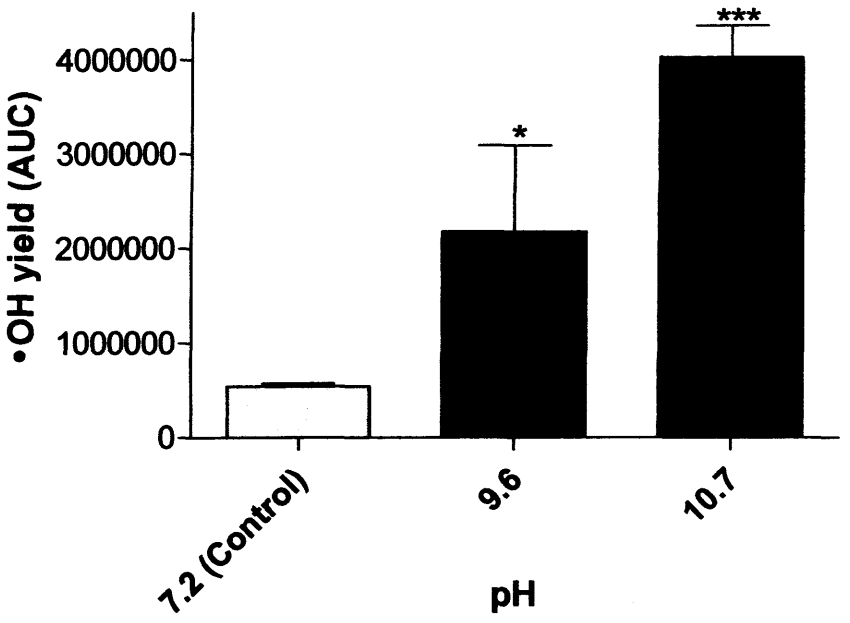


Figure 68 Effect of pH on hydroxyl radical production from Fenton chemistry. Concentration of copper sulphate = 36μM, concentration of hydrogen peroxide =352.8mM. Data expressed as mean±SEM. n=4-6.

4.3.4.3 Effect of protonation state on the redox activity of L-arginine and L-citrulline

Concentrations of $1x10^{-1}$M L-arginine and $1x10^{-2}$M L-citrulline were chosen to investigate the effect of protonation state on redox activity as both molecules demonstrated antioxidant activity at these concentrations (Chapter three).

$1x10^{-1}$M L-arginine exerted both an antioxidant and pro-oxidant effect which was dependent upon pH. At pH 7.0, when the α-amino group of L-arginine is in the NH_3 from, a strong antioxidant effect was seen with 98% of the control being inhibited (the control being Fenton chemistry at pH 7.2) (Figure). When pH was increased to 9.9, the α-amino group was in the NH_2 form and a strong pro-oxidative effect was seen with signal increasing to 81% more than the control (Fenton Chemistry at pH9.6) (Figure).

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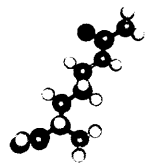
Upon incubation of $1x10^{-2}$M L-citrulline with the Fenton system both an antioxidant and pro-oxidant effect was seen which was again dependent upon pH. At pH 7.1±0.025, when the L-citrulline α-amino group is in the NH_3 from, a strong antioxidant effect was seen with 97% of the control being inhibited (the control being Fenton chemistry at pH 7.2) (Figure). When pH was increased to 10.8±0.05, when the α-amino group is in its NH_2 form, strong pro-oxidative effect was seen with signal increasing to 74.24% more than that control (the control being Fenton Chemistry at pH10.7) (Figure).

69



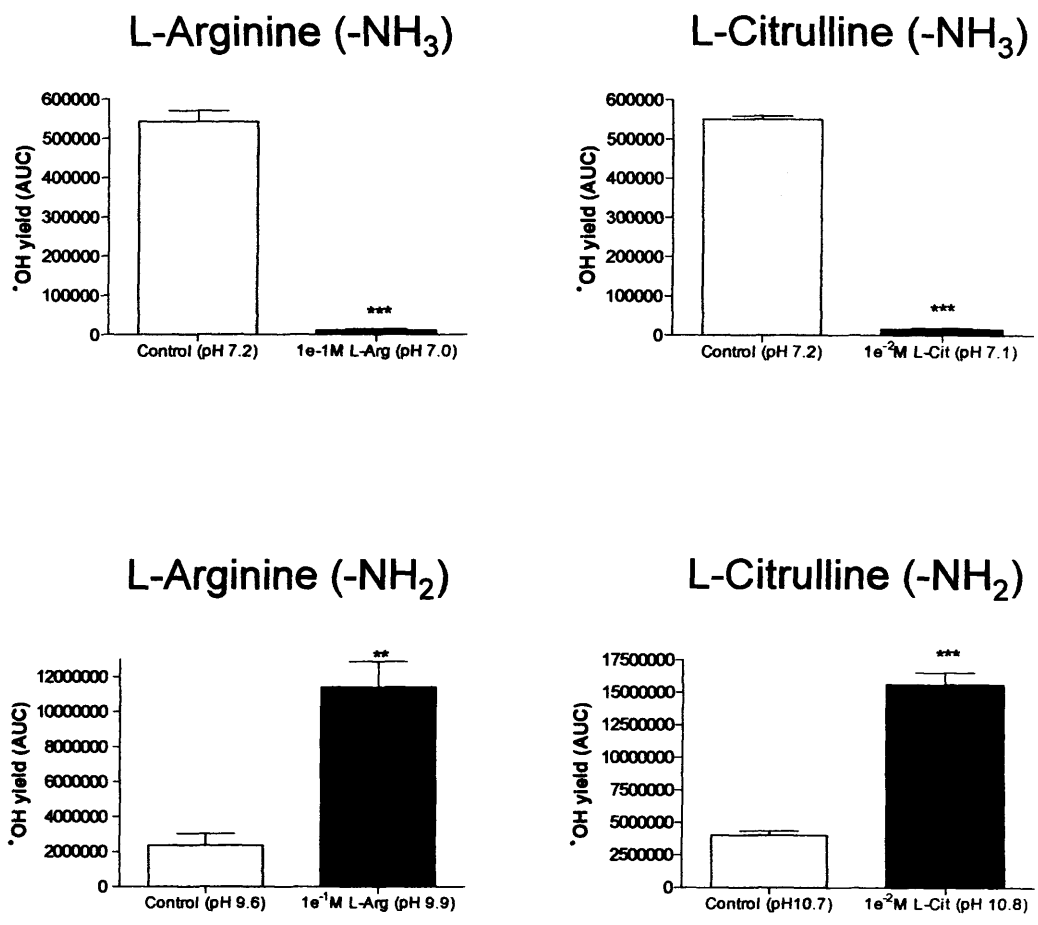


Figure 69 Effect of α-amino group protonation state on the redox activity of L-arginine and L-citrulline. Data expressed as mean±SEM. ** represents p<0.01; * represents p<0.001. n=4-6.**

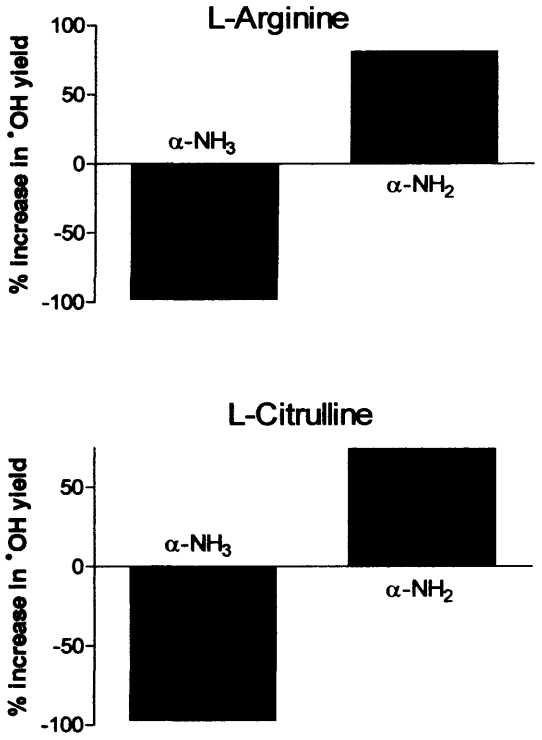
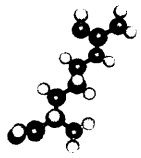


Figure 70 Effect of the protonation state of the α-amino group on the redox chemistry of $1x10^{-1}$M L-arginine and $1x10^{-2}$M L-citrulline from anti-oxidants to pro-oxidants respectively. Data expressed as mean±SEM. n=4-6.



4.3.5 Pro-oxidant effect of L-arginine and L-citrulline

In order to investigate how L-arginine was exerting its pro-oxidant effect, L-arginine was incubated in its NH_3 and NH_2 form with 352.8mM hydrogen peroxide alone. The effect of pH on H_2O_2 alone was run as a control and no significant difference was seen in signal (between pH 7.2 and pH 9.0 ($P>0.05$) (**Figure**).

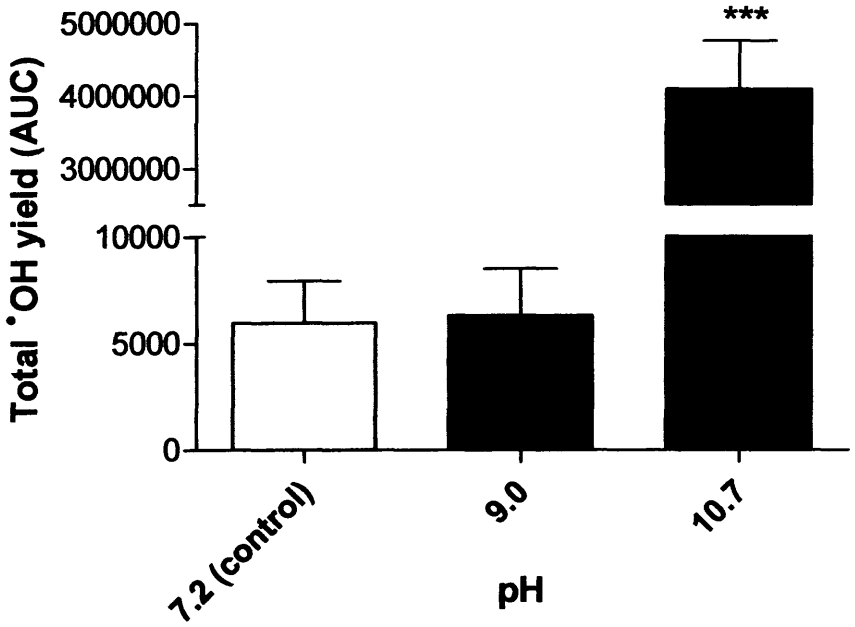


Figure 71 Effect of pH on the baseline signal from 353.8mM hydrogen peroxide Data expressed as mean±SEM n=4-6.

No significant change in signal was seen in the presence of the NH_3 group but the NH_2 group on L-arginine mediated a significant increase in signal (Figure). It was not possible to look at the effect of L-citrulline on hydrogen peroxide as at the pH required to convert the L-citrulline α-amino group into its NH_2 form (pH 10.7), a significant interaction between luminol and hydrogen peroxide was seen ($p<0.001$ cf luminol and hydrogen peroxide at pH 7.2) (Figure).

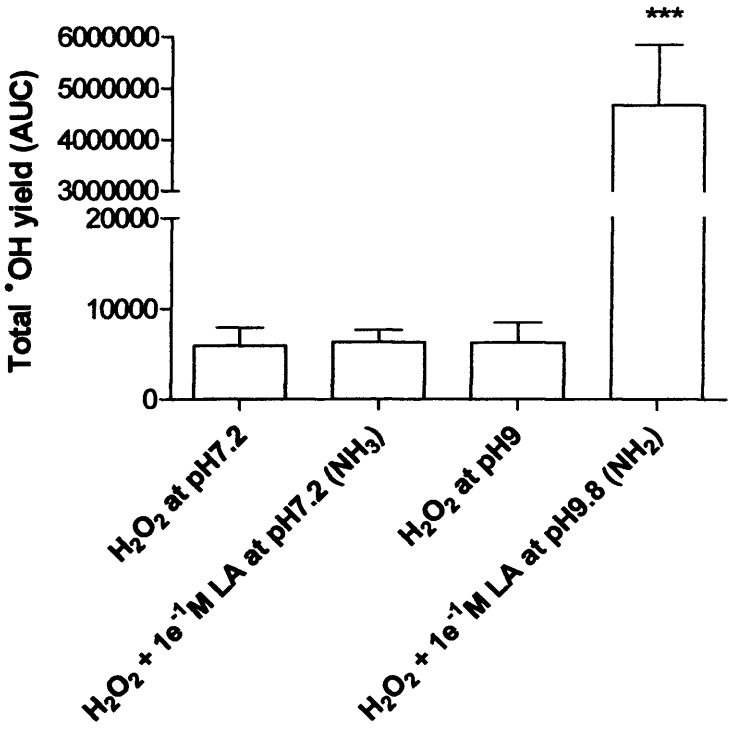


Figure 72 Effect of the α-amino group protonation state of L-arginine (LA) on interaction with hydrogen peroxide * represents p<0.001 compared with all other groups. P>0.05 between all other groups Data expressed as mean±SEM n=6.**

4.4 Discussion

In chapter three the ability of physiological concentrations of L-arginine and L-citrulline to scavenge hydroxyl radicals was established. This chapter follows directly on from that in that it aimed to characterise the exact mechanism by which L-arginine and L-citrulline were exerting their antioxidant action.

There are two main mechanisms by which an antioxidant may reduce hydroxyl radical formation, and in relation to the adapted Fenton system these are:

1. Indirect reduction of hydroxyl radicals via reduction of substrate – copper
2. Direct scavenging of hydroxyl radicals

4.4.1 Indirect reduction of hydroxyl radical levels

The first aim of this chapter was to test the hypothesis that L-arginine and L-citrulline were able to decrease hydroxyl radical production through the reduction of the substrate of Fenton chemistry, copper. The first technique used to test this was ozone based chemiluminescence. This technique allowed measurement of NO release from S-nitrosoglutathione a reaction known to be dependent on the presence of transition metals. NO release from s-nitrosothiols occurs via degradation by heat, u.v. light, pH and transition metal ions [331,337,338]. In the absence of light and transition metals, s-nitrosothiols are stable at physiological pH and temperature. As the system was kept at physiological pH (7.4) and temperature (37°C), it could therefore be said that NO release was proportional to the concentration of transition metal ions present. Injection of 10nmoles and 80nmoles of GSNO into PBS resulted in NO liberation (Figure 60 & Figure 61). In order to test the dependency of transition metal ions on this process, 10nmoles and 80nmoles of GSNO was added to a range of EDTA concentrations resulting in a significant decrease in NO release (Figure 59). The fact that L-arginine and L-citrulline both significantly decreased NO release to a similar

degree to EDTA (Table 24 and Table 25) strongly suggested that both L-arginine and L-citrulline were able to chelate Cu(II). It was possible to ascertain that Cu(II) and not Cu(I) was being chelated based on the following reasons:

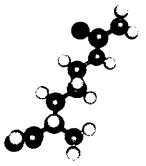
1. Cu(I) does not exist in aqueous solutions as it is rapidly oxidised to Cu(II)
2. Although Cu(II) is not commonly thought to catalyse NO release from GSNO, it has been shown that Cu(II) can catalyse a certain degree of GSNO degradation, and it is simply the formation of GSSG, a metal chelator, that inhibits further NO release. Within this system, it would appear that this NO release is detectable.

$2GSNO + Cu(II) \rightarrow GSSG + Cu(II) + 2NO$ **Equation 33**

3. Ascorbic acid, a reducing agent that has been shown to convert Cu(II) to Cu(I) [174,326,409], significantly increases NO release from GSNO (Figure 60 & Figure). As the increase of NO release is so large (~3000%) one can assume that there is little Cu(I) present in PBS alone

$GSNO + Cu(I) \rightarrow GS^- + NO + Cu(II)$ **Equation 34**

The EPR based assay on the other hand, in which Cu(II) was detected using MGD, showed little Cu(II) chelation, with L-citrulline exerting no effect on Cu(II) levels ($p>0.05$). L-arginine was only effective at supraphysiological concentrations of 1M ($p<0.01$ and $p<0.05$ respectively) (Figure 64). This is in complete contrast to the ozone based chemiluminescence study. One potential explanation for this difference is that in the EPR based study it was assumed that if L-arginine and L-citrulline were bound to copper then it would not be detectable by EPR. However it is possible that even when bound to L-arginine and L-citrulline, copper(II) was still able to interact with MGD and so a signal was still seen. As the chelation of copper by the amino acids would have no

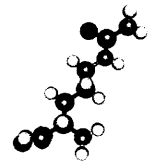


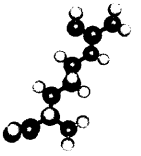
effect on total copper concentration, this may explain why no change in copper levels was seen. Indeed, the literature would support a chelating effect of the amino acids on copper [258] and these results suggested that both L-arginine and L-citrulline have the capability to reduce total hydroxyl radical levels through decreasing their production by Fenton chemistry.

4.4.2 The effect of various structural moieties on redox activity

The next stage of this study was to elucidate the moiety that controlled the antioxidant properties of L-arginine and L-citrulline, be that through a direct or indirect action. Three structurally related compounds were chosen in order to answer this question. glycine was chosen because structurally it is the simplest amino acid and allows the effect of the α-amino group to be studied in isolation. This molecule was chosen as it contains both the α-amino group and carboxylic acid group required for dative binding required for copper chelation. Aminoguanidine was chosen due to its structural similarity to the guanidinium group contained within L-arginine allowing us to investigate the role of this group in the antioxidant properties of L-arginine. As it is lacking in both an α-amino group and carboxylic acid any antioxidant actions seen are independent to copper chelation. Finally the effects of N-α-acetyl-L-arginine were investigated as within this molecule the α-amino group is replaced with an acetyl group, and so allows the effect of the guanidinium group in the absence of the α-amino group to be investigated.

Two main contradictory studies exist on this topic. The first is a study by Wallner *et al* [379] who attributed the antioxidant action of L-arginine to the α-amino group based on the fact that N-α-acetyl-L-arginine and the guanidinium group, involved in NO synthesis, had no antioxidant activity and glycine did. The second was carried out by Lass *et al* [199] who attributed the antioxidant capacity of L-arginine to its guanidinium group due to its ability to scavenge superoxide and did not support a radical scavenging activity of glycine. Both of these studies focused on the superoxide radical and so care must be taken when drawing comparisons.



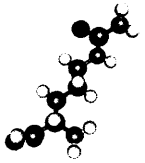


4.4.2.1 Antioxidant properties of L-arginine

These results demonstrated that both glycine and aminoguanidine were capable of reducing hydroxyl radical levels but that N-α-acetyl-L-arginine was not (Figure 65). This suggested that in theory, both the alpha amino group and the guanidinium group were capable of conferring antioxidant activity to L-arginine. Another interesting finding was the fact that although only the alpha amino group is replaced in N-α-acetyl-L-arginine and the guanidinium group remains unchanged, all antioxidant activity was inhibited (Figure 65). As aminoguanidine was demonstrated to be an effective hydroxyl radical scavenger, this would suggest that although aminoguanidine is an antioxidant, for some reason when bound in to a molecule, in this case L-arginine, it loses all antioxidant ability.

4.4.2.2 Antioxidant properties of L-citrulline

It was more difficult to determine the mechanism of action of L-citrulline, as it was not possible to isolate the functional groups contained within this amino acid. Based on the results obtained for L-arginine that assigned the α-amino group as the antioxidant moiety, and the fact that L-citrulline also had an almost identical redox profile to glycine (Figure 65), it is likely that L-citrulline redox activity was also controlled by the α-amino group. Only one other study has looked at the mechanism of antioxidant action of L-citrulline and that is a study by Akashi *et al* [8] that used the techniques of HPLC and LCMS to propose two mechanism of action: 1) abstraction of a hydrogen ion from the α-carbon forming a carbon centred radical as an intermediate 2) side chain attach by the hydroxyl radical, resulting in decomposition to several radical derivatives and eventual formation of unknown secondary products of various molecular weights via polymerisation and/or condensation reactions. Although the results presented herein support a role for the α-amino group it does not rule out a possible interaction of the α-carbon or side chains of either L-citrulline with the hydroxyl



radical. Indeed, further studies investigating the actual products of these interactions would no doubt shed further light onto this topic.

This study strongly suggests that the α-amino group is responsible for the antioxidant activity of L-arginine and L-citrulline; however it offers no evidence pertaining to how this structural group is exerting its antioxidant effect as it supports both a direct and indirect interaction with the hydroxyl radical. In terms of copper chelation, amino acids form a chelate with copper through dative bonding between the α-amino and carboxylate groups (Figure 56). Removal of the α-amino group would therefore potentially remove all copper chelating ability and potentially all antioxidant capacity. Alternatively, the structure of the α-amino group suggests hydroxyl radical levels may be reduced through a direct interaction of the α-amino group with the hydroxyl radicals via donation of a hydrogen ion and therefore removal of this group would remove antioxidant capacity. The following section discusses the potential of the α-amino group to directly interact with hydroxyl radicals.

4.4.3 Direct hydroxyl radical scavenging - Effect of protonation state of the α-amino group on antioxidant activity

As mentioned previously the most likely mechanism of action for direct interaction between the α-amino group and hydroxyl radical is via the donation of a hydrogen ion from the protonated form of the α-amino group (Figure 73).

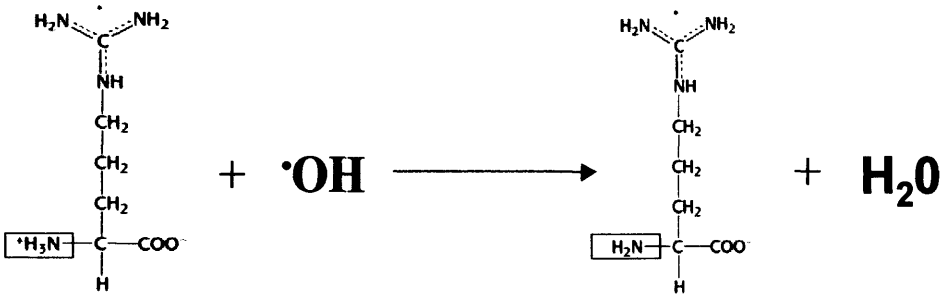
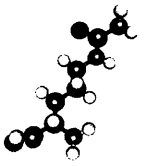
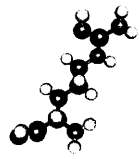


Figure 73 Hypothesis reaction between hydroxyl radical and L-arginine.





To test this hypothesis, the effect of pH on the α-amino group was investigated. By analysing the pKa values of the subgroups of L-arginine and L-citrulline, it was rationalised that pH could be used to modify the protonation state i.e. the number of hydrogen ions attached to the α-amino group, thus allowing us to further analyse the role of this group in the control of antioxidant function. By converting the α-amino group from $-NH_3$ to $-NH_2$, and analysing the effect this has on redox activity, it was possible to assign a mechanistic action to this group. Analysis of both L-arginine and L-citrulline pKa values showed that the α-amino group had a pKa of 8.99 and 9.69 respectively. Therefore by altering the pH to values either side of this value, the α-amino acid was predominantly in either in its NH_2 or NH_3 state. The theory being tested was that antioxidant activity would be lost when NH_3 was converted into NH_2. Also as conversion of the α-amino group from the protonated to the un-protonated form was not expected to affect the chelating abilities of the amino acids, any change in antioxidant capacity could be attributed to a 'chelation' independent effect i.e. a direct interaction between the α-amino group and the hydroxyl radical.

At neutral pH when the α-amino group of the amino acids were in the NH_3 from, a strong antioxidant effect was seen with 98% and 97% of the control being inhibited for L-arginine and L-citrulline respectively (Figure). At alkaline pH, when the α-amino group was in the NH_2 form, not only was antioxidant activity abolished, but a strong pro-oxidative effect was seen with signal increasing to 81% and 74.% above the control for L-arginine and L-citrulline respectively (Figure). These results strongly support the hypothesis that both L-arginine and L-citrulline are able to directly interact with hydroxyl radicals via the α-amino group. As the protonation state should not affect the ability of the amino acids to chelate copper, the reduction in antioxidant capacity can only be attributable to a direct interaction between the α-amino group and hydroxyl radical as illustrated in Figure . It does however raise the issue of why, if the chelating abilities of the amino acids remained unchanged, all antioxidant activity was lost. Based on the assumption that both direct and indirect hydroxyl radical interactions are occurring, then the loss of one should not completely remove all antioxidant

properties from the amino acids, merely reduce total antioxidant capacity. Why a complete loss of all hydroxyl radical scavenging capacity was lost is unclear. It may be that copper chelation only plays a small part in the observed reduction in hydroxyl radicals or that the change in pH effects copper chelation in unforeseen ways. Determining the precise mechanism of action by which L-arginine reduce hydroxyl radical levels would appear more difficult than first anticipated.

4.4.4 Pro-oxidative effect of L-arginine and L-citrulline

In chapter three it was demonstrated that L-arginine and L-citrulline have the ability to act as pro-oxidants under certain experimental conditions and based on this, an investigation to elucidate the moiety that controls this pro-oxidant action was carried out. $1x10^{-4}$M glycine, but not aminoguanidine or N-α-acetyl-L-arginine exhibited an identical pro-oxidant response as L-arginine and L-citrulline (Figure 65). This shows that the pro-oxidant effect is mediated via the alpha amino group. As previously discussed, conversion of the α-amino group into its unprotonated NH_2 form converts both amino acids from antioxidants into pro-oxidants (Figure 69) It is unlikely that this 'switch' from NH_3 to NH_2 would occur under physiological conditions due to the high pKa value for these groups. However, following the reaction scheme depicted in Figure , it is possible that at certain concentrations of hydroxyl radicals and amino acid, the formation of the de-protonated, NH_2 form of the amino acids is sufficient that pro-oxidative activity outweighs antioxidant activity. This may explain the pro-oxidative effect witnessed with both amino acids in chapter three.

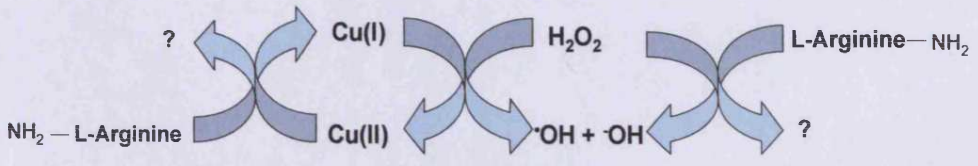


Figure 74 Proposed mechanism for the pro-oxidant activity demonstrated by L-arginine and L-citrulline.

Within the system used in this assay, the only potential pro-oxidant mechanisms

are interaction of the amino acids with hydrogen peroxide or Cu(II). To test this L-arginine was incubated in its NH_3 (pH 7.0±0.16) and NH_2 form (9.9±0.08) with hydrogen peroxide alone. No significant change in signal was seen with the deprotonated NH_2 group but the NH_3 group mediated a significant increase in signal (Figure 69). It would appear that L-arginine is able to directly interact with hydrogen peroxide to produce an oxidising species, possibly the hydroxyl radical. The mechanism by which L-arginine is converting hydrogen peroxide into a radical is still unclear, but the fact that only the α-amino group is changing between pH 7.2 and pH 9.8 suggests that this moiety is involved.

The interaction with hydrogen peroxide does not fully account for the increase in hydroxyl radical levels seen. The interaction with hydrogen peroxide accounts for 59% of the signal increase (Figure 70 vs Figure 71) but the mechanism by which the signal is further increased remains unresolved.

It was not possible to investigate at the effect of L-citrulline on hydrogen peroxide as at the pH required to convert the α-amino group into its $-NH_2$ form (pH 10.7), a significant interaction between luminol and hydrogen peroxide was seen (Figure) ($p<0.001$ cf luminol and hydrogen peroxide at pH 7.2). One would assume however that as L-arginine and L-citrulline have displayed almost identical redox profiles in all other aspects, this would remain true in relation to the interaction with hydrogen peroxide. Indeed, the results from this chapter would suggest that all amino acids would exert some degree of antioxidant/pro-oxidant activity as the α-amino group is integral to all amino acid structures.

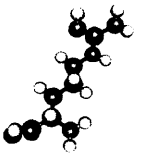
4.4.5 Conclusions

The results herein demonstrate that the antioxidant activity seen with both L-arginine and L-citrulline is mediated through both a direct and indirect interaction with the hydroxyl radical. It would appear that the α-amino group in its protonated NH_3 form is responsible for controlling the redox activity of both amino acids through two mechanisms:

1. Inhibition of production through the Fenton reaction via chelation of copper

2. Direct scavenging of hydroxyl radicals through the donation of a hydrogen ion to form water.

The relative importance of each interaction remains uncertain, however one can assume that if the concentration of amino acid exceeds that of copper, few hydroxyl radicals would be synthesised. However, if the concentration of copper greatly exceeds the local concentration of amino acid then the direct antioxidant actions of L-arginine and L-citrulline would come into play. In conclusion, this study is the first to characterise the antioxidant mechanism for both L-arginine and L-citrulline against hydroxyl radicals and the first to demonstrate that chelation of copper can potentially lead to decreased levels of oxidative stress under certain conditions.

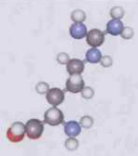


4.5 Summary

- L-arginine and L-citrulline reduce hydroxyl radical levels via a direct and indirect action.
    - Both amino acids are capable of chelating the substrate for hydroxyl radical production, copper (II).
    - L-arginine and L-citrulline can directly interact with the hydroxyl radicals via the α-amino group in its protonated NH_3 state leading to the formation of a non-toxic product, most likely water.

- Pro-oxidant activity is also controlled via the α-amino group in its non-protonated NH_2 state.

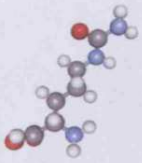
- Pro-oxidative activity is, in part, mediated via an interaction with hydrogen peroxide to form a luminol detectable radical, most likely the hydroxyl radical.



Ex Vivo studies

Effects on endogenous ROS

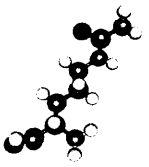
Chapter Five



5.1 Introduction

This thesis has so far focused on defining and characterising the antioxidant properties of L-arginine and L-citrulline in a series of *in vitro* systems. This chapter aimed to clarify whether the antioxidant properties previously demonstrated by L-Arginine and L-Citrulline were relevant in a cellular system. To investigate this a platelet model was chosen as platelets are known to synthesise ROS under normal conditions and it has been shown that ROS levels increase upon activation by agonists such as collagen [46,186,284,376]. These endogenous ROS are thought to be involved in platelet activation and aggregation and so a platelet model provided an ideal system to investigate the antioxidant activity of both L-arginine and L-citrulline in a physiologically relevant system. Indeed several studies have been carried out investigating potential antioxidants and their effect on oxidative stress in platelets [268-271,414].

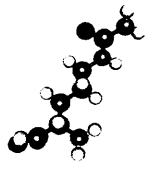
To date there have been several studies investigating the effects of L-arginine on platelets. It has been hypothesised that in human platelets extracellular L-arginine modulates intracellular NO synthesis by providing a substrate for NOS found in platelets [2,11,230,268,293,398]. This hypothesis is based on the fact that upon collagen induced platelet activation, NO production is increased [34,104,197,230] through stimulations of extracellular L-arginine uptake through y^+ [371] and y^+L [240] transporters located in the platelet membrane. This mechanism is thought to represent a negative feedback loop preventing hyperaggregation. The fact that L-arginine has been shown to increase platelet NO production [2,11,230,268,293,398], which is well known to effect platelet aggregation, meant that great care was taken to limit the effect of NO on this study. Although there is significant evidence to suggest that L-arginine is operating through NO, this does not rule out an antioxidant role for L-arginine and indeed a large section of this chapter sets about distinguishing between an NO dependent and independent effect. It is important to reiterate that the aim of this chapter was not to fully elucidate the mechanism by which L-arginine inhibits platelet aggregation, as that would be a



project worthy of a thesis in itself. The aim of this body of work was to assess whether the antioxidant properties demonstrated by L-arginine and L-citrulline in the previous two chapters had any function in a biological system.

5.2 Aims

- To establish a bioassay that allows the anti-aggregatory effects of L-arginine and L-citrulline to be measured.
- To identify whether L-arginine and L-citrulline are working via an antioxidant action by:
  - Ruling out NO synthesis through the use of D-arginine, the inactive enantiomer of L-arginine
  - Comparing the effects of both amino acids to the well known antioxidant ascorbic acid
  - Comparing the effects of both amino acids to specific radical scavengers, superoxide dismutase, catalase and mannitol.



5.3 Materials and Methods

5.3.1 Platelet aggregation

Blood was taken by standard venepuncture from the antecubital vein using the anticoagulant trisodium citrate (3.8%), 1:9 v/v (Sodium Citrate : Blood). Blood was then centrifuged at 150g and 4 ^{0}C for 10 minutes and platelet rich plasma (PRP) kept at 37 ^{0}C (i.e. body temperature – to return platelet metabolism to normal rates after cooling).

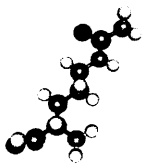
Platelet aggregation was measured using a dual channel aggregometer (Chrono-log, Model 560). Electrodes were calibrated before each measurement and aggregation consequently measured for 10 minutes at 37°C with continuous stirring at 1000rpm. The effect of a range of compounds, L-arginine, L-citrulline, D-arginine, Ascorbic acid, superoxide dismutase, catalase and mannitol on collagen induced platelet aggregation were analysed (see Table 29 for exact concentrations) following the methodology described in chapter two.

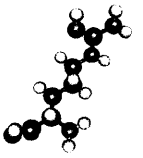
Table 29 Concentration of test compounds in platelet aggregation assay

Test Compound	Final concentration
L-arginine	$1x10^{-4}$M – 1M
D-arginine	$1x10^{-4}$M – 1M
L-citrulline	$1x10^{-3}$M - $1.6x10^{-1}$M
Ascorbic acid	$1x10^{-3}$M – 1M
Mannitol	$1x10^{-3}$M – $5x10^{-1}$M
Superoxide dismutase	10, 100 & 500 Units
Catalase	100, 1000 & 2000 Units

5.3.1.1 Optimum amino acid incubation time

In order to investigate the effects of L-arginine and L-citrulline on collagen induced aggregation, an appropriate incubation period was required. A study by Anfossi *et al* [11] demonstrated that collagen-induced aggregation was decreased after only a 10 minute incubation with 0.1mM-6mM L-arginine. Preliminary





studies demonstrated no significant difference in the inhibitory effect of the amino acids ($1x10^{-1}M$) after incubation with PRP for 1, 5, 10, 20, 30 and 60 minutes (data not shown). Taking into consideration that platelets are only viable for a limited time (all studies were performed within two hours of obtaining blood) a 10-minute incubation time was chosen. As a control samples were incubated with PBS in place of amino acids.

5.3.1.2 Characterisation of the antioxidant properties of D-arginine

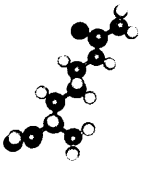
In order to characterise the antioxidant properties of D-arginine, the luminol based chemiluminescence assay used in chapter three was utilised. D-arginine was tested against the lowest and highest concentration of hydroxyl radical (Table 30) described in chapter two and three.

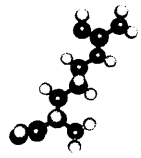
Table 30 Concentration of copper sulphate and hydrogen peroxide found in fenton chemistry systems 1 and 4.

System	Concentration of copper sulphate	Concentration of hydrogen peroxide
1	4.5µM	44.1mM
4	36µM	352.8mM

The effect of D-arginine on hydroxyl radicals was measured via chemiluminescence utilising a luminol probe as previously described in chapter two. The antioxidant capacity of D-arginine was recorded in the presence (AUC_{AA}) and absence ($AUC_{Control}$) of the amino acid, and inhibition of control (I) was calculated as:

$I=((AUC_{control} - AUC_{AA}) / AUC_{control}) * 100.$





5.3.2 Detection of ROS in Platelet Rich Plasma

Blood was taken as previously described, separated into PRP stored at room temperature. A round-bottomed glass tube was then coated with $5x10^{-5}$moles of PBN and a total volume of 500µL added to the tube to give a final concentration of 100mM PBN. The composition of the 500µL varied as described in Table 15. All solutions were vortexed to ensure full mixing of PBN with solution. Samples were placed in a quartz glass cell and EPR spectra recorded at room temperature using a Varian 104 EPR spectrometer.

The instrument settings were as follows; Gain 8x10, modulation 1.6 Gauss, time constant 0.128sec, gauss scan width 200Gauss, power 5mW. Samples were run for 10 minutes (10 x 1 minute scans), with no initial incubation period.

5.3.3 Statistical Analysis

Data expressed as mean ± SEM. One way analysis of variance with a Bonferroni *post hoc* test were used to calculate significance which was considered at $p<0.05$ unless otherwise stated.

5.4 Results

5.4.1 Development of a collagen induced platelet aggregation bioassay

5.4.1.1 Optimising collagen concentration

In order to initiate platelet aggregation, a study into finding the optimum concentration of collagen was undertaken (Figure 75). No response was seen at concentrations less than 1µg/ml and only modest changes in aggregation were seen between concentrations higher than 1µg/ml. For these reasons a final concentration of 1µg/ml collagen was chosen for use in all other aggregation experiments.

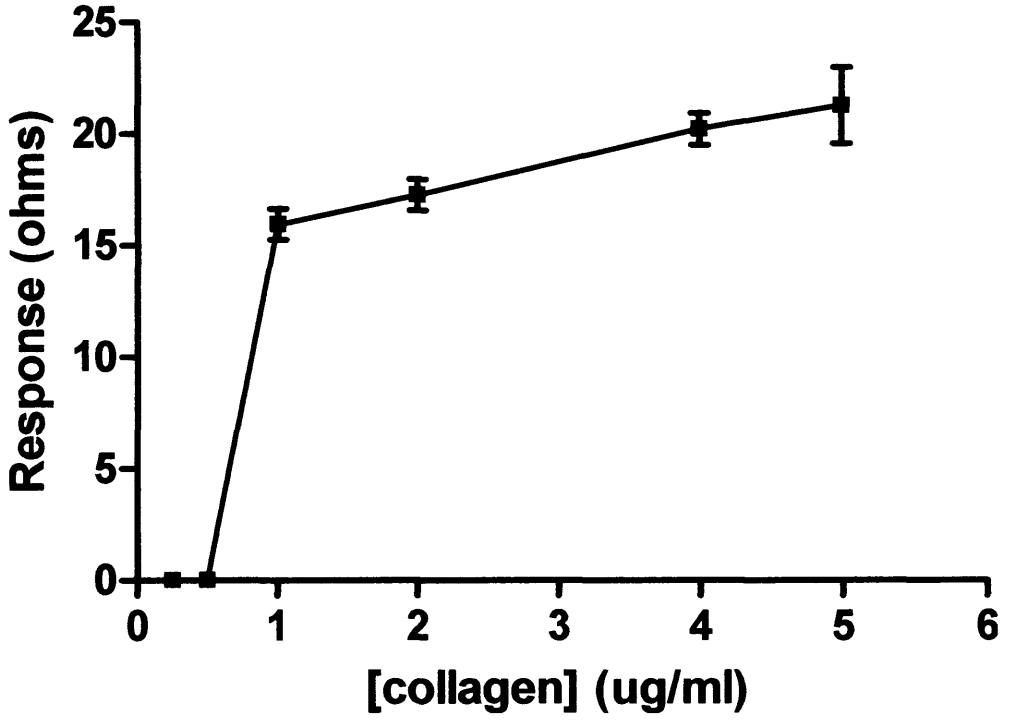
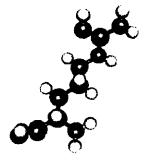


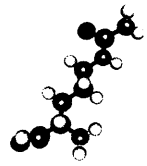
Figure 75 Collagen concentration curve in PRP over 10 minutes. n=8



5.4.2 Effect of L-arginine, L-citrulline and D-arginine on collagen induced platelet aggregation

Upon incubation of PRP with L-arginine and L-citrulline a significant decrease in the degree of collagen induced aggregation was observed. This inhibition was only seen with supraphysiological concentrations of L-arginine and L-citrulline (EC_{50} values of 15.7mM &128mM respectively) (Figure).

In order to rule out the involvement of NO in the anti-aggregatory effects of L-arginine, the inactive enantiomer, D-arginine was also tested. Its inability to act as a substrate for NOS and the fact that it possesses a similar antioxidant profile to L-arginine (Table 31) enabled NO-dependence or independence to be elucidated. Although not as effective as L-arginine at inhibiting aggregation, D-arginine did exert an anti-aggregatory effect (EC_{50}=72.77mM).



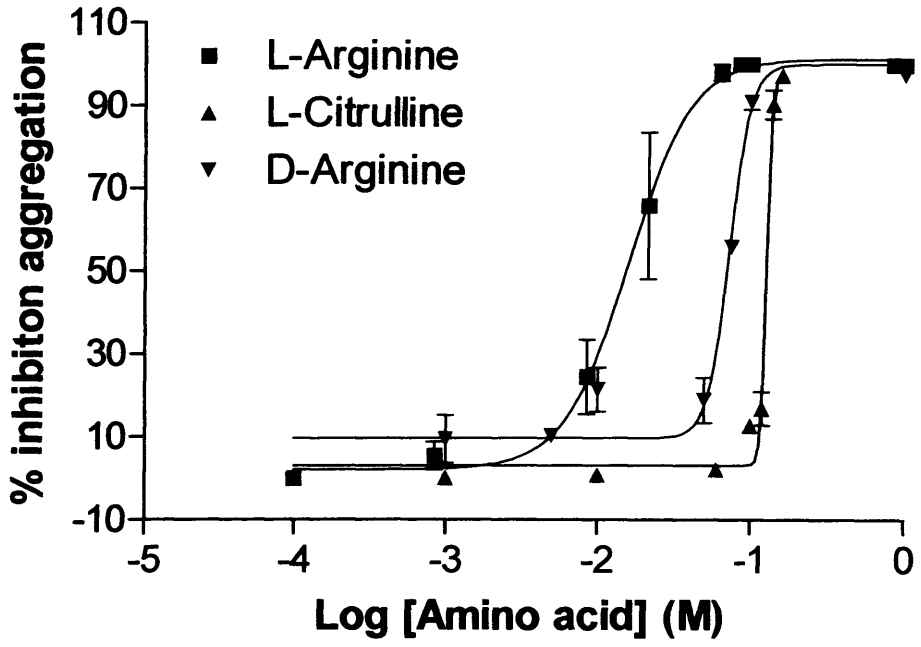


Figure 76 Effect of L-arginine, L-citrulline and D-arginine on 1µg/ml collagen induced aggregation n=4-7

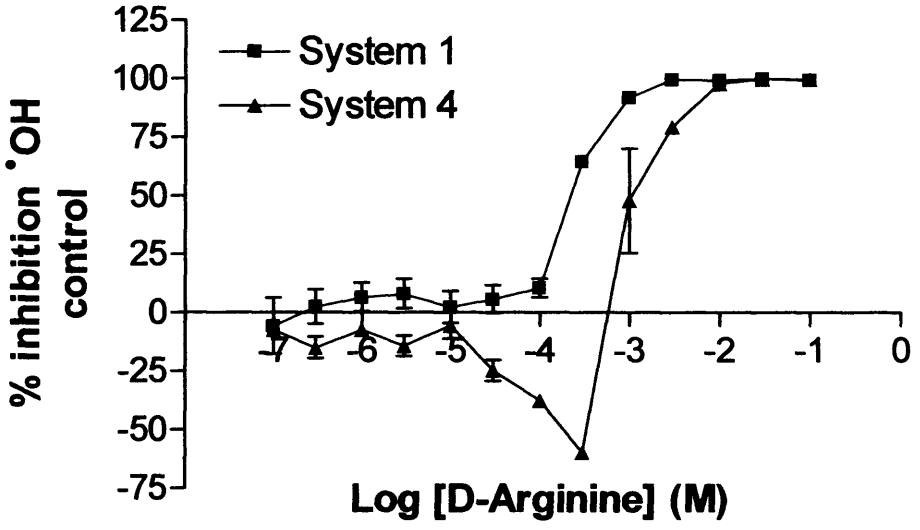


Figure 77 Antioxidant properties of D-arginine against hydroxyl radicals. System 1 represents 4.5µM copper Sulphate and 44.1mM hydrogen peroxide. System 4 represents 36µM copper Sulphate and 352.8mM hydrogen peroxide n=9

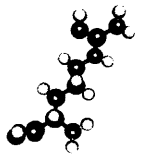


Table 31 EC_{50} values for L-arginine, L-citrulline and D-arginine against a range of hydroxyl radical concentrations. (L-arginine and L-citrulline values taken from chapter three).

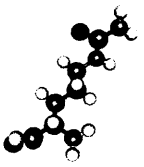
Amino Acid	System 1	System 4
L-arginine	375.6μM	1363μM
L-citrulline	226.3μM	1038μM
D-arginine	242.1μM	962.2μM

Due to supraphysiological concentrations of amino acids required to inhibit collagen induced aggregation, an investigation into the whether this effect was mediated through an antioxidant action was carried out.

5.4.3 Detection of ROS from stimulated and unstimulated platelets

In order to measure radical production from collagen induced aggregation EPR was used. As described in chapter two, this allows specific detection of low levels of ROS. The spin trap PBN was chosen due to its lipid soluble properties that would allow entry into the platelets. Platelet rich plasma was used in order to mimic the conditions in the previously described aggregation studies.
Initially platelets were stimulated with the same concentration of collagen used in the aggregation studies (1μg/ml), however no ROS could be detected upon stimulation. The concentration of collagen was therefore increased to 10μg/ml in an attempt to increase the ROS synthesis to detectable levels. However even at 10μg/ml no ROS were detected.

PBN is well known to preferentially bind carbon centred radicals over oxygen centred radicals and so DSMO was added to the solution to pre-emptively convert oxygen radicals produced into carbon centred radicals. Once again no radicals were detected (Figure).
78



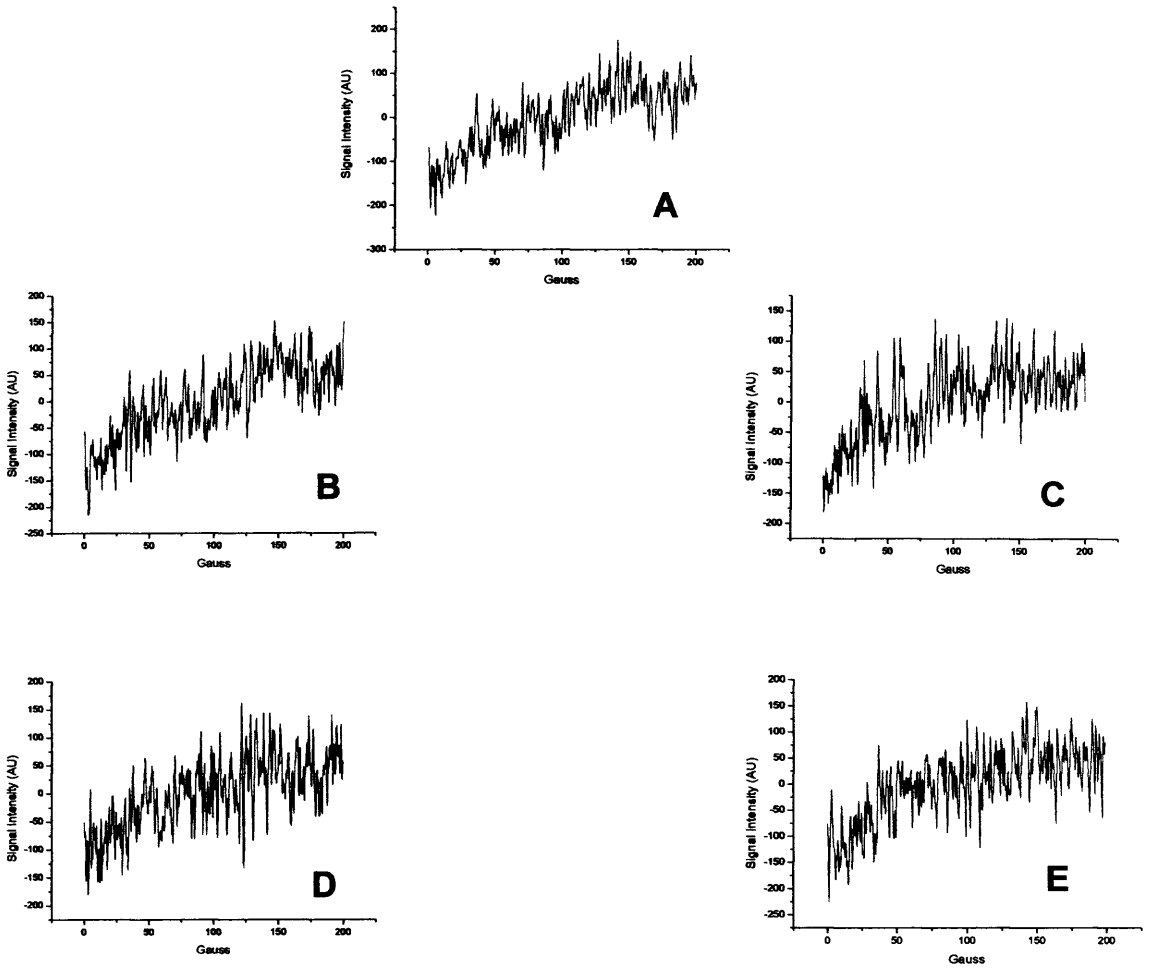


Figure 78 Detection of radicals in PRP by EPR. No radicals were seen under any conditions. A: Control (PBS + PBN only) B: ROS in unstimulated platelets C: ROS in 10μg/ml collagen stimulated platelets D: Carbon radicals in unstimulated platelets D: Carbon radicals in 10μg/ml collagen stimulated platelets.

5.4.4 Effect of Ascorbic acid on platelet aggregation

In order to investigate the effects of a well-known antioxidant on platelet aggregation, ascorbic acid was chosen. As with L-arginine and L-citrulline, ascorbic acid exerted an anti-aggregatory effect but only at supraphysiological concentrations (EC_{50}=63.2mM) (Figure 79). This does however suggest a role for free radical scavenging in the modulation of platelet aggregation.

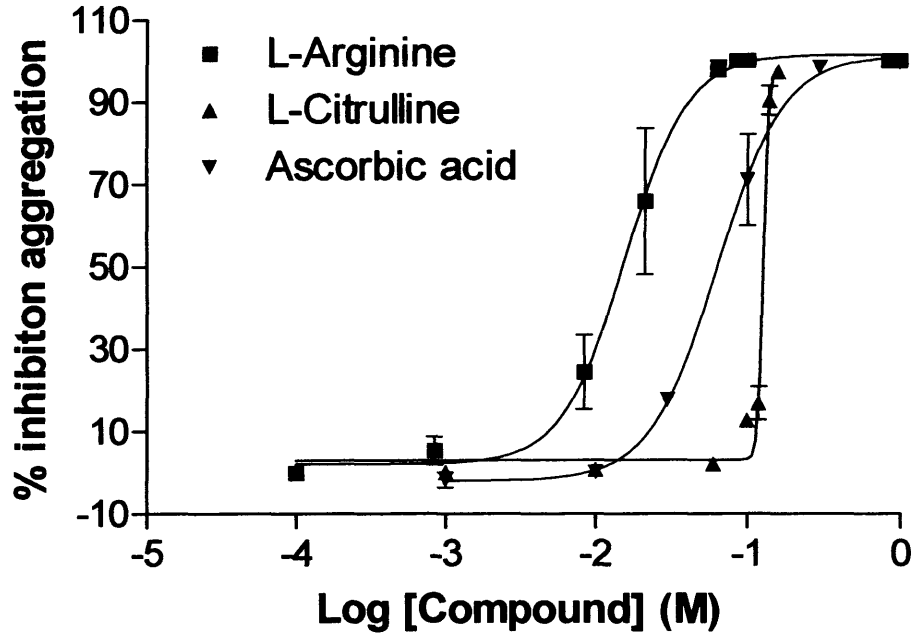
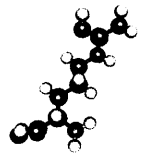


Figure 79 Effect of L-arginine, L-citrulline and ascorbic acid on 1µg/ml collagen induced platelet aggregation. n=4-7



5.4.5 Effect of SOD, catalase and mannitol on platelet aggregation.

In order to investigate the role of individual radical types on collagen induced aggregation and to further investigate the effect of scavenging free radicals, SOD (a superoxide scavenger), catalase (a hydrogen peroxide scavenger) and mannitol (a widely used hydroxyl radical scavenger) were incubated with PRP. Neither SOD nor catalase had any significant effect on collagen induced platelet aggregation ($p>0.05$ compared with control) (Figure).

Mannitol exerted a similar anti-aggregatory profile to that seen with the amino acids and ascorbic acid (Figure). The EC_{50} value for mannitol was 98.27mM.

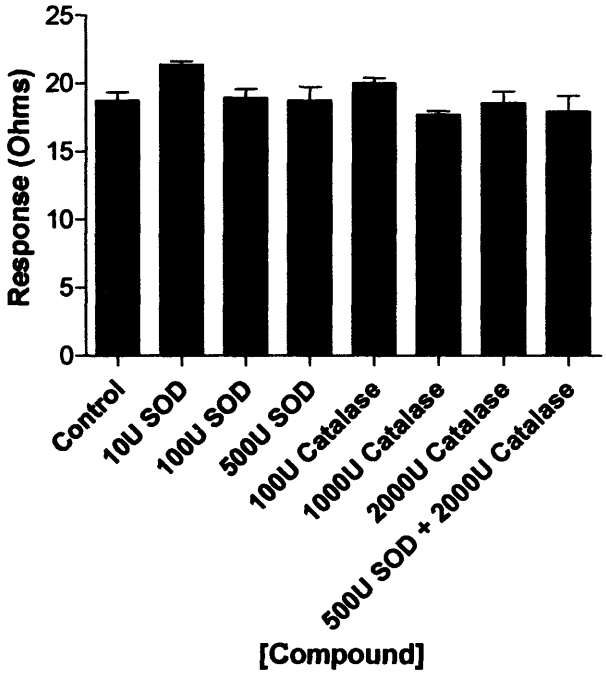


Figure 80 Effect of SOD and catalase on collagen induced platelet aggregation. For control n=16. For all other treatment groups n=4. p>0.05 for all groups cf control.

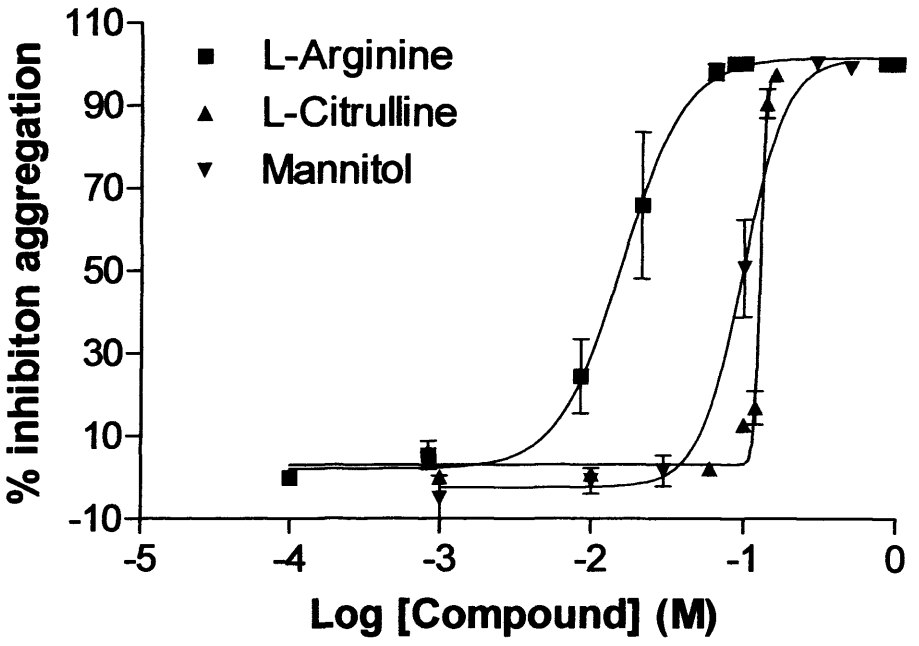
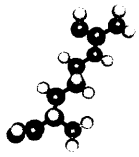


Figure 81 Effect of L-arginine, L-citrulline and mannitol on collagen induced platelet aggregation. Data expressed as mean ± SEM. n=4-7



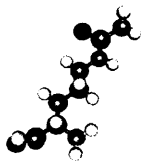
5.5 Discussion

The aim of this chapter was to establish a bioassay capable of elucidating the physiological relevance of the previously demonstrated antioxidant properties of L-arginine and L-citrulline (chapters three and four) i.e. are the amino acids able to bring about physiological change through the scavenging of ROS? To answer this question a platelet model was chosen based on the following:

1. Activation of platelets by agonists such as collagen results in increased production of ROS including superoxide, hydroxyl radicals and hydrogen peroxide [46,186,284,376].
2. Endogenous and exogenous ROS modulate platelet function including aggregation [98,160,208,311] [159,372] [80,151,266].
3. Aggregation can be easily measured using the technique of platelet aggregometry.
4. Numerous studies have demonstrated the ability of antioxidants such as ascorbic acid and α-tocopherol to modulate platelet function via scavenging of ROS [1,192,268,373].
5. The inhibitory effects of L-arginine on platelet function are well described [2,11,230,268,293,398] suggesting that this amino acid is able to modulate platelet function potentially via an antioxidative action.

5.5.1 Assay development

Collagen was the agonist of choice based on studies showing that stimulation of platelets with collagen results in increased levels of superoxide, hydroxyl radicals and hydrogen peroxide [46,186,284,376], a factor vital in an assay designed to investigate antioxidant function. In order to find the optimum concentration of collagen to initiate aggregation, a range of concentrations were tested (0.25,0.5,1,2,4,5 μg/ml). 1μg/ml was chosen as lower concentrations failed to initiate aggregation and little increase in aggregation was seen at higher



concentrations.

An incubation time of 10 minutes was chosen initially based on findings in a study by Anfossi *et al* ([11] who demonstrated that collagen induced aggregation was decreased after only 10 minutes with L-arginine. Preliminary studies supported this as no change in the inhibitory aggregation effect of L-arginine and L-citrulline was seen with varying incubation times (1,5,10,20,30 & 60 minutes) prior to stimulation with 1μg/ml collagen.

5.5.2 Effect of L-arginine and L-citrulline on platelet aggregation

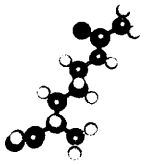
5.5.2.1 Comparisons to other studies

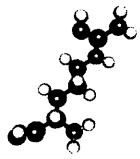
L-arginine and L-citrulline only exerted an anti-aggregatory effect at millimolar concentrations (EC_{50} values of 15.7mM and 128mM respectively) (Figure). This was unexpected because although no work has been carried out investigating the effects of L-citrulline, several studies have demonstrated an anti-aggregatory effect at physiological concentrations of L-arginine [2,11,398]. Indeed the premise of this study was that platelets synthesise ROS which modulate platelet function and as L-arginine has been shown to inhibit platelet aggregation at physiological concentrations this may be mediated via an antioxidant action.

A probable explanation for such a difference between published results and the results within this chapter are that different test conditions were employed including:

1. Choice of anticoagulant

   Anticoagulants vary in their mechanism of action. For example hirudin inhibits coagulation via inhibition of thrombin and citrate inhibits coagulation through the chelation of calcium. Citrate was chosen as the agonist of choice in this assay based on numerous other studies that also use citrate in aggregation studies.



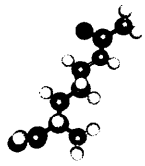


2. Use of whole blood, PRP or washed platelets

   Different published studies have employed the use of different types of platelet suspensions including whole blood, platelet rich plasma and washed platelets re-suspended in a variety of buffers including modified Tyrodes buffer [268,270] , PBS [197] and Ca^{2+} free HEPES buffer ([107,335]. PRP was chosen for this study as it allows the effect of the amino acids to be investigated in a more physiological setting. Whole blood was avoided because results can be biased through interactions of test compounds with other cell types such as neutrophils and red blood cells.

3. Route of L-arginine administration e.g. intravenous/oral vs. addition after blood is taken.

   Although the administration of oral or i.v. L-arginine may be more clinically relevant it makes it more difficult to assign a mechanism of action to L-arginine. As the substrate for NOS, L-arginine could be inhibiting platelet aggregation through increasing endothelial NO production.



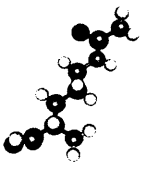
5.5.3 Hypothesis for a two fold mechanism of action for L-arginine

Two studies exist that closely mimic the experimental conditions employed within this assay however these present opposite findings. Anfossi *et al* [11] demonstrated that L-arginine was able to inhibit platelet aggregation in citrated PRP stimulated with 8µg/ml collagen. At 100µM a 10% decrease in aggregation was seen, at 500µM a 23% decrease and at 1mM a 31% decrease was observed. This was concomitant with an increase in cGMP levels suggesting an NO dependent role. This is in contrast to the results obtained herein in which no anti-aggregatory effect was seen at these concentrations.

In a separate study by Bode-Boger *et al* [34], L-arginine in citrated PRP had no effect on 1µg/ml collagen-induced platelet aggregation. This correlates with the results from this study where at a concentration of 2.5mM and less no anti-aggregatory effect was seen. Of particular interest in the study by Bode-Boger *et al* was that when hirudin, an anticoagulant that does not effect extracellular calcium levels, was used, L-arginine exerted an anti-aggregatory effect. This suggests that the anti-coagulant used has a strong effect on the effect of L-arginine, and the differences seen between citrate and hirudin would suggest that the inhibitory action of L-arginine is dependent on calcium.

This theory correlates with the results obtained in this study if it is assumed that the mechanism by which L-arginine inhibits platelet aggregation is two fold.:

1. At physiological concentrations of L-arginine:
   Stimulation by collagen leads to an increase in intracellular calcium resulting in NOS stimulation and therefore an increase in NO production. In the presence of exogenous L-arginine NO synthesis is increased leading to a concentration dependent disaggregation. This has been demonstrated in several studies that show administration of L-arginine results in an increase in cGMP levels [2,11,34,126].



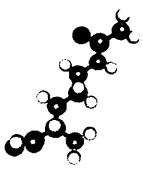
2. At supraphysiologcal concentrations of L-arginine
   As well as increasing NO production L-arginine is able to scavenge ROS produced upon platelet activation leading to further disaggregation.

5.5.3.1 NO dependent inhibition of platelet aggregation

In this system physiological levels of amino acids were unable to inhibit platelet aggregation suggesting the NO dependent inhibition of aggregation was absent, or at least was not enhanced further. As previously mentioned, the production of NO within platelets is thought to be calcium dependent [293]. As the use of citrate as an anticoagulant chelates extracellular calcium, NOS activation may be decreased leading to reduced NO production. Increasing L-arginine concentration therefore has no effect on aggregation because the inhibition of NOS activity means that excess L-arginine is not utilised as a substrate. This may explain why only millimolar concentrations of L-arginine exerted an anti-aggregatory effect as in this system the NO component has been knocked out and L-arginine can therefore only function in an antioxidant capacity.

This may also explain why in the two studies previously discussed (Bode-Boger *et al* [34] vs Anfossi *et al* [11] such different results were seen upon administration of L-arginine. Although both studies used citrate as the anticoagulant, the study carried out by Anfossi *et al* used eight fold higher concentrations of collagen than both this study and the study by Bode-Boger *et al*. Collagen stimulates calcium release from both extracellular (70%) and intracellular (30%) stores. In a citrated sample, the presence of higher concentrations of collagen could lead to up to eight times more calcium being released from intracellular stores. This may be sufficient to stimulate NOS and so exogenous L-arginine will be utilised to synthesise sufficient levels of NO to inhibit platelet aggregation. This may explain why Anfossi *et al* saw an inhibitory effect with micromolar amounts of L-arginine.

As the aim of the chapter was to assess antioxidant capacity in a biological system, the fact that the NO component is removed allows clearer analysis of the



results and suggests that the antiaggregatory effects observed in this study are NO-independent. As ROS are known to be involved in modulating platelet function, the anti-aggregatory effect seen at millimolar concentrations by all three amino acids may be in part due to an antioxidant mediated effect. This could explain why L-citrulline, an amino acid that has no effect on platelet NO production, was also able to inhibit platelet aggregation. The reason that L-citrulline, demonstrated to have an almost identical antioxidant profile to L-arginine, was less effective at inhibiting aggregation (EC_{50} values of 128mM &15.7mM respectively) may be due to the differences in platelet uptake of L-citrulline compared to L-arginine. The maximum uptake of L-arginine is thought to occur within 1 minute and remain unchanged for 60 minutes whereas L-citrulline is significantly slower [420].

5.5.3.2 NO independent inhibition of platelet aggregation – an antioxidant effect

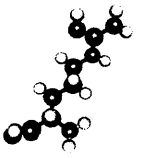
In order to investigate whether the effects of L-arginine and L-citrulline on platelet aggregation were mediated via an antioxidant effect based on the following criteria:

1. Comparison to D-arginine – an inactive enantiomer of L-arginine incapable of synthesising NO but that has been demonstrated to be capable of scavenging hydroxyl radicals.

2. Comparison to the well known antioxidant ascorbic acid which has been shown to inhibit platelet aggregation

3. Measurement of ROS production in collagen stimulated platelets

4. Comparison to specific ROS scavengers, SOD, catalase and mannitol.



5.5.4 Evidence of an antioxidant effect on platelet aggregation

5.5.4.1 Comparison to D-arginine

D-arginine exerted a similar antiaggregatory effect to L-arginine and L-citrulline (EC_{50} value of 72.77mM vs 15.7mM & 128mM respectively). This supports the hypothesis that the antiaggregatory effects are NO-independent at millimolar concentrations.

5.5.4.2 Comparison to ascorbic acid and detection of ROS using EPR

Ascorbic acid appeared no more potent in the attenuation of aggregation than L-arginine or L-citrulline. This was unexpected as several studies have demonstrated a role of antioxidants in regulating platelet function [268,373]. The reason for this discrepancy may once again be due to the differences in assay conditions. In the majority of studies that look at ROS, washed platelets are used, whereas in this study PRP was the media of choice. Although the use of washed platelets provides a simpler system in which ROS can be detected using techniques such as cytochrome C reduction it does not represent physiological conditions. The assay used in this study was developed using PRP which allows the effects of plasma on ROS to be taking into consideration. Plasma contains numerous antioxidants including transferrin, α-tocopherol, bilirubin, ascorbic acid and ceruloplasmin. The presence of these in the platelet system would therefore regulate the levels of ROS present. This may explain why, in the EPR study using the lipid soluble spin trap PBN, no ROS was detected upon stimulation with up to 10μg/ml collagen. The presence of endogenous antioxidants may explain why such high concentrations of amino acids and ascorbic acid are required to inhibit platelet aggregation. Another factor to take into account is the fact that in this system, the NO component, involved in the inhibition of platelet aggregation, was effectively 'knocked out'. It is possible that synthesised, antioxidants such as L-arginine are able to inhibit platelet

aggregation via prevention of ROS mediated NO degradation, an element effectively blocked in this system. This would also explain the relatively small antiaggregatory effect witnessed upon administration of ascorbic acid (Figure).
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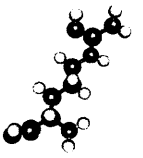
5.5.4.3 Comparison to superoxide, hydroxyl radical and hydrogen peroxide scavengers

Finally the effect of specific radical scavengers on platelet function was elucidated. SOD and catalase had no effect, which may suggest that superoxide and hydrogen peroxide are not involved in platelet aggregation, however it is more likely that due to their size, they were unable to enter the platelets, an issue not immediately realised at the start of this study. Mannitol on the other hand is a significantly smaller molecule and so should be able to enter the platelets. Indeed mannitol demonstrated similar anti-aggregating properties to L-arginine, L-citrulline and ascorbic acid. This suggests that the inhibition of aggregation in this model is due to hydroxyl radical scavenging. This supports data from the previous two chapters, which demonstrated that both amino acids were significantly better at scavenging hydroxyl radicals in comparison to superoxide. The fact that ascorbic acid, which is known to scavenge both superoxide and hydroxyl radicals is no more effective at inhibiting aggregation further supports this.

5.5.5 Conclusions

This study highlights the difficulties in using a biological system to evaluate antioxidant function. The inability of physiological concentrations of antioxidants to modulate platelet function may not be due to a lack of antioxidant activity rather it may be that modulation of platelet ROS has little effect on platelet function due to the presence of other signaling pathways e.g. arachidonate metabolism. Therefore even if 100% of ROS produced were scavenged, little overall change in platelet function may be detected. Alternatively, due to the presence of endogenous antioxidants which have evolved to control ROS production, the exogenous antioxidants used may be unable to effectively compete, resulting in no overall change in redox status.



Indeed maybe the biggest drawback of this bioassay is the fact that the platelets were not under conditions of oxidative stress i.e. there was no exogenous source of ROS as would be expected in disease states associated with oxidative stress. It is important to consider that ROS are involved in physiological processes as well as pathophysiological ones. In this case the ROS are involved in modulating *healthy* platelet function. To this end platelets have evolved endogenous antioxidants to maintain 'healthy, non-toxic' levels of ROS. The exogenous antioxidants used in this study have to compete with these which may explain why such high concentrations were needed to elicit an effect.

Maybe the true test of the effectiveness of an antioxidant is to test it in a system of true oxidative stress in which the body's endogenous antioxidants have been overwhelmed, a line of investigation pursued in the following chapter. In conclusion, it would appear that both L-arginine and L-citrulline are able to modulate platelet function through an NO-independent effect that would appear to be mediated through scavenging of hydroxyl radicals. The presence of endogenous antioxidants may explain why such high concentrations of amino acids were required to initiate an effect.

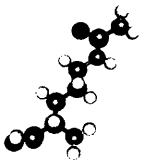
5.6 Summary

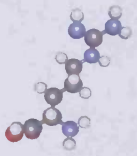
- L-arginine and L-citrulline inhibit collagen induced platelet aggregation at millimolar levels (EC_{50} values of 15.7mM and 128mM respectively).

- Within this system this inhibitory effect is mediated mainly through an NO independent effect based on the following:
    - NO production is likely to be inhibited due to the chelation of extracellular calcium by citrate
    - D-arginine, an inactive enantiomer of L-arginine, inhibits platelet aggregation to a similar degree as L-arginine and L-citrulline

- The inhibition of platelet aggregation is mediated through the scavenging of hydroxyl radicals based on the fact that ascorbic acid and mannitol, a specific hydroxyl radical scavenger, inhibit platelet aggregation to a similar degree as L-arginine and L-citrulline.

- The high concentrations of antioxidants required to inhibit platelet aggregation are likely to be due to:
    - The presence of endogenous antioxidants
    - The fact that ROS may only play a minor role in platelet aggregation





Ex Vivo studies

Effects on exogenous ROS

Chapter Six

6.0 Introduction

The overall aim of this final results chapter was to use the superoxide and hydroxyl radical generating systems characterised in chapter three to investigate the antioxidant capacity of L-arginine and L-citrulline in biological systems. In the previous chapter (chapter five), only high concentrations of L-arginine and L-citrulline were demonstrated effective at scavenging endogenously generated free radicals, most likely due to the presence of endogenous plasma antioxidants. Consequently the exogenous production of ROS, as described previously, was used to overcome the actions of endogenous antioxidants and create an environment representative of the oxidative stress conditions seen in disease states such as hypertension, hypercholesterolaemia and diabetes.

Two separate systems were set up based on the superoxide and hydroxyl radical assays developed in chapter three. Accordingly the results of this chapter are divided into two sections:

Study One

The overall aim of this study was to examine the ability of L-arginine and L-citrulline to scavenge ROS, specifically superoxide, and thus prevent ROS mediated endothelial dysfunction in vessels. ROS are known to exert numerous deleterious effects on the endothelium which can be broadly split into nitric oxide dependent and independent effects, as discussed in detail in 1.3. More specifically ROS have been demonstrated to effect basal vessel tone, agonist induced contraction and endothelium dependent relaxation [82,111,122,203,305,399,410]. In this chapter the effect of ROS on rabbit aorta endothelial function was explored using a xanthine oxidase / hypoxanthine system. Furthermore the specific ROS involved in mediating these effects was investigated. Following on from this, the effects of L-arginine and L-citrulline on the ROS mediated endothelial dysfunction was studied.

Study Two

The second study involved the development of an assay capable of measuring hydroxyl radical mediated lipid peroxidation, a phenomenon involved in disease states such as atherosclerosis and associated with established risk factors. Upon establishing this assay the ability of L-arginine and L-citrulline to inhibit lipid peroxidation was measured in this system.

Ultimately, this chapter was designed to bring together systems first developed in chapter three to investigate the ability of L-arginine and L-citrulline to prevent ROS mediated damage in a biological systems. The effects against both hydroxyl radicals and superoxide were studied with the overall aim of establishing whether L-arginine and L-citrulline have sufficient antioxidant capacity to protect against ROS mediated damage in a biological setting.

6.1 Aims

System One - The ability of L-arginine and L-citrulline to protect against ROS mediated endothelial dysfunction in vessels.

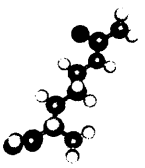
- To establish an *ex vivo* assay capable of quantifying the effects of ROS on the endothelium. To include:
    - Effect of ROS on quiescent tone i.e. in the absence of phenylephrine.
    - Effect of ROS on contractile function i.e. in the presence of phenylephrine.
    - Effect of ROS on endothelium dependent relaxation
    - Investigation into the specific ROS responsible for inhibiting endothelium dependent relaxation.

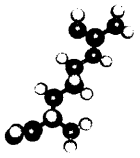
- To characterise the ability of L-arginine and L-citrulline to attenuate ROS mediated endothelial dysfunction.

System Two - The ability of L-arginine and L-citrulline to prevent hydroxyl radical mediated lipid peroxidation.

- To establish an *in vitro* assay capable of quantifying hydroxyl radical mediated lipid peroxidation.

- To characterise the ability of L-arginine and L-citrulline to prevent hydroxyl radical mediated lipid oxidation.





6.2 Materials and Methods

System One

6.2.1 ROS mediated endothelial dysfunction model

6.2.1.1 Set-up of aortic tissue

As described in chapter two

6.2.1.2 Effect of Reactive oxygen species on endothelial function

After assessment of endothelial function the effect of three concentrations of reactive oxygen species on endothelial function was tested, referred to as ROS 1, 2 & 3 (Table 32). Tissue was incubated for five minutes with hypoxanthine and for a following five minutes with xanthine oxidase. Tissues were then exposed to phenylephrine (PE, $1x10^{-6}M$) for ten minutes, a time period sufficient to allow a constriction plateau. At this point the rings were exposed to cumulative additions of acetylcholine at 5-minute intervals ($1x10^{-9}M$ – $1x10^{-5}M$). As a control, constriction and subsequent acetylcholine induced relaxation was run in the absence of reactive oxygen species. Adiagrammatic represenation of this protocol can be found in Figure .

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Table 32 Concentrations of substrates used to produce reactive oxygen species

System	[Hypoxanthine]	[Xanthine oxidase]
1	5μM	0.5 mU / ml
2	200μM	4 mU / ml
3	1mM	40 mU / ml

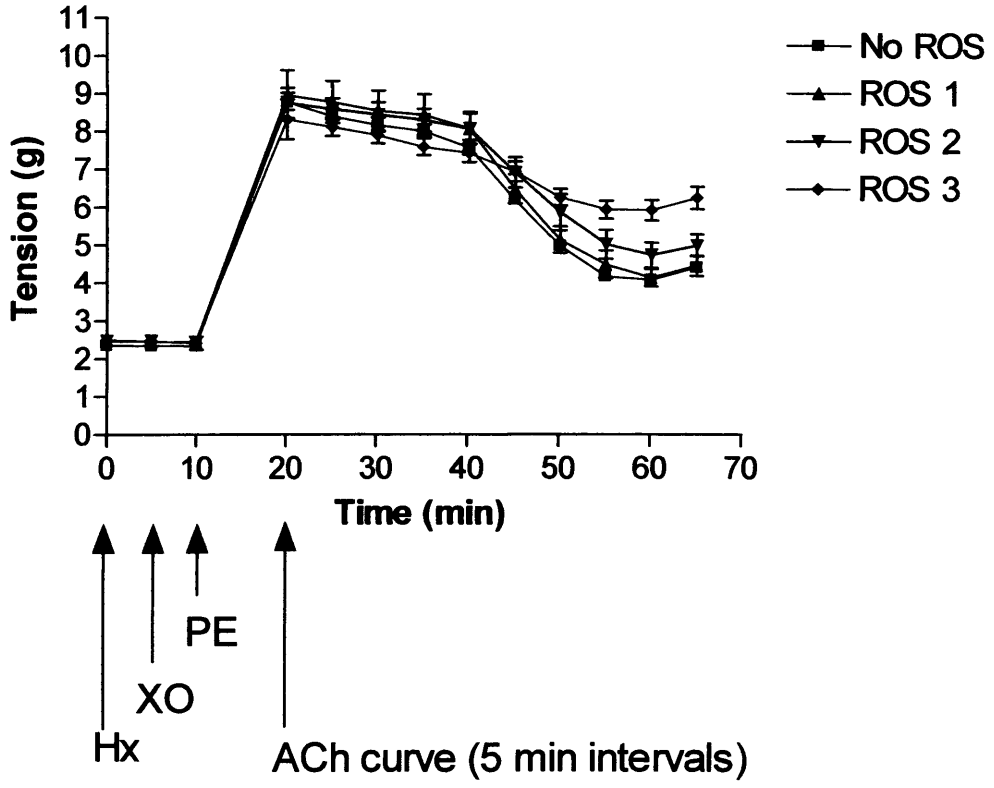


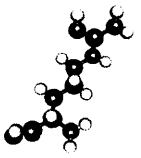
Figure 82 Aortic ring preparation: effect of ROS on endothelial function. ROS 1, 2& 3 refer to the concentrations of hypoxanthine/xanthine oxidase used in each system. For details refer to Table 32. At twenty minutes increasing concentrations of acetylcholine were added at five minute intervals ($1x10^{-5}M - 1x10^{-9}M$).

6.2.1.3 Investigation into radical specificity

In order to investigate the ROS involved in xanthine oxidase / hypoxanthine mediated endothelial dysfunction, tissues were incubated with 100 Units/ml SOD, 1200 Units /ml catalase or a combination of both SOD and catalase five minutes prior to the addition of 1mM hypoxanthine.

6.2.1.4 Investigation into the ability of L-arginine and L-citrulline to prevent ROS mediated endothelial dysfunction

Tissue was incubated for 10 minutes with $1x10^{-4}M$, $3x10^{-3}M$ or $3x10^{-2}M$ L-



arginine or L-citrulline prior to the addition of 1mM hypoxanthine. The concentrations of amino acids were chosen on the basis that $1x10^{-4}$M represents plasma concentrations of both amino acids, 3mM represents an average L-arginine concentrations experienced in plasma after exogenous administration (0.8mM to 8mM) [52,162,210,280] and 30mM represents the highest concentration achievable in this system.

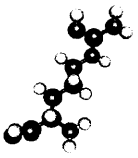
Rings were then exposed to phenylephrine (PE, $1x10^{-6}$M) for 10 minutes and then cumulative additions of acetylcholine at 5-minute intervals ($1x10^{-9}$M – $3x10^{-2}$M). As a control, constriction and subsequent acetylcholine induced relaxation was run in the absence of ROS.

System Two

6.2.2 Hydroxyl radical mediated lipid peroxidation model

6.2.2.1 TBARS assay

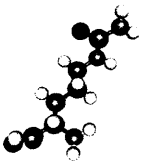
4mg/ml phosphatidylcholine was incubated with hydrogen peroxide (final concentration 44.1mM) and copper sulphate (final concentration 4.5μM) in order to initiate oxidation (for full protocol see chapter 2). A range of L-arginine and L-citrulline concentrations were added to investigate the extent with which they inhibited lipid oxidation. ($1x10^{-7}$M – $2x10^{-1}$M & $5x10^{-1}$M for L-citrulline and L-arginine respectively). All samples were incubated at 37°C, pH 7.4 for 150 minutes. After the incubation period, samples were placed on ice and 250μM EDTA was added to terminate the oxidation reaction (adapted from Wasowicz *et al* [384]). 100μl of sample or the appropriate volume of MDA standard was added to glass tubes containing 1ml of HPLC grade water. 1ml of the TBA solution was then added and all samples heated at 100°C for 1 hour. TBA-MDA fluorescence was measured at 525nm for excitation and 547nm for emission. The calibration curve was prepared using 0-0.3nmol/ml MDA.



6.2.3 Statistical Analysis

All data expressed as mean± SEM. "n" refers to the number of aortic ring segments used in each experiment. Concentration response curves were fitted to sigmoid curves using GraphPad Prism software. Maximum relaxation (R_{MAX}) and the effective concentration (EC_{50}) that gives 50% of the maximal response were then calculated and statistical analysis performed.

Comparisons of R_{MAX} values between control and pre-treated groups were performed using a one-way ANOVA followed by a Bonferroni *post hoc* test.



6.3 Results

6.3.1 System One

6.3.1.1 Assay development

In order to investigate the ability of L-arginine and L-citrulline to protect against ROS mediated endothelial dysfunction an assay capable of demonstrating the effects of ROS had to be set-up. An aortic ring preparation was chosen as it is classically used to determine the effects of various mediators on endothelium dependent relaxation.

6.3.1.1.1 Choice of vasoconstrictor

Phenylephrine was chosen to constrict the vessels based on a study that demonstrated that norepinephrine, but not phenylephrine, was subject to oxidation by xanthine oxidase / hypoxanthine derived ROS [399]. In order to investigate the stability of phenylephrine in the presence of ROS, $1x10^{-6}$M PE was added to the tissue in the presence of the highest concentration of ROS (Table 32). No significant difference was seen in the ability of PE to maintain contraction over one hour in the presence or absence of ROS ($p>0.05$) (Figure).

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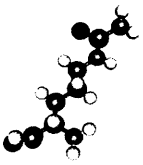


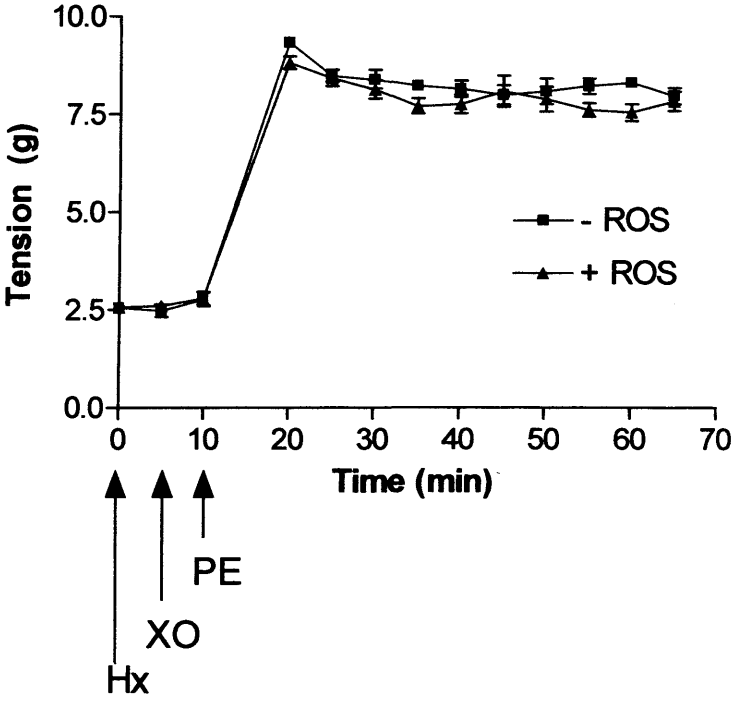


Figure 83 Effect of ROS on PE induced contraction over one hour. ROS refers to the concentration of hypoxanthine and xanthine oxidase used (1mM and 40mU/ml) see Table 32.P>0.05 between groups. Data expressed as mean±SEM. n=4.

6.3.1.1.2 Generation of ROS

In order to generate superoxide, a XO/HX system was utilised. This system was chosen because xanthine oxidase is known to be up regulated in disease states such as coronary artery disease, hypertension and hypercholesterolaemia [390]. Furthermore, as demonstrated in chapter 3 this system synthesised detectable amounts of superoxide.

6.3.1.1.3 Time course of radical production

The experiment was tightly time controlled due to the nature of radical production from the HX/XO system. In a previous study, superoxide production from 0.005Units/ml xanthine oxidase and 5μM hypoxanthine was shown to

increase in a linear fashion over twenty minutes (r^2=0.8628) (Figure). This highlighted the need for a tightly controlled timed protocol and all compounds were added at exactly the same time after initiation of radical production in order to make results comparable.

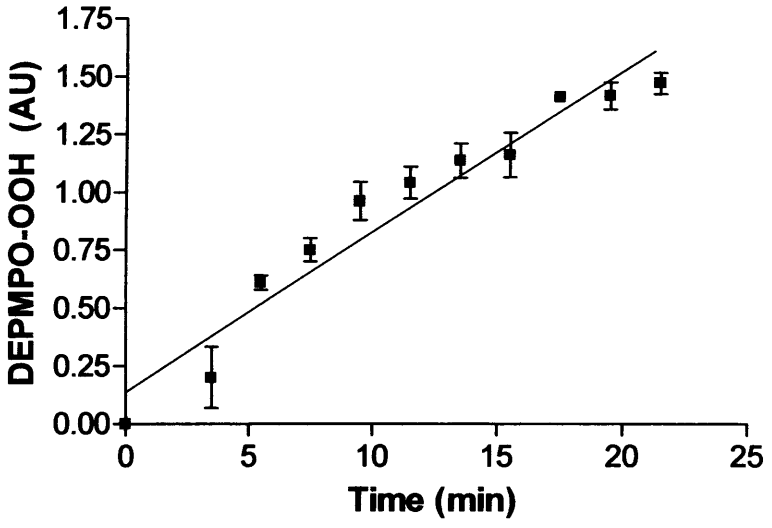
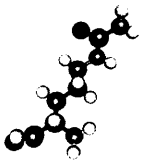


Figure 84 Timecourse of superoxide production as detected using EPR from 5μM hypoxanthine and 0.005U/ml xanthine oxidase. R^2 = 0.8628. Data expressed as mean±SEM. n=5.

6.3.1.1.4 Choice of radical concentration

Three different concentrations of xanthine oxidase and hypoxanthine were used (termed ROS 1,2 & 3). In order to find the optimum concentration of ROS to inhibit endothelial function. The lowest concentration of ROS (ROS 1) had no effect on acetylcholine induced relaxation ($p>0.05$) but the two highest concentrations of XO/HX (ROS 2 and ROS 3) both significantly inhibited relaxation ($p<0.0001$ for both groups as compared by R_{MAX}) (Figure 85). The highest concentration of radical (ROS 3) was therefore chosen for use in all other experiments.



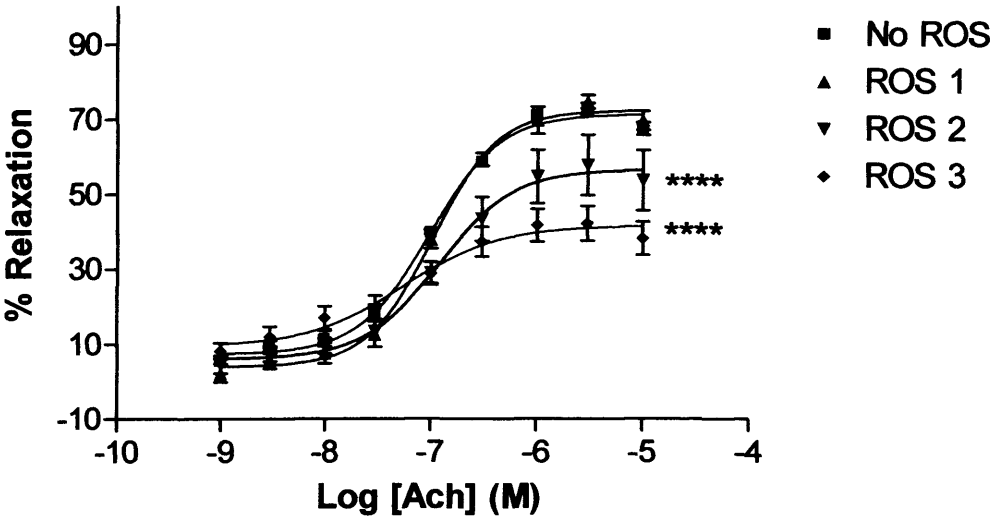


Figure 85 Effect of varying concentrations of XO/HX generated ROS on endothelial function as assessed by ACh mediated relaxation. ROS 1, 2& 3 refer to the concentrations of hypoxanthine/xanthine oxidase used in each system. For details refer to Table 32 ** represents p<0.0001 cf control (No ROS). Data expressed as mean±SEM. For control n=16. For all other treatment groups n=5.**

Table 33 Effect of ROS in ACh mediated relaxation. **** represents p<0.0001 ns represents no significant difference cf control. Details of ROS 1,2 and 3 can be found in Table 32. Data refers to Figure .

Group	R_{MAX}		EC_{50}
	Value	Significance cf control	
Control	71.23±1.203		$9.7x10^{-8}M$
ROS 1	72.23±1.748	Ns	$1.04x10^{-7}M$
ROS 2	56.53±3.717	****	$1.18x10^{-7}M$
ROS 3	41.47±2.77	****	$5.6x10^{-8}M$

6.3.1.2 Effect of ROS on quiescent vessel tone

Within the literature there exists evidence that ROS, specifically those derived from a XO/HX system, are able to elicit smooth muscle contraction in the presence of endothelium [203,410]. Our results demonstrated that ROS had no effect on quiescent vessel tone over a time period of one hour (Figure).

86

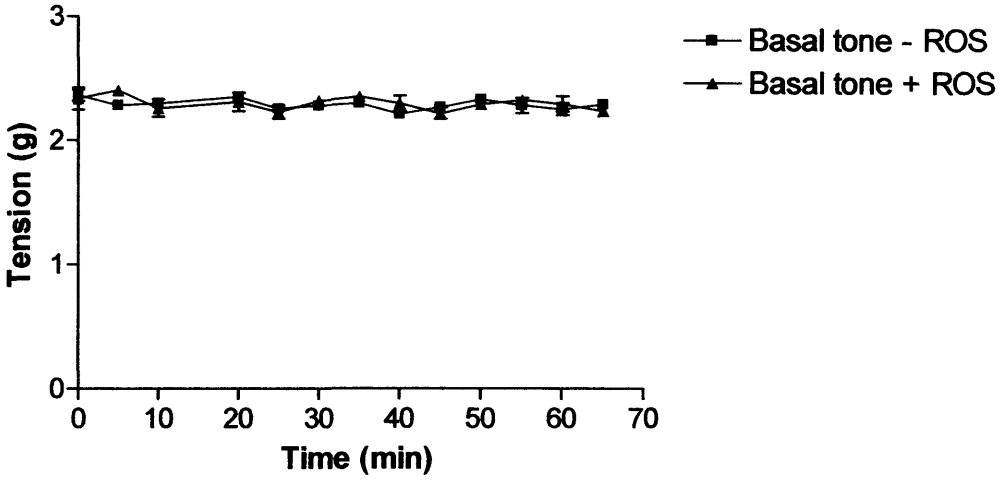


Figure 86 Effect of ROS on quiescent vessel tone. ROS refers to the concentration of hypoxanthine and xanthine oxidase used (1mM and 40mU/ml) see Table 32. No significant difference between groups (p>0.05). Data expressed as mean±SEM. n=4

6.3.1.3 Effect of ROS on contractile function

ROS generated from a XO/HX system have been shown to have an effect on normal contractile function as demonstrated by their ability to increase the degree of tension achieved upon administration of norepinephrine [410], epinephrine [203] and attenuate the level of tension achieved with phenylephrine [111]. Our results however demonstrated that that XO/HX generated ROS had no effect on the tension achieved upon addition of PE (Figure).

87

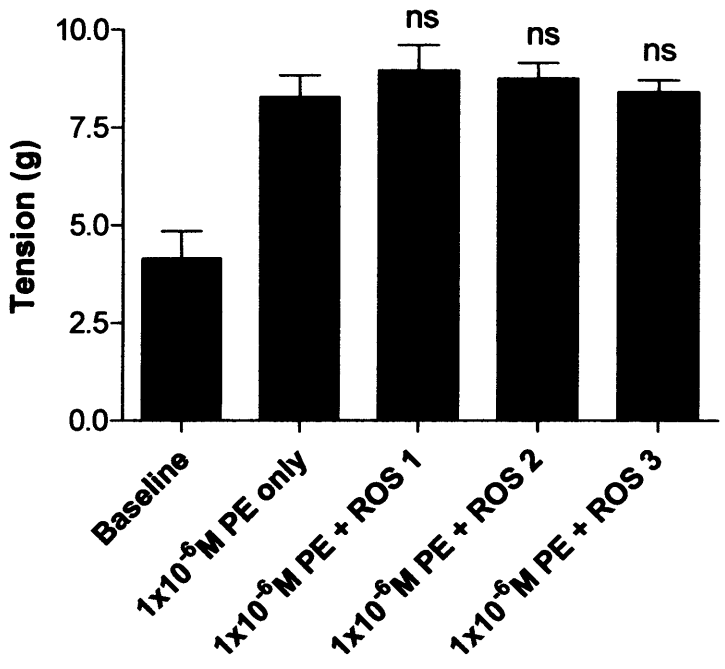


Figure 87 Effect of varying concentrations of ROS on PE induced contraction. For details on ROS 1,2, and 3 see Table 32. ns represents p>0.05 ($1x10^{-6}$M PE only). Data expressed as mean±SEM. For baseline group n= 19. All other treatment groups n=5 except for $1x10^{-6}$M + ROS 3 where n=13.

6.3.1.4 Investigation into the ROS responsible for inhibiting endothelium dependent relaxation

In order to determine the ROS responsible for inhibiting endothelium dependent relaxation, SOD, catalase and a mixture of SOD and catalase was added to the aortic ring 5 minutes prior to the addition of the radical generating system. As a control, the effect of these antioxidants on acetylcholine meditated relaxation in the absence of ROS was investigated with no significant difference seen between groups (Figure 88 and Table 34).

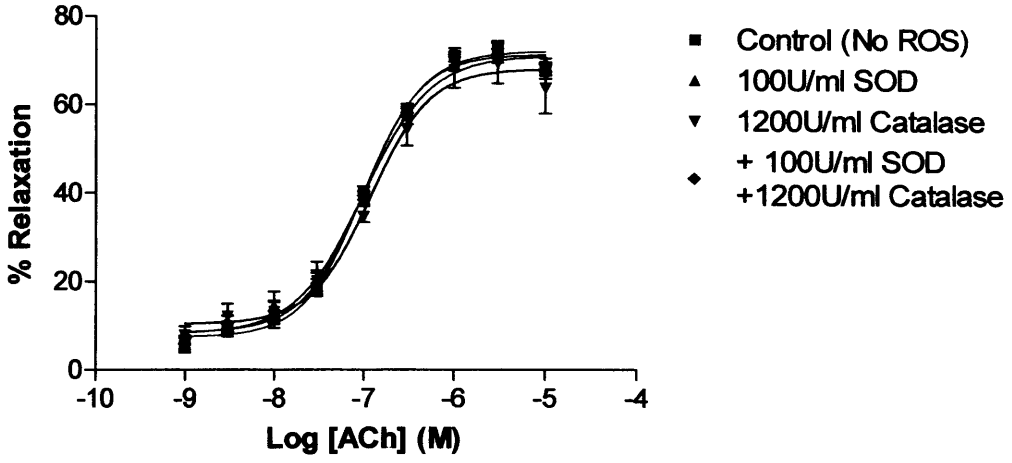


Figure 88 Effect of SOD and catalase on endothelial function in the absence of ROS. No significant difference between groups ($p>0.05$). Data expressed as mean±SEM. For control n=16. For all other treatment groups n=5-6.

Table 34 Effect of SOD and catalase on ACh mediated relaxation. ns represents no significant difference cf control. Data refers to Figure .

Group	R_{MAX}		EC_{50}
	Value	Significance cf control	
Control	71.23±1.203		$9.7x10^{-8}M$
100U/ml SOD	70.95±1.708	ns	$9.94 x10^{-8}M$
1200U/ml CAT	67.93±2.501	ns	$1.2 x10^{-7}M$
100U/ml SOD + 1200U/ml CAT	71.99±1.264	ns	$1.0 x 10^{-7}M$

In the presence of the highest concentration of ROS, inhibition of acetylcholine induced relaxation was seen as previously demonstrated (Figure 89). Comparisons between the R_{MAX} achieved in the presence and absence of ROS were made. This allowed us to tell if the antioxidant response was significantly different to the normal endothelial response and to the ROS exposed endothelial response.

Pre-treatment with SOD appeared to completely inhibit ROS mediated endothelial dysfunction as there was no significant difference compared with normal endothelial response ($p>0.05$) (Figure 89). catalase was less effective at preventing ROS mediated endothelial dysfunction, although catalase improved relaxation in comparison to tissue exposed to ROS only ($p<0.01$), there was also a significant difference compared with the normal endothelial response ($p<0.05$) (Figure 89). A combination of SOD and catalase did not improve upon the protective effects of SOD alone ($p>0.05$) (Figure).

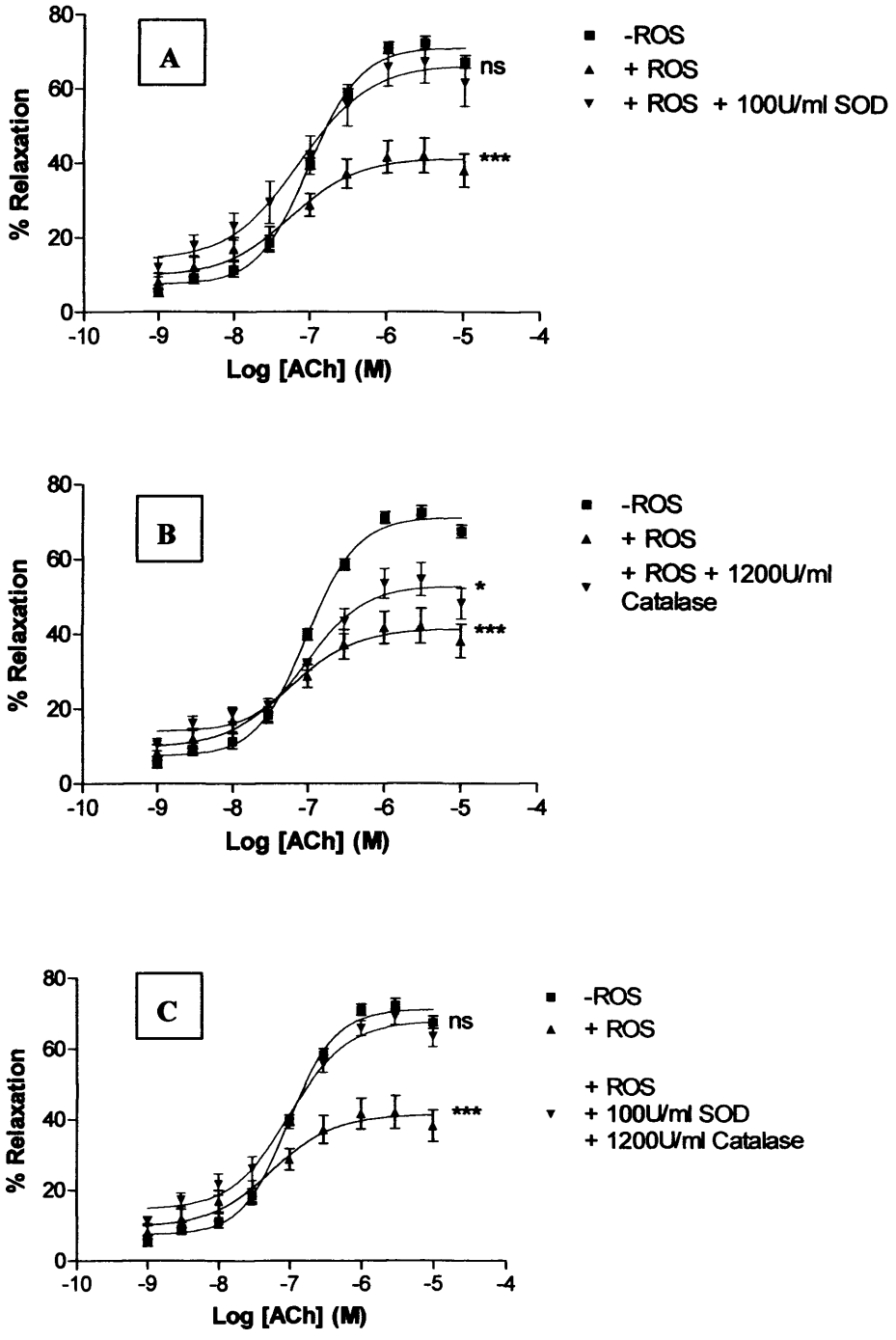


Figure 89 Effect of SOD and catalase on ROS mediated endothelial dysfunction. ROS refers to the concentration of hypoxanthine and xanthine oxidase used (1mM and 40mU/ml) see Table 32. A) illustrates the effects of 100U/ml SOD, B) illustrates the effects of 1200U/ml catalase, C) illustrates the effects of combining 100U/ml SOD and 1200U/ml catalase. * represents $p<0.001$, * represents $p<0.05$, ns represents $p>0.05$ cf control (-ROS). Data expressed as mean±SEM. N=4-16.**

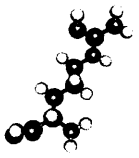


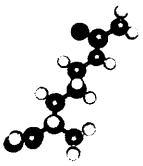
Table 35 Effect of SOD and catalase on ROS mediated inhibition of ACh mediated relaxation. ns represents no significant difference, * $p<0.05$, ** $p<0.01$, *** $p<0.001$ cf control. Data refers to figure Figure .

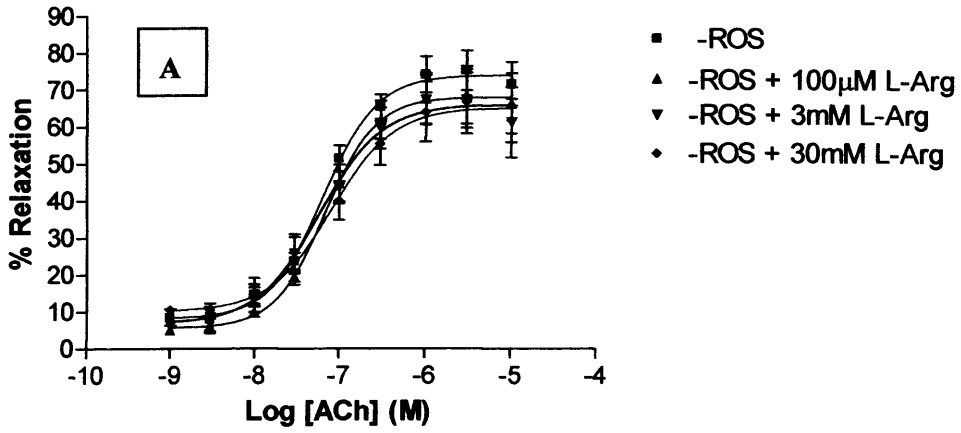
Group	R_{MAX}			EC_{50}
	Value	cf control	cf ROS 3	
Control	71.23±1.203		***	$9.7x10^{-8}M$
ROS	41.47±2.77	***		$5.6x10^{-8}M$
ROS + 100U/ml SOD	66.62±3.959	ns	**	$7.3x10^{-8}M$
ROS + 1200U/ml catalase	58.82±2.306	*	**	$1.1x10^{-7}M$
ROS + 100U/ml SOD & 1200U/ml catalase	67.92±2.129	ns	***	$9.9x10^{-8}M$

6.3.1.5 Investigation into the ability of L-arginine and L-citrulline to attenuate ROS mediated endothelial dysfunction

In order to investigate the ability of L-arginine and L-citrulline to inhibit ROS mediated endothelial dysfunction, three concentrations of both amino acids were pre-incubated with the aortic tissue before exposure to ROS. As a control, the effect of these amino acids on acetylcholine meditated relaxation in the absence of ROS was first investigated. L-citrulline and L-arginine had no effect on acetylcholine induced relaxation ($p>0.05$) at all concentrations (Figure).

90





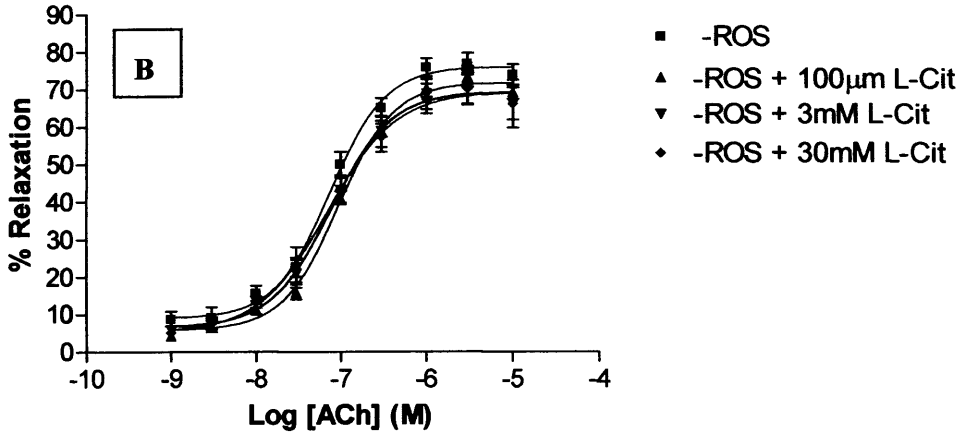


Figure 90 Effect of varying concentrations of A) L-arginine and B) L-citrulline on endothelial function in the absence of ROS. Concentrations of hypoxanthine and xanthine oxidase are 1mM and 40mU respectively. No significant difference between groups ($p>0.05$) Data expressed as mean±SEM. N=4-6

Table 36 Effect of L-arginine on ACh mediated relaxation. ns represents no significant difference. Data refers to Figure .

Group	RMAX		EC_{50}	N
	Value	cf control		
Control	74.08±2.377		6.6×10^{-8} M	5
100µm L-Arg	68.13±2.919	ns	6.9×10^{-8} M	4
3mM L-Arg	66.09±3.712	ns	5.9×10^{-8} M	4
30mM L-Arg	65.28±3.737	ns	8.2×10^{-8} M	4

Table 37 Effect of L-citrulline on ACh mediated relaxation. ns represents no significant difference cf control. Data refers to Figure .

Group	RMAX		EC_{50}	N
	Value	cf control		
Control	76.08±1.687		7.5×10^{-8} M	6
100µm L-Cit	71.89±1.328	ns	9.4×10^{-8} M	4
3mM L-Cit	69.33±2.493	ns	7.7×10^{-8} M	5
30mM L-Cit	69.32±2.487	ns	7.0×10^{-8} M	5

The ability of L-arginine and L-citrulline to protect against ROS mediated endothelial dysfunction varied and the effect of each amino acid shall be discussed in turn. L-arginine was unable to inhibit the ROS mediated endothelial dysfunction at any concentrations ($p<0.001$ cf control by not cf ROS) (Figure 91). L-citrulline on the other hand completely inhibited ROS mediated endothelial dysfunction at the two lowest concentrations (100μM and 3mM) but at the highest concentration of 30mM all protective actions were lost ($p<0.001$ cf control by ns cf ROS) (Figure 92).

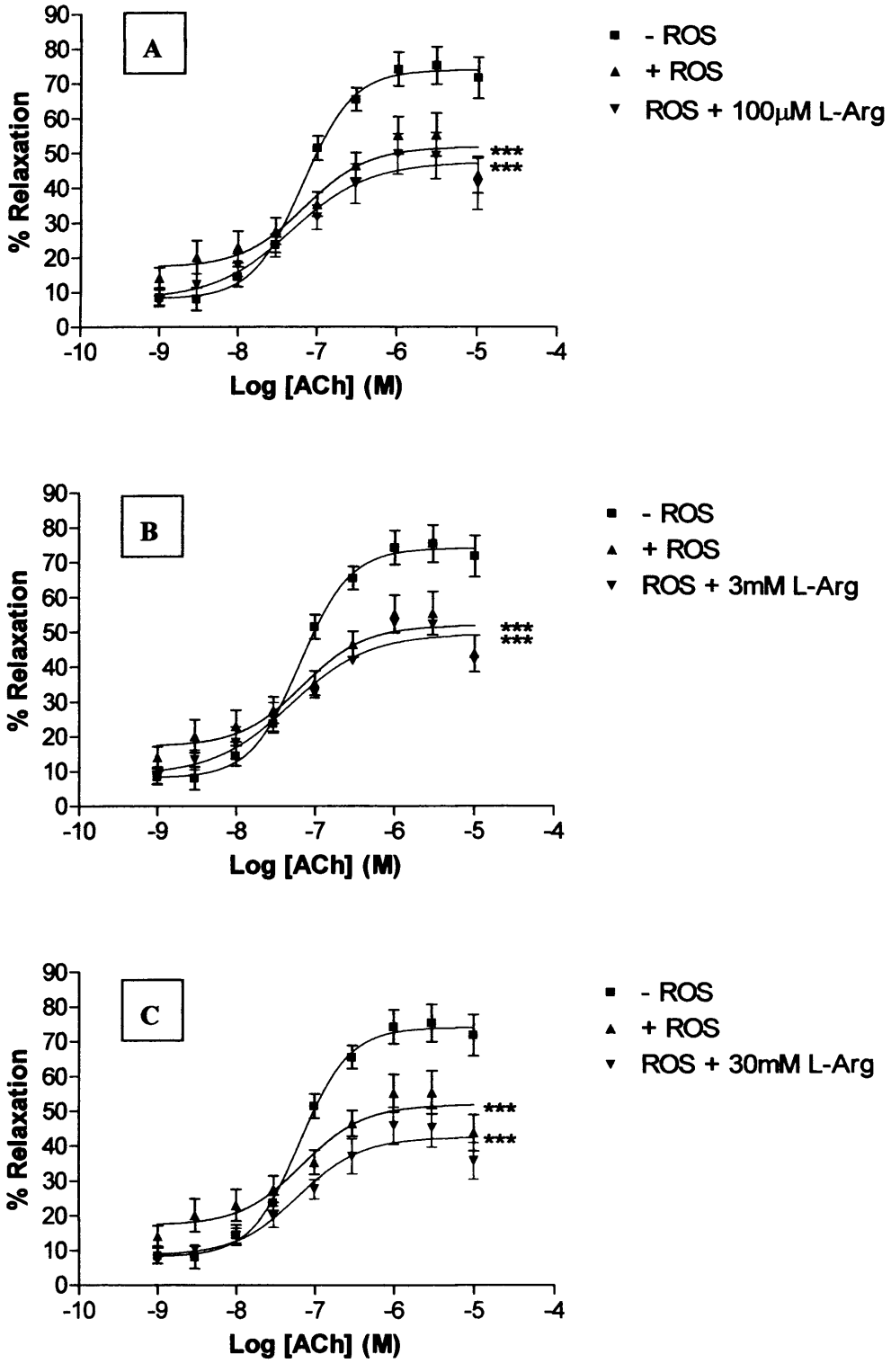


Figure 91 Effect of A) 100μM B) 3mM C) 30mM L-arginine on ROS mediated endothelial dysfunction. ROS refers to the concentration of hypoxanthine and xanthine oxidase used (1mM and 40mU/ml) see Table 32. * represents p<0.001 cf control –ROS). Data expressed as mean±SEM. N=4-5.**

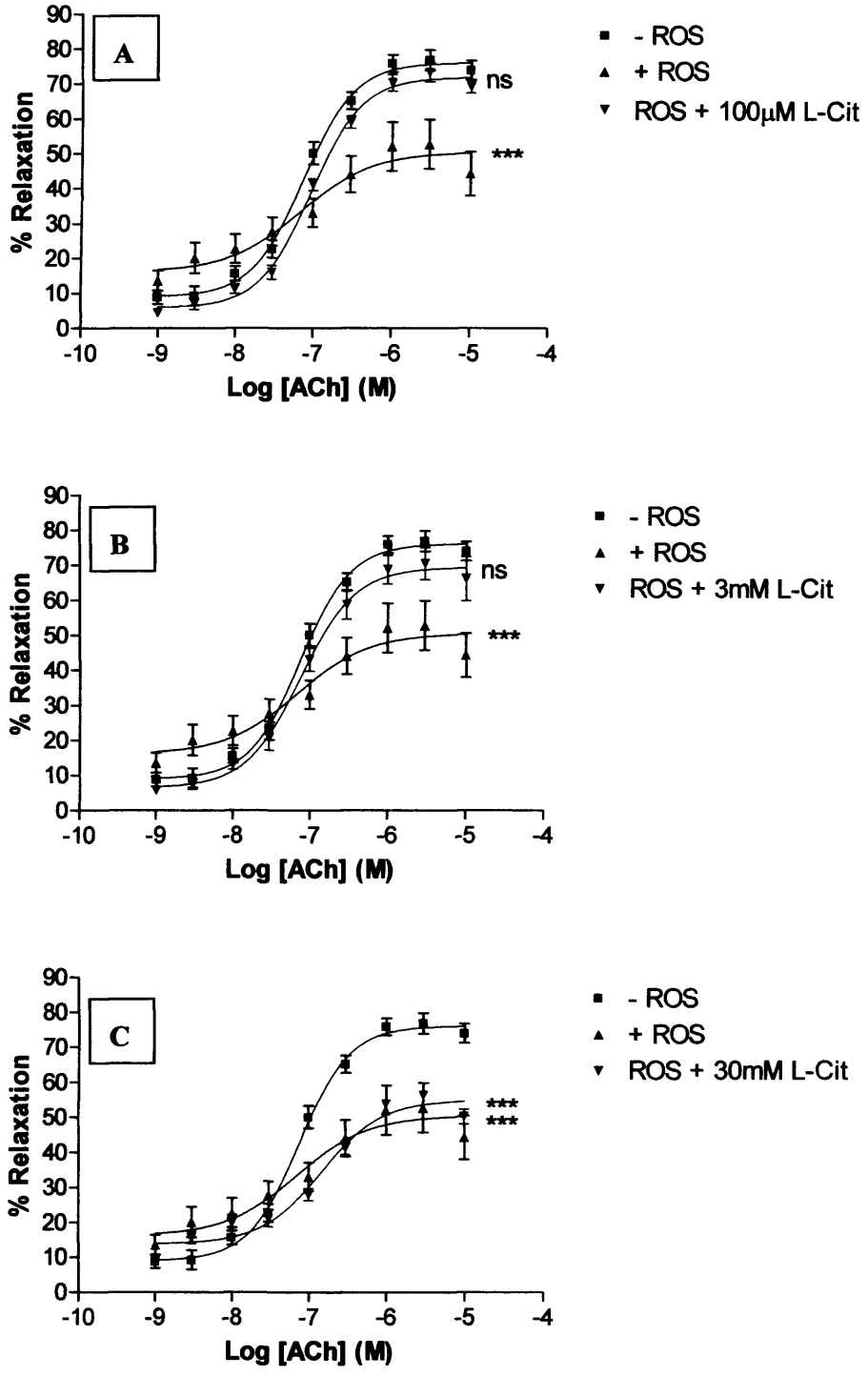


Figure 92 Effect of A) 100μM B) 3mM C) 30mM L-citrulline on ROS mediated endothelial dysfunction. ROS refers to the concentration of hypoxanthine and xanthine oxidase used (1mM and 40mU/ml) see Table 32.* represents $p<0.001$, ns represents $p>0.05$ cf control (–ROS). Data expressed as mean±SEM. N=4-5**

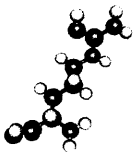


Table 38 Effect of L-arginine on ROS mediated inhibition of ACh mediated relaxation. ns represents no significant difference, *** $p<0.001$ cf control. Data refers to figure Figure .

Group	RMAX			EC_{50}
	Value	cf control	cf ROS	
Control	74.08± 2.377		***	6.6×10^{-8}M
ROS	51.91± 3.355	***		7.0×10^{-8} M
ROS + 100µM L-arg	47.47± 3.862	***	ns	4.8×10^{-8} M
ROS + 3mM L-arg	49.45± 2.470	***	ns	5.0×10^{-8} M
ROS +30mM L-arg	42.53± 2.89	***	ns	6.2×10^{-8} M

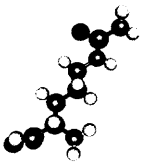
Table 39 Effect of L-citrulline on ROS mediated inhibition of ACh mediated relaxation. ns represents no significant difference, *** $p<0.001$ cf control. Data refers to Figure .

Group	RMAX			$EC_{50\,(m)}$
	Value	cf control	cf ROS	
Control	76.08± 1.687		***	7.5×10^{-8} M
ROS	50.51± 4.224	***		6.9×10^{-8} M
ROS + 100µM L-cit	71.89± 1.328	ns	***	9.4×10^{-8} M
ROS + 3mM L-cit	69.33± 2.493	ns	***	7.7×10^{-8} M
ROS +30mM L-cit	54.97± 2.033	***	ns	1.5×10^{-7} M

6.3.2 System Two – Lipid peroxidation system

6.3.2.1 Effect of copper sulphate / hydrogen peroxide concentration on lipid oxidation

The Fenton chemistry system adopted in chapter three was used to generate hydroxyl radicals and the effect of a range of hydroxyl radicals on phosphatidylcholine peroxidation was tested. Malondialdehyde, a downstream product of lipid peroxidation was used as an indicator of the degree of lipid peroxidation. Basal PC i.e. PC in the absence of hydroxyl radicals, was shown to exist in a partially oxidised state (0.043±0.002nmoles/ml MDA). Upon addition of four increasing concentrations of copper sulphate / hydrogen peroxide mixes, a significant increase in lipid peroxidation was seen (Figure 93). Concentrations of 4.5μM copper sulphate and 44.1mM hydrogen peroxide were chosen for use in all other experiments as this Fenton chemistry mix allowed both increases or decreases in lipid peroxidation to be detected.



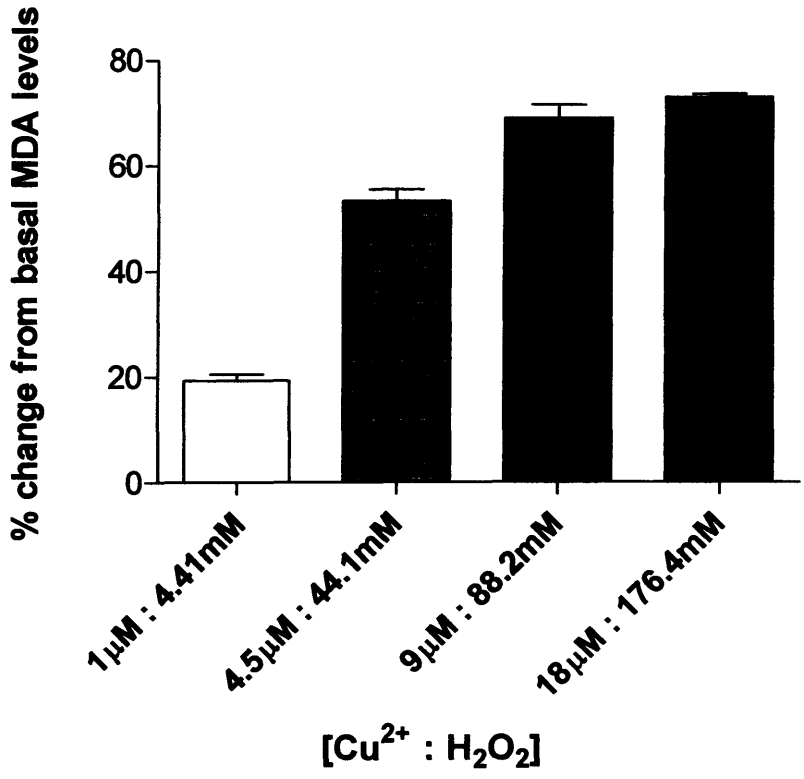
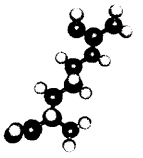


Figure 93 Effect of varying concentrations of Cu^{2+}/H_2O_2 on hydroxyl radical mediated PC peroxidation. All values significantly different ($p<0.001$) except 9μM vs. 18μM ($p>0.05$). Data expressed as ± SEM n=4.



6.3.2.2 Effect of L-arginine and L-citrulline on lipid peroxidation

Exposure of phosphatidylcholine to hydroxyl radicals resulted in lipid peroxidation and an increase in MDA level from 0.047±0.008nmol/ml to 0.072±0.008nmol/ml ($p<0.001$). Both L-arginine and L-citrulline were able to significantly inhibit lipid peroxidation in a concentration dependent manner (Figure 94). L-arginine significantly inhibited lipid peroxidation at concentrations of $1x10^{-6}$M ($p<0.05$) and $>1x10^{-4}$ M ($p<0.001$). L-citrulline was able to inhibit lipid peroxidation at concentrations as low as $1x10^{-7}$M ($p<0.05$) and $1x10^{-6}$M ($p<0.01$). At concentrations $>1x10^{-4}$M, L-citrulline significantly inhibited phosphatidylcholine peroxidation ($p<0.001$). Antioxidant activity was proportional to the concentration of L-arginine and L-citrulline present with EC_{50} values 53.16µM and 87.25µM respectively (Figure 94).

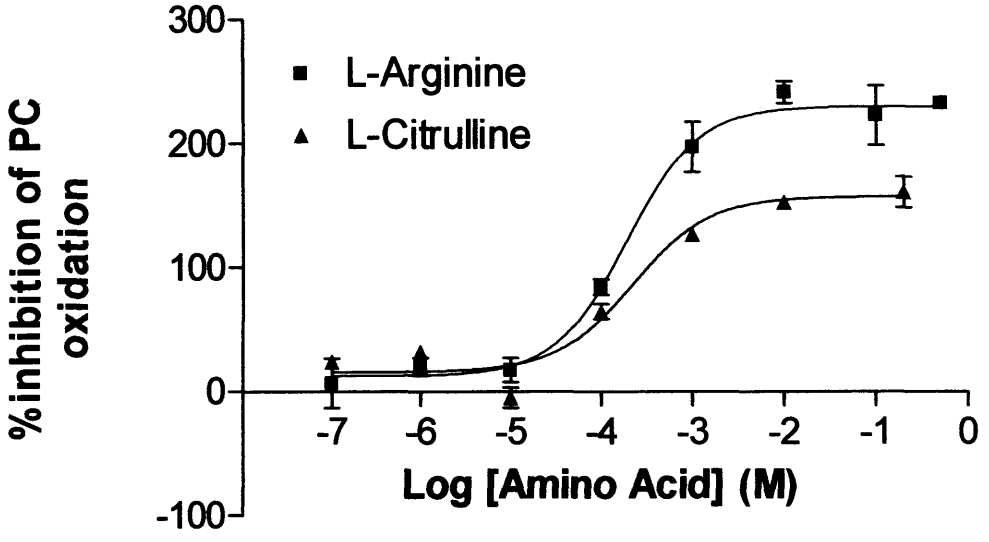
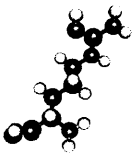
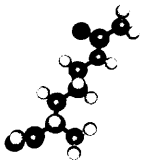


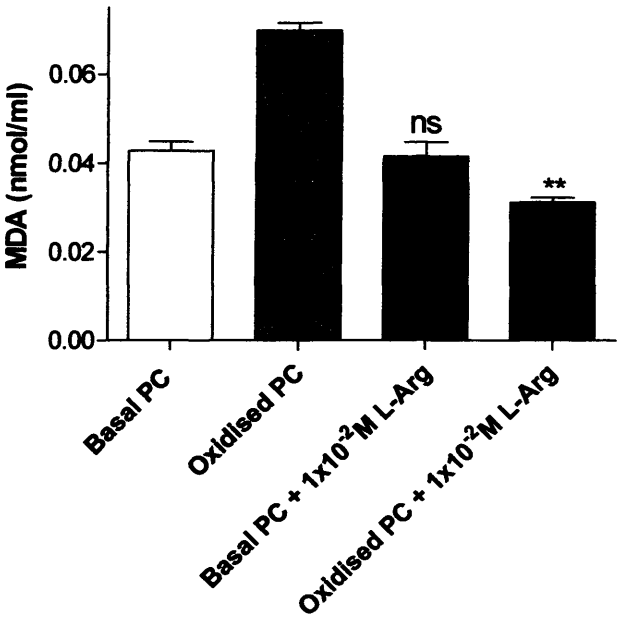
Figure 94 Effect of L-arginine and L-citrulline on hydroxyl radical mediated PC peroxidation. EC_{50} values 53.16µM and 87.25µM respectively. Data expressed as mean ± SEM. N=7-9 except $1x10^{-2}$M where n=4.



6.3.2.3 Effect of L-arginine and L-citrulline on basal phosphatidylcholine peroxidation levels

Basal phosphatidylcholine (i.e. phosphatidylcholine MDA levels before addition of hydroxyl radicals) was demonstrated to exist in a partially oxidised state (0.047±0.008nmol/ml MDA). At concentrations > $1x10^{-3}$M, both L-arginine and L-citrulline not only significantly inhibited oxidation of phosphatidylcholine ($p<0.001$) but also significantly reduced basal phosphatidylcholine MDA levels. At $1x10^{-2}$M, L-arginine significantly reduced MDA levels from 0.0427±0.0046 nmol/ml to 0.031±0.0026 ($P<0.05$). L-citrulline significantly reduced levels from 0.0441±0.0013 to 0.02537±0.0013 ($p<0.001$). This occurred in the presence of 4.5μM Cu^{2+}/44.1mM H_2O_2 but not in their absence (Figure 95) i.e. addition of amino acid alone had no significant effect on MDA levels.





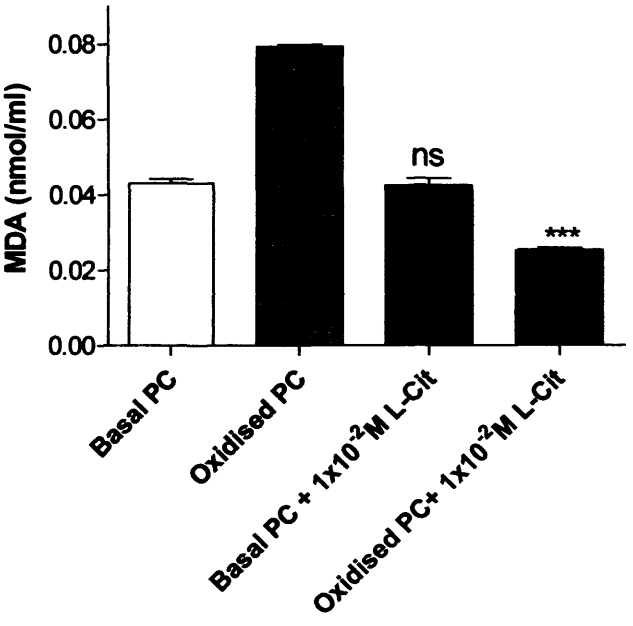
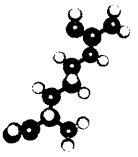


Figure 95 Effect of L-arginine and L-citrulline on basal PC peroxidation levels in the presence and absence of hydroxyl radicals. * represents p<0.001, ** represents p<0.01, ns represents p>0.05. Data expressed as mean ± SEM. n=4-6.**



6.4 Discussion

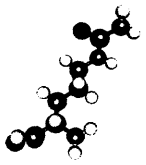
The overall aim of this chapter was to investigate the ability of L-arginine and L-citrulline to prevent ROS mediated damage in biological systems. In the previous chapter the ability of these amino acids to modulate endogenously produced ROS was investigated with mixed results. Inhibition was only seen at high concentrations of amino acids and this was proposed to be due to the presence of either high concentrations of endogenous antioxidants or the relatively small impact of ROS on platelet function. To overcome these problems this chapter employed the exposure of different biological systems to exogenously produced ROS. This allowed full characterisation of the effects of ROS on the system under investigation and the actions of endogenous antioxidants to be overwhelmed. Two systems were employed to do this 1) exposure of rabbit aortic rings to a XO/HX system and 2) exposure of phosphatidylcholine to hydroxyl radicals and each system shall be discussed separatley.

6.4.1 System One

The aim of this system was to develop a xanthine oxidase / hypoxanthine system, first described in chapter three, to investigate the effect of ROS on endothelial function. Assuming ROS resulted in dysfunction, the second aim was to investigate the ability of L-arginine and L-citrulline to scavenge the ROS and inhibit endothelial function.

6.4.1.1 Development of ROS mediated endothelial dysfunction model

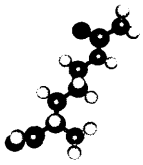
The assay developed in chapter three (capable of producing superoxide from xanthine oxidase and hypoxanthine) was used to establish a model of ROS mediated endothelial dysfunction. There exists a large body of work linking the generation of ROS to the development of cardiovascular disease [47,116,117]. Nearly every risk factor for cardiovascular disease is associated with enhanced ROS production and it therefore seems likely that oxidative stress is a common



pathway in the development of cardiovascular disease. Increased levels of reactive oxygen species are associated with up-regulation or increased activity of several enzymes including xanthine oxidase. Xanthine oxidase itself has been linked to atherosclerosis, coronary artery disease, hypertension and heart failure [47,49,72,92,123,242,253,254,265,342,351]. This made xanthine oxidase a very relevant choice for the development of a ROS mediated endothelial dysfunction model and indeed other xanthine oxidase assays have been used to similar effect ([28,82,83,111,122,203,305]. Phenylephrine was the agonist of choice based on evidence that norepinephrine (but not phenylephrine) is subject to oxidative degradation [399], supported by my own preliminary studies (Figure 83). The next step in the set-up of this assay was the choice of xanthine oxidase / hypoxanthine concentration. Three concentrations were chosen as illustrated in Table 32. Only the two highest concentrations of XO/HX significantly inhibited acetylcholine induced relaxation ($p<0.0001$) (Figure 85), so the highest concentration of XO/HX was chosen for use in all other experiments.

As the aim of this experiment was to investigate the effect of ROS on endothelium dependent relaxation, control experiments were run to investigate the effects of ROS on quiescent vessel tone and on contractile function. It has been suggested that ROS, particularly those derived from XO/HX, are able to elicit smooth muscle contraction in the presence of endothelium [203,410]. The results herein demonstrated no effect on vessel tone (Figure 86). It has also been suggested that XO/HX generated ROS effect contractile function with a number studies demonstrating that ROS are able to increase the degree of tension achieved upon administration of norepinephrine [410], epinephrine [203] and attenuate the level of tension achieved with phenylephrine [111]. These results however demonstrate no such effect on the actions of phenylephrine (Figure 87). In terms of the development of our assay, this meant that the inhibitory effects of ROS on vessel tone were due to an effect on relaxation alone.

The specific radical responsible for attenuating acetylcholine induced relaxation was then investigated. Tissues were pre-incubated with SOD, catalase and a combination of both in order to rule out the effects of primary superoxide and



hydrogen peroxide formation i.e. formation through xanthine oxidase and secondary hydrogen peroxide formation i.e. through the dismutation of superoxide (Figure 96).

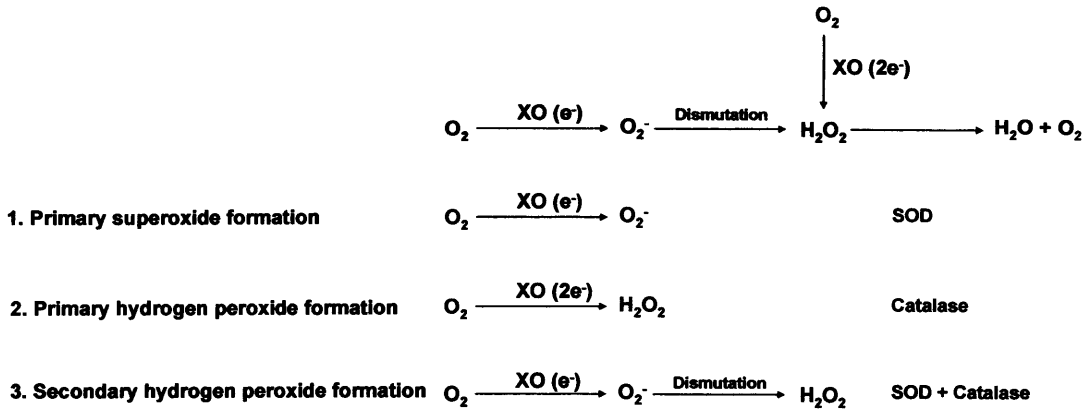
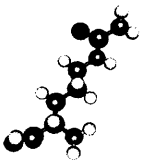
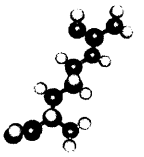


Figure 96 Source of ROS from XO/HX and the sites of action of SOD and catalase

Results with SOD and catalase suggested that the endothelial dysfunction caused by the XO/HX system was due to a combined effect of superoxide and hydrogen peroxide. The ability of SOD to completely attenuate the inhibition caused by XO/HX, suggested that hydrogen peroxide was being synthesised through the dismutation of SOD (Figure 89).

Based on these findings the following hypothesis was reached concerning the mechanism of action of XO/HX mediated endothelial dysfunction. Xanthine oxidase has been shown to bind to endothelial cells via glycosoaminoglycans and to be taken up into the cells through endocytosis [152]. The uptake of xanthine oxidase at 37 °C is time- and concentration-dependent. The cell-associated XO remained active and maximum cellular XO catalytic activity and binding thought to occur within 1 h of XO addition. There is thought to be an initial rapid increase, followed by a slower increase implying ongoing endocytosis of XO while binding to cells [152]. In our system however, addition of extracellular SOD completely attenuated the loss of endothelial function seen with ROS, suggesting





that in the time course of our study the majority of xanthine oxidise remained outside the cell. NO produced within the endothelial cell diffuses equally in all directions. In the absence of any external factors a set percentage diffuses to the smooth muscle resulting in relaxation whilst the rest moves into the blood stream (in this case buffer filled lumen). In the presence of extracellular superoxide, the concentration of NO in the lumen will be reduced resulting in the formation of a diffusion gradient towards the lumen. Thus less NO will reach the smooth muscle resulting in a decrease in relaxation. In our system relaxation was inhibited by ~30% in the presence of superoxide and hydrogen peroxide (Figure 89).

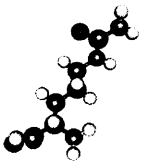
ROS inhibit vessel relaxation through NO dependent and independent effects. superoxide not only reduces NO bioavailability but also through its reaction with NO leads to the production of peroxynitrite which itself may cause any of the following:

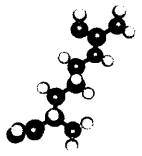
1. Inhibition of soluble guanylate cyclase [386]
2. Oxidation of zinc thiolate clusters and tetrahydrobiopterin leading to NOS monomerisation and therefore eNOS uncoupling [196,201,421]
3. Inactivation of prostacyclin synthase [422]

As well as interacting with NO, the ROS could also mediate their effects through an NO independent mechanism including any combination of the following factors:

1. Activation of endothelial enzymes e.g. Cyclooxygenase (COX) which releases endothelial derived contacting factors [18,167]
2. Free radical-catalysed peroxidation of arachidonic acid leading to the formation of prostaglandin F2 like compounds [246]
3. Extensive endothelial disruption [152,203,410]

In summary, it is likely that hydrogen peroxide and superoxide are able to inhibit acetylcholine induced relaxation through NO dependent and independent





mechanisms.

6.4.1.2 Investigation into the ability of L-arginine and L-citrulline to attenuate ROS mediated endothelial dysfunction

Upon development of a model of ROS mediated endothelial dysfunction, the ability of L-arginine and L-citrulline to prevent this dysfunction was investigated. This was based on the theory that if the amino acids were capable of directly scavenging superoxide they should be able to prevent the loss in endothelial function. Based on previous results (chapter three), where no superoxide scavenging ability was evident, it seemed unlikely that either L-arginine or L-citrulline would have any significant protective effect. However numerous studies have shown that L-arginine is capable of inhibiting ROS mediated endothelial dysfunction [37,122,137,228,408] and so it was important to investigate whether this was the case in our study as *in vitro* results do not necessarily predict *in vivo* responses.

6.4.1.2.1 Effects of L-arginine

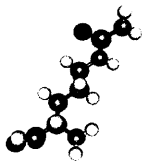
Upon pre-incubation of aortic tissue with L-arginine, no change in ROS mediated dysfunction was seen (Figure 90). This was in accordance with our results from chapter three that demonstrated that L-arginine lacked any superoxide scavenging activity below concentrations of $1x10^{-1}$M (Figure 44) Therefore although exogenous L-arginine has been shown to improve endothelial function in several studies through the reduction of oxidative stress [37,38,137] this study would suggest that this is unlikely to be mediated through a direct superoxide scavenging effect. This is not to say that L-arginine is not capable of lowering levels of superoxide in the body, just that reduction in superoxide is not mediated through a direct superoxide scavenging action. In clinical trials involving the administration of L-arginine, the decrease in superoxide levels seen is most likely due to inhibition of superoxide producing enzymes such as xanthine oxidase [223] and through improvement of endogenous antioxidant defence systems by increasing glutathione levels [223] and SOD and catalase activity [89]. In our

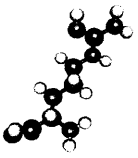
system, L-arginine is added directly to the tissue only ten minutes before the onset of oxidative stress. It is therefore not possible for L-arginine to regulate enzyme activity, whether that is through up regulation of antioxidant enzymes or down regulation of superoxide producing enzymes. This is to our advantage however as it means any effects demonstrated by L-arginine are directly attributable to direct superoxide scavenging or lack of as the case might be. This is something that long-term exogenous administration of L-arginine does not investigate.

Effects of L-citrulline

The results obtained with L-citrulline were more complicated that those achieved with L-arginine. L-citrulline demonstrated protective properties at 100μM and 3mM, i.e. was able to prevent ROS mediated endothelial dysfunction (Figure). This is in direct contrast to the results obtained from L-arginine where no protective effect was seen. This was unexpected as in all other chapters L-citrulline has acted in an almost identical fashion to L-arginine. In chapter three both were able to scavenge hydroxyl radicals but not superoxide at physiological concentrations (Figure 44 & Figure 51). In chapter four, both demonstrated the same mechanism of hydroxyl radical scavenging and in the previous chapter both exerted similar inhibitory effects on platelet aggregation (Figure 76). This led to the question as to why L-citrulline was effective at protecting against ROS mediated endothelial dysfunction whereas L-arginine was not.

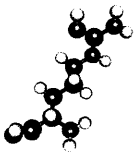
First of all it is unlikely that L-citrulline is able to scavenge superoxide as it also demonstrated a lack of superoxide scavenging ability at concentrations lower than $1x10^{-1}$M in the *in vitro* system (Figure 44). Although it is true that an *in vitro* result is not entirely predictive of an *in vivo* result, the fact that L-arginine failed to protect against ROS mediated endothelial dysfunction and both have demonstrated identical antioxidant properties so far, suggests that L-citrulline is not working through a direct scavenging action.





One possible explanation is that L-citrulline is working in a ROS independent manner. L-citrulline has been shown to directly relax smooth muscle through activation of particulate guanylate cyclase in smooth muscle [308]. This is NO independent, as it can not be blocked by L-NAME. Activation of pGC resulted in increased levels of cGMP, which act in part through the opening of K_{ca} channels leading to hyperpolarisation of vascular smooth muscle cells and reduction of intracellular calcium. This would explain why even in the presence of ROS, L-citrulline was able to relax the vessel.

The second unexpected finding was that at the highest concentration of L-citrulline, all protective properties were lost. As demonstrated with the same concentration of L-arginine (30mM), L-citrulline offered no protection against ROS mediated endothelial dysfunction. It is unlikely that this is due to an inhibitory effect on NOS, or even a toxic effect on the cell itself, as 30mM L-citrulline had no effect on acetylcholine mediated relaxation (Figure 90). It may therefore be that such high concentrations of L-citrulline may inhibit pGC and so the NO independent component of relaxation is lost.

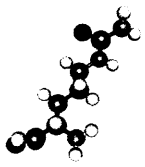


6.4.2 Study Two

6.4.2.1 Development of hydroxyl radical mediated lipid peroxidation model

In chapter three, the EPR based assay demonstrated that L-arginine and L-citrulline were able to scavenge hydroxyl radicals with an EC_{50} of 17.09μM and 44.06μM respectively (Figure 51). This provides strong evidence for the role of L-citrulline and L-arginine as hydroxyl radical scavengers and to test this, the ability of both amino acids to protect against hydroxyl radical mediated lipid peroxidation was investigated. Polyunsaturated fatty acids (PUFA) are the main constituent of mammalian cells and easily suffer are easily oxidised oxidation leading to the formation of lipid peroxyl radicals ($LOO^{\bullet}$). Due to their high reactivity, they abstract the closest available hydrogen from molecules including adjacent PUFAs, proteins, sugars, nucleic acids and neighbouring cell membrane PUFA's [336]. They can also abstract a hydrogen from cholesterol and oleic acid [380]. They are therefore capable of causing severe cell damage.

In order to investigate the effects of L-arginine and L-citrulline on hydroxyl radical mediated lipid peroxidation we first had to set up a system capable of measuring changes in hydroxyl radical levels. To do this a range of hydrogen peroxide and copper sulphate concentrations were incubated with phosphatidylcholine. The concentrations of copper sulphate and hydrogen peroxide were chosen based results from chapter three that demonstrated that each system produced significantly different levels of hydroxyl radical (Figure 52). The next step was to incubate these systems with PC and to test whether varying levels of hydroxyl radicals resulted in significantly different degrees of lipid peroxidation. Our results showed that as the concentration of hydroxyl radical increased so did the degree of lipid peroxidation (Figure 93). Concentrations of 4.5μM copper sulphate and 44.1mM hydrogen peroxide were chosen for use in all other experiments as these allowed potential increases and decreases in lipid peroxidation to be detected.



6.4.2.2. Investigation into the ability of L-arginine and L-citrulline to attenuate hydroxyl radical mediated lipid peroxidation

L-arginine and L-citrulline were both capable of preventing hydroxyl radical mediated peroxidation of phosphatidylcholine at concentrations found within the plasma (EC_{50} values of 53.16μM and 87.25μM) (Figure 94). As demonstrated in chapter four, this may be through direct hydroxyl radial scavenging or through decreased production of hydroxyl radicals through chelation of copper. Both mechanisms are valid in a biological system where Fenton chemistry is one of the main routes of hydroxyl radical generation. These results show that both amino acids are capable of preventing peroxidation and thus may be able to prevent oxidation of lipoproteins, a problem contributing to the development of atherosclerosis and involved in risk factors for atherosclerosis such as diabetes [24,168], hypertension [309], hyperlipidemia [367] and ageing [166,171,343].

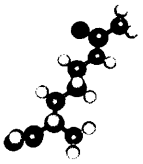
Also of interest was the fact that at concentrations of $>1x10^{-3}$M, L-arginine and L-citrulline were able to reduce oxidised phosphatidylcholine to levels lower than seen under basal conditions (Figure 95). This reduction only occurred in the presence of hydroxyl radicals. The mechanism behind this is unclear but the requirement for hydroxyl radicals suggests that a switch exists which is needed to convert both amino acids from redox inactive molecules to antioxidants. In this case, the switch would appear to be an oxidative insult. It is also possible that the product formed from the reaction between the amino acids and hydroxyl radicals is able to exert further reductive activity, not possessed by the parent amino acid.

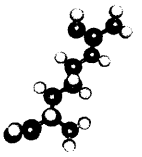
6.4.3 Conclusions

This study would suggest that the ability of L-arginine and L-citrulline to protect biological systems against exogenously derived ROS i.e. conditions of oxidative stress, varies greatly and is dependent on the radical involved. The results obtained from the superoxide-mediated model of endothelial dysfunction suggest that L-arginine is unable to directly scavenge superoxide and therefore is unable

to prevent ROS mediated endothelial dysfunction. L-citrulline however was protective at low concentrations, not through scavenging of superoxide, but through a ROS independent action, potentially through a direct relaxant action on vascular smooth muscle.

In the system of hydroxyl radical mediated lipid peroxidation, physiological concentrations of L-arginine and L-citrulline i.e. concentrations found in plasma, were able to prevent lipid peroxidation. In terms of disease states, oxidative stress is used as a blanket term to include an increase in several types of ROS, including both superoxide and hydroxyl radicals. This study highlights that administration of these amino acids in an antioxidant capacity would have varying results depending on the disease state in question. Disease states associated with hydroxyl radical mediated damage e.g. atherosclerosis, would benefit from administration of exogenous L-arginine and L-citrulline whereas superoxide mediated pathologies would be unlikely to see any improvement. Also the ability of L-citrulline to restore endothelial function through a superoxide independent mechanism is an important finding as it suggests that, like L-arginine, it may be able to improve vascular function through a ROS independent mechanism.





6.5 Summary

System One

- Superoxide and hydrogen peroxide generated from a xanthine oxidase / hypoxanthine system cause endothelial dysfunction as characterised by a loss in acetylcholine induced relaxation

- L-arginine has no superoxide scavenging effects demonstrated through its inability to prevent ROS mediated endothelial dysfunction

- L-citrulline is able to prevent ROS mediated endothelial dysfunction at 100μM and 3mM although this is unlikely to be mediated through a ROS dependent effect

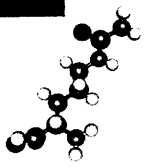
System Two

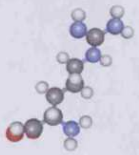
- Fenton chemistry derived hydroxyl radicals mediate phosphatidylcholine peroxidation in a concentration dependent manner

- L-arginine and L-citrulline are able to significantly inhibit this peroxidation at concentrations found in human plasma (53.16μM and 87.25μM respectively).

Conclusion

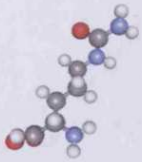
- The ability of L-arginine and L-citrulline to attenuate damage caused during oxidative stress will depend entirely on the relative importance of superoxide vs. hydroxyl radicals on the pathology of the disease in question.





General Discussion

Chapter Seven



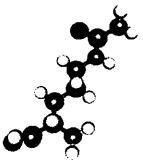
7.1 General Background

The overall aim of this thesis was to investigate whether the improvement in endothelial function seen upon administration of L-arginine and L-citrulline *in vivo* could be attributed to a ROS scavenging action. Oxidative stress plays a prominent role in the development of endothelial dysfunction through numerous NO dependent and independent mechanisms and it is therefore vital to characterise potential antioxidant molecules that could prevent the development of cardiovascular disease.

L-arginine and L-citrulline act in a protective manner in several disease states including hypertension, hypercholesterolaemia and diabetes mellitus [37,51,58,65,137,358] which are characterised by an increase in ROS. This has led to investigations into whether this protective action could be mediated through direct ROS scavenging. A significant amount of work has been carried out investigating the antioxidant effects of L-arginine, but contradictory results [6,199,379,383] have meant that the question of whether L-arginine is actually capable of scavenging ROS remains unanswered. Also, the vast majority of studies have focused on superoxide radicals with only a handful investigating its efficiency against hydroxyl radicals [290,298]. Even within studies concluding that L-arginine has an antioxidant effect, there exists discord concerning the mechanism of its action [199,379]. Investigations into the antioxidant properties of L-citrulline are far fewer and focus mainly on the ability of L-citrulline to reduce hydroxyl radical levels in plants [8].

With the current contradictions that exist concerning the antioxidant properties of L-arginine, and the majority of working concerning L-citrulline having been carried out in plants, a study aimed at fully characterising the antioxidant profile of both molecules was needed and this thesis fulfilled this criteria in four well defined chapters which answered:

- Are L-arginine and L-citrulline capable of scavenging ROS and if so, is this action specific to ROS type e.g. superoxide vs. hydroxyl radical?



(Chapter Three)

- What is the mechanism for the scavenging of the hydroxyl radical? **(Chapter Four)**

- Are these amino acids capable of modulating *endogenous* ROS levels and what effect does this have on physiological function? **(Chapter Five)**

- Are L-arginine and L-citrulline capable of modulating *exogenous* ROS in
    - a model of superoxide mediated endothelial dysfunction?
    - a model of hydroxyl radical mediated lipid peroxidation?

    (Chapter Six)

The following table summarises these results

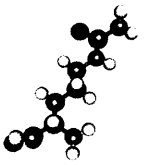


Table 40 Summary of thesis results.

Chapter	Overall aim	Results summary
Chapter Three	To establish whether L-arginine and L-citrulline were capable of scavenging ROS and if so was this action specific to ROS type e.g. superoxide vs. hydroxyl radical.	• No antioxidant activity against $O_2^{\bullet-}$ at concentrations less than $1x10^{-1}M$ • Inhibition of $^{\bullet}OH$ at concentrations found in human plasma (EC_{50} of 16.19μM and 44.06μM for L-Arg and L-Cit respectively). • Pro-oxidative effect seen at $1x10^{-4}M$ expressed as an ability to increase the level of $^{\bullet}OH$ above that of the control.
Chapter Four	To characterise the mechanism responsible for the scavenging of hydroxyl radicals	• Reduction in $^{\bullet}OH$ mediated via two mechanisms 1) direct scavenging action mediated via the α-amino group 2) indirect mechanism mediated through the ability to chelate Cu(II). • The α-amino group in its NH_3 form mediates an antioxidant effect and in its NH_2 form, a pro-oxidant effect
Chapter Five	To investigate the effects of L-arginine and L-citrulline on *endogenous* ROS levels in platelets and to establish what affect this had on physiological function.	• Millimolar concentrations required to inhibit collagen induced platelet aggregation ($EC_{50\ of}$ 157.7mM and 128mM for L-Arg and L-Cit respectively) • Inhibition of aggregation mediated via scavenging of $^{\bullet}OH$. • The high concentrations of antioxidants required to inhibit platelet aggregation likely to be due to the presence of endogenous antioxidants and the fact that ROS may only play a minor role in platelet aggregation
Chapter Six	To investigate the effects of L-arginine and L-citrulline on *exogenous* ROS in a vascular model of superoxide mediated endothelial dysfunction.	• L-citrulline was able to prevent ROS mediated endothelial dysfunction in aortic vessels at 100μM and 3mM although this is unlikely to be mediated via a ROS dependent effect. • L-arginine had no protective effects at any concentration.
	To investigate the effects of L-arginine and L-citrulline on *exogenous* ROS in a model of hydroxyl radical mediated lipid peroxidation.	• L-arginine and L-citrulline were able to significantly inhibit $^{\bullet}OH$ mediated lipid peroxidation at concentrations of amino acid found in human plasma (53.16μM and 87.25μM respectively).

In summary, the main findings from this body of work predict that administration of L-arginine or L-citrulline in an antioxidant capacity *in vivo* would have varying results depending upon the relative role of superoxide vs. hydroxyl radicals in the progression of the disease state. The lack of superoxide scavenging effects would suggest that in disease states characterised by increased superoxide such as diabetes [21,92,147,226,415], heart failure [21,92,226] or hypertension [67,253,254,351] L-arginine and L-citrulline would afford little protective effect via direct scavenging of superoxide.

Studies in which L-arginine and L-citrulline have been shown to improve endothelial function with a concomitant decrease in superoxide levels are most likely due to indirect reduction of superoxide levels through the following potential mechanisms:

1. Inhibition of leukocyte adhesion to the vessel wall.

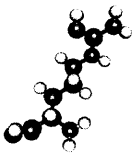
   L-arginine is capable of inhibiting leukocyte adhesion to the vessel wall [3,4,356,361]. In this scenario the reduction in superoxide is not due to radical scavenging by L-arginine, merely that the number of superoxide generating 'cells' is reduced.

2. Recoupling of NOS

   ADMA is an endogenous inhibitor of NOS and competes for the L-arginine transporter found in endothelial cell membranes and so can uncouple NOS leading to the production of superoxide. Exogenous L-arginine would increase the concentration of L-arginine to a level sufficient to successfully compete with ADMA. This would result in the re-coupling of NOS thus increasing NO• production and decreasing superoxide production.

3. Improving redox status

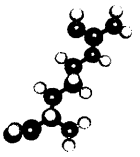
   L-arginine has also been shown to protect against oxidative stress via less direct actions such as decreasing xanthine oxidase activity [223] and improving antioxidant defence systems by increasing glutathione levels [223] and SOD



and catalase activity [89]. This would result in an overall decrease in superoxide levels without L-arginine having to directly interact with the free radical

The beneficial effects of L–citrulline in disease states such as hypercholesterolaemia [137] may in part be mediated through the ability of cell types such as macrophages and endothelial cells to convert L-citrulline into L-arginine through the actions of ASS and ASL [139,365,404,407]. Indeed, in a study by Hayashi *et al* [137], L-citrulline was demonstrated to produce pharmacological effects that closely resembled those seen upon L-arginine administration and NO action. This evidence combined suggests that although neither amino acid is able to directly interact with superoxide, exogenous administration may be beneficial in the long term rather than directly.

Both amino acids were able to inhibit hydroxyl radicals at amino acid concentrations found in plasma (~100μM) (Chapter Three). This antioxidant action transferred to an *ex vivo* system which demonstrated prevention of lipid peroxidation at physiological concentrations (chapter Six). It has been suggested that due to the highly reactive nature of the hydroxyl radical (its reaction rate is only limited by diffusion), it will react indiscriminately and therefore no one molecule is better that the other when it comes to their ability to scavenge hydroxyl radicals, put another way, most organic molecules will have demonstrate antioxidant capacity against the hydroxyl radical. It is important to factor in however two things when considering L-arginine and L-citrulline. The first is that through their ability to chelate copper (chapter Four); these amino acids possess the capability to actually prevent hydroxyl radical synthesis through Fenton chemistry. Secondly we have also demonstrated that upon direct interaction with the hydroxyl radical the most likely product is ultimately non-toxic water (chapter Four). This is in contrast with the majority of other molecules, which upon interaction with hydroxyl radicals often form toxic products such as lipid peroxides and protein carbonyls, which are themselves detrimental to the surrounding environment. This is one test of a physiologically effective antioxidant, that the product formed upon scavenging the ROS in



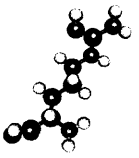
question is less toxic than the parent radical. In the case of L-arginine and L-citrulline, this would appear to be true.

Through the scavenging of hydroxyl radicals, L-arginine and L-citrulline would potentially be able to inhibit effects such as aortic contraction [215], induction of calcium mobilisation [83] and cell death mediated through damage to cell membranes, nucleic acid and proteins. Another mechanism through which hydroxyl radicals interact with their environment is through their ability to oxidise lipids. Lipid peroxides are able to react with proteins, sugars, nucleic acids and neighbouring cell membrane PUFA's [336]. They can also abstract a hydrogen from cholesterol and oleic acid [380] and are therefore capable of causing severe cell damage. Lipid peroxidation is also associated with endothelial dysfunction and reduced bioactivity of NO through the mechanisms described below:

1. Ox-LDL is cytotoxic to endothelial cells [257].
2. Ox-LDL promotes the recruitment of inflammatory cells to the vessel wall which is thought to be the initiating step in the development of atherosclerosis and also results in increased local production of ROS from the accumulated cells [291].
3. Ox-LDL and other products of lipid peroxidation react with NO and decrease its production [62,263].
4. Ox-LDL decreases eNOS protein levels in endothelial cells [222].
5. Products of lipid peroxidation e.g. lysophosphatidylcholine, may interfere with signal transduction and receptor mediated stimulation of NOS activity [180,188] and activation of sGC [323].

The ability of L-arginine and L-citrulline to prevent endothelial dysfunction *in vivo* may therefore be in part through inhibition of lipid peroxidation.

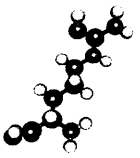
The question therefore remains, are L-arginine and L-citrulline exerting their protective effects through a purely antioxidant action? The answer to this question is almost certainly not. Although both amino acids are able to reduce



mediated through an ability to inhibit hydroxyl radical production and to scavenge free hydroxyl radicals. The ability of both molecules to scavenge hydroxyl radicals gives insight into the known capability of L-arginine to inhibit LDL oxidation and prevent lipid peroxidation and L-citrulline to decrease atheromatous lesion size. The ability of both amino acids to inhibit lipid peroxidation may also explain why exogenous administration of these amino acids results in improved endothelial function as oxidised lipids negatively influence the NO signaling pathway in the long term. Their inability to scavenge superoxide radicals however would suggest that their protective effects are only in part mediated via scavenging of ROS and that a certain degree of protection is mediated via an antioxidant independent effect.

7.3 Implications for future work

Based on the results of this thesis, I feel that the next logical step, in terms of L-arginine / L-citrulline research, is to investigate the effects of hydroxyl radical scavenging on cell / tissue function in conditions of oxidative stress. It is commonly assumed that a number of the protective effects seen upon administration of L-arginine e.g. inhibition of inflammatory cell adhesion to the endothelium and potentiation of endothelium dependent vascular smooth relaxation are mediated via its ability to increase NO levels. In a similar vein, studies that have investigated the effects of L-citrulline on vascular function have commonly used L-citrulline for its role as a potential precursor for L-arginine [137]. This thesis would suggest that these effects may, in part be mediated through the ability of the amino acids to both directly scavenge hydroxyl radicals and to inhibit their production. Specifically, an investigation into the ability of L-arginine and L-citrulline to inhibit hydroxyl radical mediated events e.g. aortic contraction and induction of calcium mobilisation would be of great interest. Also the ability of both amino acids to prevent LDL oxidation and the damaging effects of this molecule e.g. its ability to recruit inflammatory cells to the vessel wall [291], decrease NO production [62,263] and decrease eNOS protein levels in endothelial cells [222] is an area I feel warrants further scrutiny. It is vital that the



beneficial effects of these amino acids demonstrated in numerous clinical trials are fully characterised specifically in terms of their mechanism of action. The ability of these amino acids to reduce hydroxyl radical levels and the downstream effects these may have warrants further investigation. These amino acids have the potential to be a safe and inexpensive treatment for disease states associated with increased levels of hydroxyl radicals.

Publications & Presentations arising from these studies

- **Papers**

  Coles K.E. and James P. E. An investigation into the antioxidative properties of L-arginine and L-citrulline - their role in the prevention of ROS mediated endothelial dysfunction' (in preparation).

- **Presentation at Cardiovascular IRG meeting.**

  Roles of L-arginine and L-citrulline in platelet function.

  July 2005, Cardiff University School of Medicine, Cardiff, UK.

- **Presentation at Minisymposium – Oxidant and NO signaling.**

  Novel properties of L-arginine and L-citrulline.

  March 2006, Cardiff University School of Medicine, Cardiff, UK.

- **Poster presentation at 2nd Joint 62nd Harden Conference/EMBO Workshop - NO; a radical in control.**

  NOS-independent roles of L-arginine and L-citrulline.

  April 2006, Cirencester, UK.

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