# **Oxidative Damage of Endothelial Cells**

Thesis submitted for the degree of Doctor of Philosophy At the University of Leicester

By

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December 1999

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# **Statement of Originality**

The accompanying thesis, submitted for the degree of Doctor of Philosophy, entitled 'Oxidative Damage of Endothelial Cells', is based on work undertaken by the author in the Department of Medicine at the University of Leicester during the period from September 1991 to April 1999. As far as the author is aware, all of the work described within is original unless otherwise acknowledged in the text or in the references. None of the work has been submitted for any other degree in this or any other university.

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# **Oxidative Damage of Endothelial Cells**

# Abstract

Most cardiovascular diseases are associated with pathophysiological alterations in endothelial cell function. According to the 'response-to-injury' hypothesis, endothelial dysfunction is the first step to atherosclerosis, which may be caused by oxidative stress, such as can occur during ischemia reperfusion, leucocyte oxidative burst or chronic exposure to oxidant stress.

This study sought to investigate the consequences of different degrees of oxidative stress on endothelial cells, using a cultured endothelial cell model; principally bovine aortic endothelial cells, subjected to oxidative stress.

High concentrations of  $H_2O_2$  or a superoxide generating system caused rapid endothelial cell death, as evidenced by increased membrane permeability, which could be partially protected by myoglobin.

Extracellular  $H_2O_2$  caused a rapid increase in intracellular peroxidation but was also eliminated by endothelial cells. However, the anti-oxidant capacity of the bovine endothelial cells was very weak and could be overcome by as little as 5 femtomol hydrogen peroxide per cell. These effects were directly related to the amount of  $H_2O_2$  available to each cell, rather than the concentration.

Exposure to relatively low amounts of  $H_2O_2$  (<0.5 picomol/cell) led to reduced endothelial cell function including prostacyclin production and mitochondrial dehydrogenase activity, and inhibited cell migration and proliferation. The cells showed gradual, partial recovery from these damaging effects.

At low amounts (0.1 to 0.5 picomol/cell)  $H_2O_2$  induced endothelial cell apoptosis within 48 hours of the exposure. After this time, some of the surviving cells showed evidence of senescence and could remain in culture for up to 30 days. Senescence was accompanied by an increase in cytoplasmic volume and accumulation of lipofuscin. Investigation of  $\beta$ -galactosidase activity suggested that the increased enzyme expression was linked to cell cycle rather than senescence.

In conclusion, endothelial cells are very sensitive to oxidative damage but the nature of the damage is related to the degree of oxidative stress. The effects of oxidative stress may play an important role in atherosclerotic and cardiovascular diseases.

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# **Chapter 1: Introduction**

# **1.1. Endothelial Cell Physiology**

The endothelial cells play an important role in all aspects of cardiovascular physiology and homeostasis (Rubanyi, 1991). Endothelium has long been viewed as an inert cellophane-like membrane that lines the circulatory system with its primary essential function being the maintenance of wall permeability. It is now recognised as a dynamic, heterogeneous, disseminated organ that possesses vital secretory, synthetic, metabolic, and immunological functions (Fishman, 1982) and that most major cardiovascular diseases are associated with pathophysiologic alterations in endothelial cell structure and/or function.

Endothelial cells line vessels in every organ system and regulate the flow of nutrient substances, diverse biologically active molecules, and have a profound influence on blood cells. Crucial to endothelial functions are membrane-bound receptors which interact with numerous molecules including proteins (e.g., growth factors, coagulant and anticoagulant proteins), lipid transporting particle (e.g., low-density lipoprotein), metabolites (e.g., nitrous oxide and serotonin), and hormones (e.g., endothelin-1), as well as through specific junctional proteins and receptors that govern cell-cell and cell-matrix interactions (Cines *et al.*, 1998)

The endothelium also plays a pivotal role in regulating blood flow. In part, this results from the capacity of quiescent endothelial cells (EC) to generate an active antithrombotic surface that facilitates transit of plasma and cellular constituents throughout the vasculature. Perturbations, such as those that may occur at sites of inflammation, or high hydrodynamic shear stress, disrupt these activities and induce EC to create a prothrombotic and antifibrinolytic microenvironment. Blood flow is also regulated, in part, through secretion and uptake of vasoactive

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substances such as nitric oxide and prostacyclin by the endothelium, which acts in a paracrine manner to constrict and dilate specific vascular beds in response to stimuli such as endotoxin (Rubanyi, 1991).

# 1.1.1. Cell Structure

The endothelium consists of a confluent monolayer of thin, flattened, polygonal or elongated cells lining the inner or abluminal surface of the heart and blood vessels between the circulating blood and the body's tissue. The endothelial cell surface in an adult human is composed of approximately 1 to 6 x  $10^{13}$  cells and covers a surface area of approximately 1 to 7 m<sup>2</sup> (Augustin *et al.*, 1994).

A typical endothelial cell is about 25 to 50  $\mu$ m long and 10 to 15  $\mu$ m wide. Besides the nucleus and a small nucleolus, it contains sparse rough endoplasmic reticulum, ribosomes and polyribosomes, a few mitochondria, some smooth endoplasmic reticulum and a small Golgi complex. Lysosomes and residual bodies (lipofuscin granules) are more frequently seen in arterial endothelium. Multivesicular bodies (a variety of lysosome) are also occasionally seen in vascular endothelial cells (Simionescu and Simionescu, 1977).

In addition to those organelles common to eukaryotic cells, endothelial cells contain a unique set of structures consisting of long, cylindrical, single membranebound, rod like bodies containing 6 to 26 microtubules set in an electron dense matrix. These specific organelles of the endothelial cell are the Weibel-Palade body, which are scattered throughout the cytoplasm, and may also be in the neighbourhood of the Golgi complex. The bodies have a maximum length at around 3.2  $\mu$ m and their diameter ranges from 0.1 to 0.5  $\mu$  (Weibel and Palade, 1964). Weibel-Palade body contains a variety of proteins, but in particular von Willebrand factor (vWF). They are also the storage site for P-selectin, which is a membrane bound receptor that is rapidly redistributed to the cell surface by a variety of stimuli which cause the Weibel-Palade bodies to release their contents to the outside.

### **Cell Membrane**

Endothelial cells are bounded by a lipid bilayer plasma membrane. Specialisation of the polysaccharide-rich cell coat (glycocalyx) at the cell surface forms the surface coat, while that at the base forms the basal lamina. The abluminal surface of endothelial cells bears a slender, fuzzy surface coat about 5 nm thick. The surface coat covers cells and extends into pinocytotic vacuoles and micropinocytotic vesicles (Ausprunk *et al.*, 1981).

Tight junctions, gap junctions and adherens junctions are the three major types of intercellular junctions between endothelial cells of blood vessels. These junctions, highly dynamic and reversible, are formed by transmembrane proteins linked to cytoplasmic and cytoskeletal proteins (Dejana *et al.*, 1995; Telo *et al.*, 1997). Tight junctions allow very close contact between adjacent cells. Gap junctions are formed by hydrophilic channels (connexons), which allow the exchange of ions and molecules between adjacent cells. Adherens junctions are formed by transmembrane cadherins, and are required for the formation of both tight junctions and gap junctions (Dejana *et al.*, 1995; Telo *et al.*, 1997).

## 1.1.2. Vasomotor

Rubanyi and Vanhoutte (Rubanyi and Vanhoutte, 1985) demonstrated that the endothelium could mediate not only rapid relaxation of the vasculature, but also rapid contraction. Endothelial cells contribute to the regulation of blood pressure and blood flow by releasing vasodilators such as nitric oxide (NO) (Furchgott, 1988) and prostacyclin (PGI<sub>2</sub>) (Zimmerman *et al.*, 1990), as well as vasoconstrictors, including endothelin (ET) and platelet-activating factor (PAF)

(Yanagisawa *et al.*, 1988). The major biological effects of those molecules are regulated by localization of specific receptors on vascular cells, through their rapid metabolism, or at the level of gene transcription. NO is constitutively secreted by EC, but its production is modulated by a number of exogenous chemical and physical stimuli, whereas the other known mediators (PGI<sub>2</sub>, ET, and PAF) are synthesized primarily in response to changes in the external environment (Balligand *et al.*, 1995) (Topper *et al.*, 1996).

### **Nitric Oxide**

In 1980 Furchgott and Zawadzki demonstrated a humoral factor that mediated endothelium-dependent relaxation in vascular tissue (Furchgott and Zawadzki, 1980), which later became known as endothelium-derived relaxing factor (EDRF). EDRF was also shown to inhibit platelet aggregation and adhesion (Griffith *et al.*, 1984). These actions, together with its vasodilator effects, are mediated by activation of the soluble guanylate cyclase, leading to increases in cyclic guanosine monophosphate (cGMP) within the platelet or smooth muscle cells (Furchgott, 1984; Griffith *et al.*, 1984; Moncada, 1987).

In 1986 it was suggested that EDRF might be nitric oxide (NO) or a closely related species (Furchgott, 1988). Moncada *et al* found the pharmacological properties of EDRF and NO on vascular smooth muscle and on platelets to be identical (Moncada *et al.*, 1988). It has also been demonstrated that cultured endothelial cells release sufficient NO to account for the effects of EDRF on vascular strips and on platelet aggregation and adhesion (Palmer *et al.*, 1987).

NO is a short-lived heterodiatomic free radical product, generated through the oxidation of L-arginine to L-citrulline by NO synthases (Stamler and Loscalzo, 1992). In endothelial cells NO is synthesised by at least two enzymes: a constitutive NO synthase (cNOS; predominantly membrane bound) and inducible

NO synthase (iNOS; predominantly cytosolic). Both enzymes are calcium- and calmodulin-dependent and utilise at least five cofactors (NADP, tetrahydrobiopterin, flavin adenine dinucleotide, flavin mononucleotide and heme) (Furchgott, 1988). Cytokines (IL-1 $\alpha$  and IFN- $\gamma$ ) have been shown to stimulate bovine microvascular endothelium in culture to express an inducible isoform of NO synthase, iNOS, or the Nos2 gene product (Balligand et al., 1995). One isoform, endothelial NOS (eNOS) or the Nos3 gene product, is constitutively active in ECs but is stimulated further by receptor-dependent agonists that increase intracellular calcium and perturb plasma membrane phospholipid asymmetry (Venema et al., 1995). Receptor-dependent agonists that stimulate eNOS include thrombin, ADP, bradykinin, substance P, and muscarinic agonists, in addition to shear stress (Topper et al., 1996) and cyclic strain (Awolesi et al., 1994). The increase in eNOS activity evoked by shear stress contributes to the phenomenon of flow-mediated vasodilatation.

EC-derived NO has several important effects on the vasculature. Firstly, NO maintains basal tone by relaxing vascular smooth muscle cells (Loscalzo and Welch, 1995) through the binding of NO to the heme prosthetic group of guanylyl cyclase. Endothelial-derived NO also inhibits platelet adhesion, activation, secretion, and aggregation, inhibits expression of P-selectin on platelets and, by inhibiting the agonist-dependent increase in intraplatelet calcium (Mendelsohn *et al.*, 1990), suppresses the calcium-sensitive conformational change in the heterodimeric integrin glycoprotein  $\alpha_{IIb}\beta_3$  (GP IIb-IIIa) required for fibrinogen binding (Michelson *et al.*, 1996).

Additionally, NO appears to promote platelet disaggregation, in part through a cyclic GMP-dependent mechanism (Mendelsohn *et al.*, 1990), and indirectly by impairing the activity of phosphoinositide 3-kinase, which normally supports conformational changes in GP IIb-IIIa, rendering its association with fibrinogen effectively irreversible.  $PGI_2$ , which does not affect platelet adhesion (Radomski

et al., 1987), acts synergistically with NO to inhibit other steps in the platelet activation cascade (Stamler et al., 1989).

In addition to these effects on the vasculature, endothelial-derived NO inhibits leukocyte adhesion to the endothelium (De Caterina *et al.*, 1995) and inhibits smooth muscle cell migration (Marks *et al.*, 1995) and proliferation (Garg and Hassid, 1989). These latter effects serve to limit neointimal proliferation that occurs after vascular injury and, combined with its stimulatory effect on EC migration and proliferation (Fukuo *et al.*, 1995) suggest that NO helps to sustain vascular reparative mechanisms.

However, Beckman *et al* have suggested that NO may not be completely benign (Beckman *et al.*, 1990). They showed that in some circumstances, NO can be converted in endothelial cell to peroxynitrite, a potentially toxic molecule. It has also been reported that NO can produce hydroxyl radicals and initiate lipid peroxidation (Hogg *et al.*, 1992).

#### **Prostacyclin and PAF**

The contribution of endothelial cells to the regulation of vasomotor tone is even more finely regulated as evidenced by the production of additional vasoactive compounds such as prostacyclin (PGI<sub>2</sub>) and PAF. Prostacyclin and PAF both are intercellular signaling molecules synthesized by stimulated EC *in vitro* and *in vivo* (Zimmerman *et al.*, 1990). Both are lipids: PGI<sub>2</sub> being an eicosanoid and PAF being a phospholipid (Meade *et al.*, 1996). Neither is constitutively present in resting human ECs nor stored within the cell. The synthesis of each is induced rapidly by humoral and mechanical stimuli via discrete, regulated pathways. Once formed, PGI<sub>2</sub> and PAF have relatively short half-lives which limits the magnitude of signals and exerts control over their biological activities (Campbell and Halushka, 1996). PAF, expressed on the surface of the endothelium, remains cell-associated even in the presence of albumin or other acceptor molecules (Whatley *et al.*, 1996) and binds to, and activates, its receptor on leukocytes and platelets to promote adhesion (Lorant *et al.*, 1995).

In contrast,  $PGI_2$  is rapidly released from ECs (Weksler *et al.*, 1977). PAF and  $PGI_2$  have spatially differentiated realms of signaling, even though both derive from a common precursor and are synthesized concurrently (Whatley *et al.*, 1996), (McIntyre *et al.*, 1985). PAF is specialized to signal leukocytes and platelets at the cell surface, whereas  $PGI_2$  acts primarily in solution to retard platelet aggregation and deposition.

 $PGI_2$  synthesis is induced at sites of vascular perturbation, where it may regulate vasoconstriction and platelet deposition (Topper *et al.*, 1996).  $PGI_2$  causes vasodilatation and altered regional blood flow by relaxing vascular smooth muscle (Campbell and Halushka, 1996).  $PGI_2$  receptor is present on both vascular smooth muscle cells and platelets (Coleman *et al.*, 1994), indicating that  $PGI_2$  acts principally to modulate the function of these two cell types (Majerus, 1983).

#### Endothelin

The ability of the endothelium to produce vasoconstrictors was established by Yanagisawa and colleagues who, in 1988, reported the discovery of the polypeptide endothelin-1 (ET-1) (Yanagisawa *et al.*, 1988). This single polypeptide is now recognised as the proband of a family of endothelins that are considered to be the most potent vasoconstrictors yet identified (Inoue *et al.*, 1989). This family of peptides consists of ET-1, ET-2 and ET-3 and their rank contractile potencies are ET-1= ET-2>ET-3. Endothelins are formed by the conversion of big-endothelin to endothelin by one or two endothelin converting enzymes. ET-1 is formed after transcription of the gene encoding

preproendothelin-1, the inactive precursor of ET-1, after stimulation by hypoxia, shear stress, and ischaemia. ET-1 released from ECs binds to the abundant G-protein-coupled ET<sub>A</sub> receptor expressed on vascular smooth muscle cells, which results in an increased intracellular calcium concentration and, in turn, increases vascular smooth muscle cell tone (Simonson and Dunn, 1990). NO shortens the duration of these effects by accelerating the restoration of intracellular calcium to basal levels (Goligorsky *et al.*, 1994). ET-1 potentiates the vasoconstrictor actions of catecholamines, which, in turn, potentiate the actions of ET-1. In states of endothelial dysfunction, such as atherosclerosis, in which concentrations of bioactive NO are reduced, the relatively unopposed actions of ET-1 promote vasoconstriction and smooth muscle proliferation (Lopez *et al.*, 1990).

Although primarily thought of as vasoconstrictive agents, endothelins can also induce transient vasodilatation. Two classes of endothelin receptor have been identified in vascular tissue, ET<sub>A</sub> and ET<sub>B</sub>, both of which appear to be G-protein linked. Recent evidence would suggest that there might be two subtypes of the ET<sub>B</sub> receptor. ET<sub>A</sub> is found in vascular smooth muscle cells and is responsible for the vasoconstrictive response, which is mediated through two distinct signal transduction systems. One is related to an enhanced influx of calcium ions through the activation of calcium channels, and the second by phospholipase C and  $A_2$  activation. ET<sub>B</sub> receptors are located on vascular endothelial cells and exert their effects, in part, by stimulation of EDRF/NO and PGI<sub>2</sub> production. However, neither the inhibition of EDRF/NO synthesis nor blockade of cyclooxygenase has been shown to reduce the vasodilatory action of endothelin. Endothelin mediated relaxation is also mediated by a calcium-activated  $K^+$ channel, which is activated by low doses of endothelin and inactivated by higher doses of endothelin. Endothelins exert long lasting effect on vascular preparations because of their prolonged receptor kinetics, suggest that endothelins are more likely to be involved in longer term responses regulating vascular tone and blood flow, and may not be involved in the acute management of vascular homeostasis.

(Katusic, 1991) Hypoxia and increased transmural pressure evoke the release of a non-prostanoid endothelium derived constricting factors (EDCF<sub>1</sub>) that is not an endothelin. This EDCF is likely to be involved in the more immediate responses required to modulate vascular tone (Katusic, 1991).

### 1.1.3. Coagulation

The endothelial cells play a primary role in the regulation of intravascular coagulation by four separate but related mechanisms: participation in procoagulant pathways, inhibition of procoagulant proteins, regulation of fibrinolysis, and production of thrombo-regulating compounds (Rubanyi, 1991). However, when the endothelium is perturbed the cells undergo programmatic biochemical changes that culminate in their transformation to a prothrombotic surface. A dynamic equilibrium exists between these two states, modulated both at the level of gene transcription and at the level of the intact cell, that often permits the injured endothelium to return to its unperturbed state once the procoagulant stimulus has dissipated (Bombeli *et al.*, 1997b).

### **Procoagulant activity**

The focal point for the coagulation cascades is the generation of the enzyme thrombin that cleaves fibrinogen to form an insoluble fibrin clot. The endothelium participates in this cascade by producing a number of cofactors, including high molecular weight kininogen (HMWK), Factor V, Factor VIII, and tissue factor (Rapaport and Rao, 1995; Nemerson, 1995). The pivotal step in transforming the EC membrane from an anticoagulant to a procoagulant surface is the induction of tissue factor (TF), which dramatically accelerates factor VIIa-dependent activation of factors X and IX. TF is not expressed by unperturbed endothelium, but can be induced by diverse agonists, including thrombin, endotoxin, several cytokines, shear, hypoxia, oxidized lipoproteins, and many

other provocations (Rapaport and Rao, 1995; Nemerson, 1995). TF expression is rapidly induced after vascular injury (Taubman *et al.*, 1997), and is found associated with ECs within atherosclerotic plaque (Thiruvikraman *et al.*, 1996) and in tumour-derived vessels. TF mRNA and protein levels decline when continually exposed to agonists. Cells in culture also shed microvesicles containing TF, and plasma levels of TF are elevated in patients with disseminated intravascular coagulation (Shimura *et al.*, 1996).

Once ECs expressing TF are exposed to plasma, prothrombinase activity is generated and fibrin is formed on the surface of the cells (Stern *et al.*, 1985). This implies that ECs express binding sites for factors IX, IXa, X. and Xa, as well as for thrombin and fibrin (Brinkman *et al.*, 1994). Factor IX has been shown to bind type IV collagen in the EC matrix (Cheung and others, 1996). Procoagulant activity is accelerated by exposure of anionic phospholipids that may occur as a consequence of apoptosis (Bombeli *et al.*, 1997a). Phospholipid exposure may account for most of the factors X and IX binding.

Generation of thrombin can have direct effects on the endothelial cells via interaction with specific thrombin receptors. The EC thrombin receptor, protease-activated receptor-1 (PAR 1), is a high-affinity G-protein-coupled protein (Woolkalis *et al.*, 1995) that is activated when a fragment derived from the amino terminus of the protein, formed as a result of cleavage by thrombin, binds to the cell-associated receptor. Binding of thrombin leads to a wide array of changes in expression of prothrombotic and antithrombotic molecules by cultured ECs, including TF, PAI-1, NO, PAF, ET, and PGI<sub>2</sub>, among others (Kanthou and Benzakour O, 1995) and disruption of cell-cell contacts (Garcia *et al.*, 1995). Thrombin is also mitogenic for ECs, fibroblasts and smooth muscle cells and is chemotactic for monocytes.

#### **Anticoagulant Activity**

Control of thrombin generation is a pivotal step in the balance between the natural antithrombotic and the induced procoagulant activities of the endothelium. Several highly regulated pathways have evolved to constrain the generation and activity of thrombin (Rosenberg and Rosenberg, 1984), so that very little enzyme activity is found in the plasma of healthy individuals (Bauer *et al.*, 1987). The matrix surrounding the endothelium contains heparan sulphate and related glycos-amino-glycans (GAGs) that promote the activity of cell/matrix associated antithrombin III (AT-III) (Marcum and Rosenberg, 1984). The subendothelium contains dermatan sulphate, which promotes the antithrombin activity of heparin cofactor II (Tollefsen and Pestka, 1985). ECs also prevent thrombin formation through the expression of tissue factor pathway inhibitor (TFPI), which binds to factor Xa within the tissue-factor/VIIa/Xa complex (Broze, 1995). TFPI is released from its EC stores by heparin. TFPI and AT-111 both contribute to physiologic haemostasis and can be depleted in acquired thrombotic states (Bombeli *et al.*, 1997a), (Jesty *et al.*, 1996).

The endothelium also helps to regulate thrombin activity through the expression of thrombomodulin (TM) (Esmon and Fukudome, 1995). Binding of thrombin to TM switches the substrate specificity from factors X (prothrombotic) to protein C (antithrombic). In turn, the activity of activated protein C (APC) is enhanced by its cofactor protein S, which is synthesized by EC, among other cell types (Fair *et al.*, 1986). ECs also express receptors for APC (Fukudome *et al.*, 1996), which regulate the activity of this pathway. APC, in turn, promotes the inactivation of activated factors V and VIII. Binding of thrombin to TM also dampens the enzyme's ability to activate platelets, factor V, factor XIII, and fibrinogen and promotes EC fibrinolytic activity. TM also inhibits prothrombinase activity indirectly by binding factor Xa (Thompson and Salem, 1986). Various inflammatory

cytokines down regulate TM gene transcription and accelerate TM internalisation (Mantovani *et al.*, 1997) while at the same time promote tissue factor expression. Soluble TM is also shed into plasma and elevated plasma levels have been identified in various disorders associated with EC injury (Cucurull and Gharavi, 1997).

#### Fibrinolysis

The endothelial surface is profibrinolytic and thus helps maintain blood in its fluid state (Hirsh *et al.*, 1994). The fibrinolytic system comprises an inactive proenzyme, plasminogen, two plasminogen activators (PA) and the plasminogen activator inhibitors (PAI). Tissue type plasminogen activator (t-PA) and urokinase-type plasminogen activator convert plasminogen into active plasmin, which degrades fibrin into soluble fibrin degradation products. The effect of plasminogen activators can be inhibited by PAI. Normally about 40% of the t-PA is in complexed form with PAI-1 and is not active (van Hinsbergh *et al.*, 1991).

Endothelial cells synthesize and secrete both plasminogen activators and their inhibitors (PAI). Many reagents can stimulate the synthesis of t-PA by endothelial cells, for example phorbol myristate acetate, activated protein C, basic fibroblast growth factor and thrombin. But most of these reagents also stimulate the production of PAI-1 (Lijnen and Collen, 1997).

Thus, endothelial cells play an important role in all aspects of cardiovascular physiology. However, endothelial cells are also constantly exposed to many hostile substances in blood, including oxidised lipids, cigarette smoke and reactive oxygen species. Such insults may lead to endothelial cell damage and dysfunction.

# **1.2.** Oxidative Damage by Reactive Oxygen Species

## **1.2.1. Introduction**

In 1954 Commoner *et al* reported the presence of hydroxyl radical in living matter using electron spin resonance spectroscopy (ESR) (Commoner *et al.*, 1954). In 1956, Denham Harman suggested that free radicals produced during aerobic respiration cause cumulative oxidative damage. Harman hypothesized that endogenous oxygen radical generation occurs *in vivo*, as a by-product of enzymatic redox chemistry (Harman, 1956). He ventured that the enzymes involved would be those "involved in the direct utilization of molecular oxygen, particularly those containing iron." Finally, he hypothesized that traces of iron and other metals would catalyse oxidative reactions *in vivo* with possible peroxidative chain reaction.

The discovery in 1969 of the enzyme superoxide dismutase (SOD) (McCord and Fridovich, 1969) provided the first compelling evidence of *in vivo* generation of superoxide anion ( $O_2$ ) The use of SOD as a tool to locate subcellular sites of  $O_2$  generation led to a realization that mitochondria are a principal source of endogenous ROS (Chance *et al.*, 1979).

There are several definitions of the term free radical. The broad one is defined as any species that has one or more unpaired electrons, which includes the atom of hydrogen, most transition metal ions and the oxygen molecule (Halliwell, 1989). However, the main free radicals produced *in vivo* are superoxide anion and hydroxyl radical, hence a more restricted definitions is adopted. That is any species that has single unpaired electron in an orbital in the outermost electron shell is defined as free radical. If this free radical is oxygen centred it is called an oxygen free radical. Hydrogen peroxide has no unpaired electrons and is not a radical. However in biological systems hydrogen peroxide is dismuted from superoxide anion and is necessary to the formation of hydroxyl radical. Therefore in this thesis hydrogen peroxide, superoxide anion and hydroxyl radical are termed reactive oxygen species (ROS).

Ground-state diatomic oxygen, despite being a radical species and the most important oxidant in aerobic organisms, is only sparingly reactive itself. It has two unpaired electrons each located in an anti-bonding orbital. The two unpaired electrons have the same spin quantum number (parallel spin), and so if oxygen attempts to oxidise another atom or molecule by accepting a pair of electrons from it, both new electrons must be of parallel spin to fit into the vacant spaces in the orbitals. Most biomolecules are covalently bonded non-radicals, and the two electrons forming a covalent bond have opposite spins and occupy the same molecular orbital. Hence the reaction of oxygen with biomolecules is spin restricted. As a result, O<sub>2</sub> preferentially accepts electrons one at a time from other radicals (such as transition metals in certain valences). Thus, in vivo, typical twoor four-electron reduction of O<sub>2</sub> relies on coordinated, serial, enzyme-catalysed one-electron reductions, and the enzymes that carry these out typically possess active-site radical species such as iron. One- and two-electron reduction of O<sub>2</sub> generates  $O_2$  and hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>), respectively, both of which are generated by numerous routes in vivo. In the presence of free transition metals (in particular iron and copper),  $O_2$  and  $H_2O_2$  together generate the extremely reactive hydroxyl radical (•OH). Ultimately, •OH is assumed to be the species responsible for initiating the oxidative destruction of biomolecules (Halliwell and Gutteridge, 1989).

## **1.2.2.** Chemistry of Reactive Oxygen Species

### Superoxide anion

In organic solvents, superoxide is a strong base and nucleophile. In aqueous solution, superoxide is extensively hydrated and much less reactive, acting as a reducing agent and as a weak oxidising agent to such molecules as adrenalin. It also undergoes the dismutation reaction, which can be written overall as:

$$2 O_2 + 2 H^+ \rightarrow H_2O_2 + O_2$$

although at physiological pH it is largely the sum of the following two stages

$$O_2$$
' + H<sup>+</sup>  $\rightarrow$  HO<sub>2</sub>'  
HO<sub>2</sub>' + O<sub>2</sub>' + H<sup>+</sup>  $\rightarrow$  H<sub>2</sub>O<sub>2</sub> + O<sub>2</sub>

The overall rate of dismutation at pH 7 is about 5 X  $10^5 \text{ M}^{-1} \text{sec}^{-1}$ , and any reaction undergone by superoxide in aqueous solution will be in competition with this dismutation reaction (Bielski and Allen, 1977).

### **Hydrogen Peroxide**

Any biological system generating superoxide anion will produce hydrogen peroxide by non-enzymatic or SOD-catalysed dismutation unless, of course, all the superoxide is intercepted by some other molecule (e.g. a high concentration of cytochrome C). Hydrogen peroxide has no unpaired electrons and is not a radical. Pure hydrogen peroxide has limited reactivity, but it can cross biological membranes, which the charged superoxide can do only very slowly unless there is an anion channel through which it can move. Hydrogen peroxide is usually reduced to  $H_2O$  *in vivo* by catalase and glutathione peroxidase (Halliwell and Gutteridge, 1989).

### **Hydroxyl Radical**

The moderate reactivity of superoxide and hydrogen peroxide in aqueous solution makes it unlikely that the damage done by superoxide-generating systems can often be attributed to direct actions of superoxide or hydrogen peroxide. In general, however, the damage done to cells is probably due to their conversion into more highly reactive hydroxyl radical.

Because of the high reactivity, hydroxyl radical produced *in vivo* would react at or close to its site of formation. Further, reaction of hydroxyl radical with a biomolecule will produce another radical, usually of lower reactivity. Such less reactive radicals can cause their own problems, since they can sometimes diffuse away from the site of formation and attack specific biomolecules.

Most of the hydroxyl radical generated *in vivo* comes from the metal-dependent breakdown of hydrogen peroxide, according to the general equation

$$M^{n+} + H_2O_2 \rightarrow M^{(n+1)} + \bullet OH + OH$$

in which  $M^{n+}$  is a metal ion. The metal can be of copper or ferrous iron. If the metal ion is Fe<sup>2+</sup> the decomposition of hydrogen peroxide is called Fenton reaction, which has a second-order rate constant of 76 M<sup>-1</sup> sec<sup>-1</sup> (Walling, 1982)

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + \bullet OH + OH$$

Some  $Fe^{3+}$  complexes may react further with  $H_2O_2$  to produce ferryl (Fe<sup>IV</sup>).

### 1.2.3. Source of Reactive Oxygen Species In vivo

There are four main sites of ROS generation within the cell: mitochondrial electron transport, peroxisomal fatty acid metabolism, cytochrome P-450 reactions, and phagocytic cells (the "respiratory burst").

Mitochondrial respiration electron transport involves a coordinated four-electron reduction of  $O_2$  to  $H_2O$ , the electrons being donated by NADH or succinate to complexes I and II, respectively, of the mitochondrial electron transport chain (ETC). Ubiquinone (coenzyme Q, or UQ), which accepts electrons from complexes I (NADH dehydrogenase) and II (succinate dehydrogenase), undergoes two sequential one-electron reductions to ubisemiquinone and ubiquinol (the Q cycle), eventually transferring reducing equivalents to the remainder of the electron transport chain: complex III (UQ-cytochrome c reductase), cytochrome c, complex IV (cytochrome-c oxidase), and finally,  $O_2$  (Halliwell and Gutteridge, 1989). However, mitochondrial electron transport is imperfect and there is some one-electron reduction leaking of  $O_2$  leading to the formation of  $O_2^{\circ}$ . The spontaneous and enzymatic dismutation of  $O_2^{\bullet}$  yields  $H_2O_2$ , so a significant by-product of the actual sequence of oxidation-reduction reactions may be the generation of  $O_2^{\circ}$  and  $H_2O_2$ .

Early studies estimated the  $H_2O_2$  generation by mitochondrial preparations amounted to 1-2% of total electron flow (Boveris and Chance, 1973; Nohl and Hegner, 1978). Recently the experiment has been repeated with more physiological conditions, and the  $H_2O_2$  flux is found to be ~10-fold lower (Hansford *et al.*, 1997). Experiments using subcellular fractions of SOD-deficient *Escherichia coli* suggest *in vivo* leakage of 0.1% from the respiratory chain (Imlay and Fridovich, 1991).

Virtually all mitochondrial  $H_2O_2$  may originate as  $O_2$  (Boveris *et al.*, 1972). Because most cellular  $H_2O_2$  originates from mitochondria,  $O_2$  from the ETC may be a cell's most significant source of ROS (Chance *et al.*, 1979).

Cytochrome c rapidly scavenges  $O_2^{\bullet}$  in vitro and is present in mitochondria at local concentrations from 0.5 to 5 mM. In mitochondria, mSOD and cytochrome c rapidly scavenge  $O_2^{\bullet}$ , in the matrix and intermembrane spaces, respectively.

mSOD may act to increase the rate of  $O_2$  generation *in vivo*, by accelerating dismutation of  $O_2$  to  $H_2O_2$ . The actual role of mSOD *in vivo* may be to increase  $H_2O_2$  generation, with  $O_2$  as a rapidly consumed intermediate (Forman and Azzi, 1997). It remains uncertain about the mechanisms, quantity, and relevance of mitochondrial  $O_2$  generation *in vivo* (Nohl *et al.*, 1996), which is complicated by recent reports of enzymatic nitric oxide (NO<sup>•</sup>) generation in mitochondria. Because  $O_2$  and NO<sup>•</sup> react to form peroxynitrite (ONOO<sup>•</sup>), mitochondrial  $O_2$  generation may need to be considered in the light of its ability to destroy NO<sup>•</sup> and form ONOO<sup>•</sup>.

Peroxisomal  $\beta$ -oxidation of fatty acids generates H<sub>2</sub>O<sub>2</sub> as a by-product. Peroxisomes possess high concentrations of catalase, so it is unclear whether or not leakage of H<sub>2</sub>O<sub>2</sub> from peroxisomes is significant (Arnaiz *et al.*, 1995; Kasai *et al.*, 1986), but during rapid cell proliferation, oxidant leakage from peroxisomes may be enhanced (Oikawa and Novikoff, 1995).

Microsomal cytochrome P-450 enzymes metabolise xenobiotic compounds by catalysing their univalent oxidation or reduction. Some of the numerous cytochrome P-450 isozymes directly reduce  $O_2$  to  $O_2$ ' (Goeptar *et al.*, 1995; Koop, 1992) and may cause oxidative stress. An alternative route for cytochrome P-450-mediated oxidation involves redox cycling, in which substrates accept single electrons from cytochrome P-450 and transfer them to oxygen. This generates  $O_2$ ' and simultaneously regenerates the substrate, allowing subsequent rounds of  $O_2$ ' generation (Halliwell and Gutteridge, 1989).

Phagocytic cells attack pathogens with a mixture of oxidants and free radicals, including  $O_2$ ,  $H_2O_2$ , NO<sup>•</sup> and hypochlorite (Chanock *et al.*, 1994; Moslen, 1994; Robinson and Badwey, 1994). Neutrophils contain an NADPH oxidase that reduces molecular oxygen to superoxide anion. Hydrogen peroxide is derived from dismutation of superoxide anion. Activated neutrophils also secrete the

enzyme myeloperoxidase, which catalyses the formation of hypochlorous acid from hydrogen peroxide and chloride ions.

In addition to these four sources of oxidants, there exist numerous other enzymes capable of generating oxidants under normal or pathological conditions, often in a tissue-specific manner (Halliwell and Gutteridge, 1989). For example, the deamination of dopamine by monoamineoxidase generates  $H_2O_2$ , in some neurons, and has been implicated in the etiology of Parkinson's disease (Fahn and Cohen, 1992). Finally, the widespread catalytic generation of NO•, achieved by various isozymes of nitric oxide synthase and central to processes as diverse as vascular regulation, immune responses, and long-term potentiation, increases the potential routes for destructive oxidative reactions. The interaction between  $O_2^{\bullet}$  and NO• results in ONOO<sup>-</sup>, which is a powerful oxidant.

# **1.2.4. Lipid Peroxidation**

In a completely peroxide-free lipid system, the attack of free radical that has sufficient reactivity to abstract a hydrogen atom from a methylene ( $-CH_2-$ ) group in a biomembrane or polyunsaturated fatty acid is called first chain initiation of a peroxidation sequence. Hydroxyl radical can abstract H from lipids:

$$-CH_2-+ \bullet OH \rightarrow -\bullet CH-+H_2O$$

The rate constant for reaction of  $\cdot$ OH with artificial lecithin bilayers is about 5 x  $10^8 \text{ M}^{-1} \text{ sec}^{-1}$  (D J W Barber and J K Thomas 1978).

Superoxide anion is insufficiently reactive to abstract H from lipids. It cannot enter the hydrophobic interior of the membranes because of its charged nature.

Abstraction of H from a  $-CH_2$ - group leaves behind an unpaired electron on the carbon. The presence of a double bond in the fatty acid weakens the C-H bonds

on the carbon atom adjacent to the double bond and so makes H removal easier. The carbon radical tends to be stabilised by a molecular rearrangement to form a conjugated diene; these can undergo various reactions. For example, if two of them came into contact within a membrane they could cross-link the fatty acid molecules. However, the most likely fate of conjugated dienes under aerobic conditions is to combine with oxygen, especially as oxygen is a hydrophobic molecule that concentrates into the interior of membranes (Halliwell and Gutteridge, 1989). This reaction produces a peroxyl radical (ROO· or  $RO_2^{\bullet}$ ).

The peroxyl radicals are capable of abstracting H from an adjacent fatty acid side chain. They might also attack membrane proteins (Hunt *et al.*, 1988). The carbon radical formed can react with oxygen to form another peroxyl radical to continue the chain reaction of lipid peroxidation. The peroxyl radical combines with the hydrogen atom that it abstracts to give a lipid hydroperoxide.

### **1.2.5. Oxidative Damage**

Most of biological macromolecules (lipids, nucleic acids, and proteins) are susceptible to oxidative damage, both *in vitro* and *in vivo*.

The lipid peroxidation chain reaction eventually produces cyclic endoperoxides and unsaturated aldehydes, which are reactive and may inactivate enzymes (Chen and Yu, 1994; Szweda *et al.*, 1993), or operate as endogenous fixatives, reacting with proteins and nucleic acids to form heterogeneous cross-links (Chio and Tappel, 1969). Moreover, a primary effect of lipid peroxidation is decreased membrane fluidity, which alters membrane properties and can significantly disrupt membrane-bound proteins.

### **Nucleic Acid**

Oxidative damage to nucleic acids includes adducts of base and sugar groups, single- and double-strand breaks in the backbone, and cross-links to other molecules. The spectrum of adducts in mammalian chromatin oxidized *in vitro* and *in vivo* includes more than 20 known products, including damage to all four bases and thymine-tyrosine cross-links (Dizdaroglu, 1992a; Dizdaroglu, 1992b; Halliwell and Dizdaroglu, 1992), the adduct 8-oxo-guanine and deoxynucleoside 8-oxo-2,7-dihydro-2'-deoxyguanosine. Specific enzymatic repair of oxidative DNA damage lesions has recently been reported (Beckman and Ames, 1997; Bohr and Anson, 1995; Epe, 1996; Rosenquist *et al.*, 1997).

### Protein

The oxidative damage of proteins includes oxidation of sulphydryl groups, reduction of disulphides, oxidative adduction of amino acid residues close to metal-binding sites via metal-catalysed oxidation, reactions with aldehydes, protein-protein cross-linking, and peptide fragmentation (Stadtman and Oliver, 1991; Starke-Reed and Oliver, 1989). Some enzymes possessing active-site iron-sulphur clusters are sensitive to inactivation by  $O_2$ . (Kuo *et al.*, 1987). Mitochondrial aconitase is inactivated *in vitro* and *in vivo* by treatments that increase mitochondrial  $O_2$  involves the release of free iron from the enzyme, which catalytically exacerbates oxygen stress by production of •OH.

Iron and copper catalyse the homolytic cleavage of ROOH (the Fenton reaction), leading to the generation of  $\bullet$ OH (Halliwell and Gutteridge, 1989). It is  $\bullet$ OH that is the most reactive oxidant, reacting at diffusion-limited rates. The catalytic properties of iron and copper explain why cells possess metal-chelating proteins such as ferritin and transferrin, which reduce the concentration of redox-active metals (Halliwell and Gutteridge, 1986; Minotti and Aust, 1987). Increasing O<sub>2</sub>

partial pressure also aggravated oxidative stress *in vivo* due to a more pronounced flux of mitochondrial  $O_2$ • (Chance *et al.*, 1979).

# **1.3. Antioxidant Defences**

Cells are equipped with an impressive repertoire of antioxidant enzymes, as well as small antioxidant molecules. These include:

- Enzymatic scavengers such as SOD, which hastens the dismutation of O<sub>2</sub>• to H<sub>2</sub>O<sub>2</sub>, and catalase and glutathione peroxidase (GPX), which convert H<sub>2</sub>O<sub>2</sub> to water;
- Hydrophilic radical scavengers such as ascorbate, urate, and glutathione (GSH);
- Lipophilic radical scavengers such as tocopherols, flavonoids, carotenoids, and ubiquinol;
- Enzymes involved in the reduction of oxidized forms of small molecular antioxidants (GSH reductase, dehydroascorbate reductase) or responsible for the maintenance of protein thiols (thioredoxin reductase); and
- 5) The cellular machinery that maintains a reducing environment (e.g., glucose-6phosphate dehydrogenase, which regenerates NADPH).

The complement of defences deployed differs not only between organisms or tissues, but also even between cellular compartments. For instance, there exist in humans three forms of SOD (cytosolic Cu, Zn-SOD, mitochondrial Mn-SOD, and extracellular SOD), encoded and regulated independently (Fridovich, 1995).

Antioxidants are both parallel (different antioxidants can play similar roles, e.g., catalase and GPX) and serial (enzymes operate in tandem to decompose free radicals to harmless products, e.g., SOD and catalase).

**Repair of Oxidative Damage** The understanding for repairing oxidative damage is relatively poor. But it is clear that cells repair oxidized lipids (e.g., phospholipase A2 cleaves lipid peroxides from phospholipids; (Pacifici and Davies, 1991), oxidized nucleic acids (e.g., glycosylases specifically recognize and excise oxidized bases from double stranded DNA; (Bohr and Anson, 1995), and oxidized proteins (Giulivi *et al.*, 1994; Stadtman, 1995).

Interaction of ROS Generation, Oxidative Damage, and Repair The relationship between three components of oxidative stress, oxidant generation, antioxidant protection, and repair of oxidative damage is illustrated schematically in Figure 1. Increases in oxidant generation, and decreases in antioxidant protection and repair systems will result in oxidative damage.



Figure 1-1 Interaction of oxidant, antioxidant and repairing.

✓ Negative feed back

---- Positive feed back

An extremely important aspect of the interactions between oxidants, antioxidants, and repair are feedback loops, positive and negative, between them. Antioxidant defences and cellular repair systems have been shown to be induced in response to oxidative challenges (Dennog *et al.*, 1996) and are potential targets of oxidative destruction. Also the generation of oxidants may be enhanced by the malfunctioning of oxidatively damaged molecules (Brunk *et al.*, 1995). Primary oxidative destruction of any target (e.g., the components of the mitochondrial ETC, scavenging enzymes such as SOD, or DNA repair enzymes) might promote further oxidative damage in what is frequently called a "catastrophic vicious cycle." The reactions described above are believed to exist in many types of cells, including endothelial cells, although there can be quite big variations from one type of cell to another.

# **1.4. Oxidative Damage of Endothelial cells**

## **1.4.1. Endothelial Cell Activation**

Endothelial activation is defined as a quantitative change in the surface properties of the endothelium that cumulatively allows endothelial cells to perform new functions (Cotran and Pober, 1990). Endothelial cell activation allows endothelial cells to localize inflammation to sites of injury or infection. Under normal circumstances, the human vascular endothelium repels neutrophils in the circulation. In response to tissue injury, such as occurs with ischaemia, neutrophils accumulate and subsequently remove necrotic material, followed by healing and scar formation. In the context of ischaemia-reperfusion, endothelial cells appear to be activated to express proinflammatory properties that include the induction of leukocyte-adhesion molecules (Harlan et al., 1991). This is evident during myocardial infarction, with complete coronary occlusion. In this setting neutrophil accumulation on the activated endothelium is seen within 3 to 6 hours and peaks by 2 days after vessel occlusion (Engler et al., 1986). When myocardium is reperfused before infarction there is a more rapid accumulation of neutrophils with initiation of neutrophil infiltration in 3 minutes and a peak in 2 to 3 hours (Mehta et al., 1988; de Lorgeril et al., 1989). The more pronounced the degree of ischaemia, the more severe the accumulation of neutrophils. Once the neutrophils adhere, they are activated to release ROS, which contribute to a positive feedback loop by further activating the host inflammatory response to injury. These neutrophils subsequently transmigrate through the endothelium and confer much of the damage associated with ischaemia-reperfusion injury.

Little is known about the mechanism of hypoxia-induced endothelial activation. It has been demonstrated the adherence of leukocytes to cultured monolayer of human endothelial cells is increased when the endothelial cells are rendered anoxic (Shreeniwas et al., 1992). Anoxia-induced adhesion was prevented by the addition of cycloheximide to the cultures, suggesting that endothelial celldependent leukocyte adherence in an anoxic environment required new protein synthesis. The intercellular adhesion molecule (ICAM) was subsequently shown to be up regulated during hypoxia. Addition of a monoclonal antibody directed against the cytokine IL-l $\alpha$  prevented leukocyte adhesion to anoxic endothelial cells, suggesting that this cytokine is released by endothelial cells in an anoxic environment and, in an autocrine fashion, contributes to the anoxia-induced adhesion of leukocytes to endothelial cells. In addition to known adhesion molecules, such as E-selectin and ICAM, there may be another class of adherence molecules that arise in hypoxic cells. Ginis *et al* characterized a novel adherence molecule that appears to be specific to hypoxic endothelial cells and underlying muscle (Ginis et al., 1995). Once the neutrophils are adherent, hypoxic endothelial cells release agents that facilitate leukocyte-mediated injury, such as interleukin-8 (IL-8). Interleukin-8 is a member of the chemokine class of cytokines, which are important activators of leukocytes. High levels of IL-8 can be demonstrated in patients during acute myocardial infarction and in patients undergoing heart operation (Abe et al., 1993; Finn et al., 1993). Interleukin-8 also appears to be particularly important for endothelial cell-neutrophil interactions in an ischaemic environment. When endothelial cells are activated under hypoxic conditions, IL-8 is released, which can feed back to increase the adhesiveness of endothelial cells for neutrophils. IL-8 is particularly important in the regulation of transendothelial neutrophil migration (Huber et al., 1991). In addition, IL-8 has an important stimulating effect on activating neutrophils to release their toxic products. Collectively these experiments suggest that hypoxia and reoxygenation

alone (without necrosis) can induce neutrophil-endothelial cell interactions,

perhaps through IL-l $\alpha$  and IL-8-dependent mechanisms.

### **1.4.2.** Neutrophil-Mediated Injury

Once the endothelium is activated, neutrophil adherence involves a highly coordinated multistep process. Neutrophils are recruited from flowing blood and begin to roll along the endothelial cell surfaces when they come in contact with E-selectin and P-selectin on the activated endothelial surface. Endothelial cell selectins are stored in cytoplasmic vacuoles and are ready to be rapidly expressed (such as P-selectin) (Hattori *et al.*, 1989), or require *de novo* synthesis and are expressed on the cell surface in a specific time sequence after the signal transduction, gene transcription, protein synthesis, and expression on the endothelial cell plasma membrane (E-selectin). Because it can be rapidly deployed, P-selectin is thought to mediate the immediate adhesion of neutrophils after brief periods of myocardial ischaemia. After endothelial cell activation by hypoxia, P-selectin is rapidly expressed (in about 5 minutes) on the surface of endothelial cells and it quickly begins to tether neutrophils from the circulation to the surface of the endothelium.

Subsequently, neutrophil-slowing allows a firm, high-affinity bond between integrins on the leukocyte and the immunoglobulin gene super family (i.e., ICAM 1 and 2, PECAM) on activated endothelial cells (Springer, 1995). Neutrophils, activated on the endothelial cells, firmly engage the endothelial cell surface through activation of the leukocyte integrin. Leukocyte-endothelial cell interactions can also be promoted by upregulation of integrin receptors (ICAM-1 and ICAM-2) on the endothelial cell. Firmly bonded between the integrins on the neutrophil and, for example, ICAM-1 on the endothelial cell junctions. This migrates into the area of tissue injury through endothelial cell junctions. This migration through the endothelium seems to be in part mediated by PECAM, which is localised in the intraendothelial cell junctions to facilitate transendothelial trafficking (Springer, 1995). Endothelial cells and subendothelial matrix proteins are particularly vulnerable to toxic neutrophil constituents at this level of the process because antioxidants and anti-proteases are excluded from the interface between endothelial cell and leukocyte (Harlan *et al.*, 1991).

ROS have been shown to stimulate the endothelial cell to express P-selectin (Lorant *et al.*, 1993). Once the inflammatory cascade is initiated and neutrophils adhere to the endothelium, more ROS are produced, and more P-selectin is expressed, resulting in augmentation of neutrophil infiltration (Patel *et al.*, 1991). Recently Pinsky and colleagues (Pinsky *et al.*, 1996) demonstrated that endothelial cells rendered hypoxic in culture release P-selectin from pre-stored storage granules in the cytoplasm. These granules, known as Weibel-Palade bodies, also release von Willebrand factor, which contributes to platelet-neutrophil aggregation and microthrombosis. Pinsk *et al* demonstrated that von Willebrand factor levels increased dramatically in the coronary effluent drawn from coronary sinus catheters at the end of aortic cross clamping in open heart surgery patients (Pinsky *et al.*, 1996).

Although the molecular signals that activate endothelial cells are initiated during the ischaemic period, it is not until reperfusion that neutrophil-mediated damage occurs. Upon adherence and transendothelial cell migration, neutrophils become activated to cause a tremendous amount of nonspecific damage. Histologically, neutrophil-mediated reperfusion injury is characterized by an abrupt release of intracellular constituents due to the sudden rupture of cell membranes resulting in the unique phenomenon of contraction band formation (Ganote, 1983). This histological picture is partially mediated by activated neutrophils that generate highly reactive ROS, which contribute to the disruption of these cellular membranes. ROS are cytotoxic due to the capacity of these molecules to react with and damage endothelial cell membrane lipids and nucleic acids, resulting in cellular dysfunction and cell death (Weiss, 1989). ROS also produce damage by reacting with polyunsaturated fatty acids, resulting in the formation of lipid peroxides and hydroperoxides, which in turn inhibit many membrane-bound
enzyme systems, damaging the sarcolemma and thereby causing disruption of cellular integrity (Braunwald and Kloner, 1982). This contributes to intracellular calcium overload and myocardial excitation-contraction uncoupling at the cellular level, recognized as the mechanical syndrome of 'stunning' at the clinical level. Furthermore, free radicals may induce significant functional alterations in endothelial cells that promote and extend the inflammatory reaction. In particular, free radicals stimulate platelet-activating factor release from the endothelium, which in turn can further activate cells of the growing neutrophil infiltrate in an amplifying feedback loop (Lewis *et al.*, 1988). Activated neutrophils may release several proteolytic enzymes that may destroy viable myocardium as well as supporting extracellular matrix. Increased levels of neutrophil-derived proteolytic enzymes have been demonstrated in ischaemic myocardium, including elastases, b-glucosaminidases, b-glucuronidases, and myeloperoxidases, all of which break down the barrier function of the endothelium, leading to swelling and impaired cardiomyocyte function (Bell *et al.*, 1990).

# **1.5.** Aim of the study

Endothelial cells play an important role in all aspects of cardiovascular physiology and homeostasis. Most major cardiovascular diseases have now been associated with pathophysiologic alterations in endothelial cell structure and/or function.

*In vivo* vascular endothelium is frequently exposed to reactive oxygen species including superoxide anion and hydrogen peroxide, for example in ischaemia reperfusion injury, or in chronic arterial atherosclerosis. The most important source of reactive oxygen species is activated polymorphonuclear leukocytes and monocytes. Other sources include cigarette smoke and oxidised lipoproteins.

The aim of this thesis is try to answer following questions:

A: It is known that exposure to large amount of reactive oxygen species leads to endothelial cell lysis. It is important to know whether this occurs under physiologically relevant conditions, and if so, what is the likely mechanism.

**B**: While it is possible for endothelial cells to be exposed to millimolar concentrations of reactive oxygen species during ischaemia/reperfusion, it is likely the cells are more often exposed to micromolar levels of reactive oxygen species. It is currently unclear whether endothelial cells will be damaged by micromolar concentrations of reactive oxygen species.

C: If the cells are indeed damaged by low level of reactive oxygen species, what is the mode of damage? Is the damage manifested as cell death or as alteration to normal endothelial cell function?

**D**: If the damage is presented as functional damage, which functions are damaged? Can the damaged cells restore normal function?

E: What is the likely fate of those cells with unrepaired function? Do they subsequently die, or survive for a relatively long time but with altered cellular function?

Those questions have been addressed in a range of cellular, cytometric and molecular studies to explore the effect of reactive oxygen species at different concentrations on cultured endothelial cells.

# **Chapter 2: General Methods**

# 2.1. Materials

## 2.1.1. Tissue Culture

Dulbecco's modified Eagle medium (DMEM), Hanks solution without phenol red, modified Hanks solution without phenol red and  $Ca^{2+}$  and  $Mg^{2+}$ , Dulbecco's phosphate buffered saline (PBS), foetal bovine serum (FBS) and trypsin-EDTA solution were obtained from Life Technologies (Paisley, Scotland). Clonetics endothelial cell basal medium (EBM) and endothelial cell growth medium (EGM) were from BioWhittaker UK (Berkshire, England). NUNC tissue culture plates, flasks and dishes were supplied from Life Technologies (Paisley, Scotland) and the university bulk store.

#### **2.1.2. Cell Analysis Reagents**

CellTiter 96 Aq<sub>ueous</sub> Non-Radioactive Cell Proliferation Assay kit and CytoTox 96 Assay kit were obtained from Promega (Southampton, England), Oncor ApopTag *in Situ* Apoptosis Detection kit was obtained from Cambridge Bioscience (Cambridge, England). Tritiated thymidine and 6-keto-prostaglandin  $F_{1\alpha}$  enzyme immunoassay system were from Amersham Life Science (Buckinghamshire, England). PeroXOquant quantitative peroxide assay was obtained from Pierce (Chester, England). Dichlorodihydrofluorescein diacetate (DCFDA), CyQUANT cell proliferation assay kit, NanoOrange protein quantification kit and SYTO green fluorescent cell-permeant stain were products of Molecular Probes and obtained from Cambridge Bioscience (Cambridge, England). AnalaR grade hydrogen peroxide was purchased from BDH (Poole, England) as 30% solution. Xanthine, xanthine oxidase, myoglobin, spin traps and all other reagents were from Sigma-Aldrich UK, unless specifically stated.

# 2.2. Endothelial Cell Culture

## 2.2.1. Introduction

Maruyama pioneered the method to isolate and culture endothelial cells from human umbilical cord vein in 1963. But due to technical difficulties at that time the cells they cultured had not been positively identified as endothelial cells and the culture could not be maintained for more than a few days.

Detailed study of endothelial function first became feasible with the development in the 1970s of techniques to culture endothelial cells *in vitro* (Jaffe *et al.*, 1973; Gimbrone *et al.*, 1974; Lewis *et al.*, 1973). Jaffe *et al* and Gimbrone established the morphologic and immunological criteria to identify endothelial cells. They also modified the method for isolating and culturing human umbilical endothelial cells, which has been widely used even to today, but they still had difficulty to culture cells for long term. The discovery by Gospodarowicz *et al* showed that vascular endothelial cells could be maintained in long term culture with supplemented bovine brain extract (BBE) (Gospodarowicz *et al.*, 1976).

In 1978 Schwartz reported the method of isolating and culturing bovine aortic endothelium, which can grow very quickly into large quantities without special growth factor supplement and is also very ease to maintain (Schwartz, 1978). In 1983 Gajdusek and Schwartz devised a simple method for cloning bovine aortic endothelial cells (Gajdusek and Schwartz, 1983). The cloning can produce a pure endothelial cell population by eliminating contaminating cells.

The techniques used in this thesis were based on the methods of Jaffe, Schwartz and Gadjusek and Schwartz.

## 2.2.2. Primary culture of Bovine Aortic Endothelium

Bovine aortas were obtained from a local abattoir as soon as possible after slaughter. Segments of descending thoracic aorta approximately 30 cm in length were transferred to the laboratory on ice and processed immediately under sterile conditions.

The aortas were examined carefully for any cuts, and the proximal end of the aorta was ligated and the distal end clamped. Fat and connective tissue were dissected carefully without damaging the root of intercostal arteries near the aorta, which were securely ligated with sutures. The clamped end of the vessel was cut off, a kwill filling tube was inserted in the distal end and the vessel tied off around it. The vessel was thoroughly washed with saline and filled with 10 ml of 0.1% collagenase in DMEM or Hanks solution using a syringe that was left in place. The vessel was wrapped in cling-film and incubated at 37 °C for 15 to 20 minutes. After gentle massaging of the vessel to aid the release of loosened cells, the collagenase/cell solution was removed by the syringe. The lumen was rinsed once with 10 ml of culture medium (DMEM with 15% fetal calf serum (FCS)). The cell suspension was centrifuged at 1000g for 10 minutes and the cell pellet was rinsed once with culture medium. The cells were finally resuspended in culture medium and plated in a 80 cm<sup>2</sup> tissue culture flask.

Collagenase treatment removed the cells from the aorta wall in large sheets which made an accurate cell count impossible, therefore the cells obtained from one such length of aorta were initially cultured in 20 ml of culture medium and plated in one 80  $\text{cm}^2$  tissue culture flask or resuspended in 40 ml of medium and plated in two flasks, depending on yield, estimated by eye under the microscope.

Most of the endothelial cells adhered to the culture flask within 2 hours. Thereafter the cells were gently washed and replaced with fresh medium after cell attachment. Generally the cells reached confluence after 2 to 3 days at density of about 8 x  $10^{6}/80$  cm<sup>2</sup> or  $10^{5}/cm^{2}$ , counted under microscope.

Some of the confluent endothelial cells were used to prepare conditioned medium. Gajdusek and Schwartz (1983) have demonstrated that the population doubling time of endothelial cell cultures, when continuously exposed to conditioned medium, is increased 40% compared to controls cultured in fresh medium. It is now known that endothelial cells can synthesis and release growth factors into culture medium. To collect conditioned medium confluent endothelial cells were maintained in growth medium for 5 to 7 days. The medium was removed from the cells, centrifuged for 10 min at 1000 g to remove cells and debris, and passed through 0.22  $\mu$ M filter before being stored at -20 °C.

# 2.2.3. Primary Culture of Human Umbilical Vein Endothelial Cell

Umbilical cords were obtained from the labour ward of Leicester Royal Infirmary and Leicester General Hospital. The procedure used for endothelial cell isolation was an adaptation of the method of Jaffe *et al* (1973).

Pieces of cord between 20 and 30 cm long were drained of as much blood as possible and placed in ice-cold, sterile PBS solution containing 2  $\mu$ g/ml gentamicin and kept refrigerated until processed. Cords which had been heavily clamped were discarded. The vein at each end of the cord was cannulated using the blunt plastic sheath of a 1.7 mm gauge intravenous cannula and held in place with surgical ties. Blood was flushed from the vein with sterile saline and then

with 0.1% collagenase solution. One end was then clamped and 2 to 5 ml of collagenase/PBS was infused into the vessel. Over-filling of the vessel was avoided since this tended to lead to rupturing and leakage and probably to contamination with non-endothelial cell types such as fibroblasts and smooth muscle cells. The cord was wrapped in cling-film and incubated at 37 °C for 15 to 20 minutes, and then the collagenase/cell suspension was flushed out with 10 ml of DMEM with 15% fetal calf serum (FCS). The cell suspension was centrifuged at 1000 g for 10 minutes and rinsed once before being resuspended in 5ml of human umbilical vein endothelial cells (HUVEC) culture medium. In early experiments HUVEC were cultured in DMEM supplemented with 15  $\mu$ g/ml of endothelial cell growth factor (ECGF) or bovine brain extract (BBE), 15% FCS, 5% adult human serum (AHS) and 2 µg/ml gentamicin. In later experiments HUVEC were cultured with Endothelial Growth Medium supplemented with 2% FCS and 15  $\mu$ g/ml BBE. The cells from one length of cord were initially cultured in a 25  $\text{cm}^2$  plastic tissue culture flask which had been previously coated with gelatin. To coat flasks, gelatin was diluted with PBS to 0.2%, 2 ml of diluted gelatin was added into each 25 cm<sup>2</sup> flask and the flask was left at 37 °C for 30 min or at 4 °C overnight. The medium was changed every other day and cells usually reached confluence after 3 to 5 days, and cell counting revealed densities about  $10^5$  cells/cm<sup>2</sup>, counted under microscope.

## 2.2.4. Subculturing

**Bovine Aortic Endothelial Cells** All uncloned cell lines were subcultured as soon as they had reached confluence. The old growth medium was removed from 80 or 75 cm<sup>2</sup> flasks, the cell monolayer was rinsed with 5 ml PBS before adding 5 ml EDTA/trypsin solution (0.02% trypsin, 1 mM EDTA and 10 mM sucrose in 20 mM HEPES buffered PBS without Ca<sup>2+</sup> and Mg<sup>2+</sup>, pH 7.4). One flask of cells were incubated with 5 ml of EDTA/trypsin solution for 2 to 5 minutes at 37 °C.

Cell detaching was facilitated by gental tapping of the flasks. As soon as the cells had detached from the culture surface of the flask 15 ml of serum-containing growth medium was added to inactivate the trypsin. The cell suspension was centrifuged at 1000g for 10 min, washed once with medium and plated out at densities about 3 - 5 x  $10^4$  cells/cm<sup>2</sup> (2.5 - 4 x  $10^6$  cells/80 cm<sup>2</sup> flask).

Human Umbilical Cord Endothelial Cells HUVECs were subcultured in a similar way as bovine cell subculture, but normally the cells would be subcultured immediately at, or just before confluence. Experience from this lab and others showed staying in confluence would reduce HUVEC plating efficiency and growth at subculture. The old growth medium was removed from  $25 \text{ cm}^2$  flask and the cell monolayer was rinsed with 3 ml of EDTA/trypsin solution. The cells were incubated with 3 ml fresh EDTA/trypsin solution for 2 to 5 minutes at 37 °C and checked under microscope. HUVECs were somewhat more difficult to detach compared with BAEC. Shaking the flask gently helped the cells to detach from the culture surface. Five millilitre Serum-containing growth medium was added into the cell suspension to inactivate the trypsin. The cell suspension was centrifuged at 1000g for 10 min, washed once with medium and plated about 5 x  $10^4$  cells/cm<sup>2</sup> (4 x  $10^6$  cells/80 cm<sup>2</sup> flask). This represented a 1:2 split.

#### **2.2.5.** Cloning of BAEC

Cloned endothelial cells were used in most of the experiments to avoid nonendothelial contamination. Primary cultures of bovine aorta endothelial cells reaching approximately 90% confluence were checked under microscope. The batches of cells with high quality and purity were suspended with 5 ml of EDTAtrypsin solution after washing. The cell suspension was aspirated through a 25 gauge needle 2 or 3 times to break up cell clumps, and diluted with 15 ml of serum-containing growth medium to inactivate the trypsin. The cells were recovered by centrifugation at 1000g for 5 minutes, washed with growth medium before being finally resuspended in 20 ml of medium and counted with a haemocytometer. The cell suspension was appropriately diluted to yield a final cell density of 10 cells/ml with growth medium and plated in a 96 well plate at 100  $\mu$ l/well, equivalent to 1 cell/well. After 24 hours, each well was visually scored for cell number and individual wells examined for the following 3 days. Those wells containing a single cell at 24 hour after plating were identified to be a possible clone. After 4 days in culture, wells containing a single colony consisting of 8 to 12 cells were considered to be derived from one single progenitor cell. The culture medium was changed every 4 to 5 days using a 50 : 50, fresh : conditioned medium mixture, until the cells formed a monolayer. It took about 12 to 18 days for the cells to reach confluence at a density of about 4 x 10<sup>4</sup> cells/well, during which time the cells were estimated to have undergone 13 to 15 cell doublings.

The cells were then transferred, on a well to well basis, to  $24 \times 16$  mm well plates in normal growth medium, using standard subculturing techniques. It took about 4 to 6 days for the cells to reach confluence at a density of about  $2 \times 10^5$  cells/well (about  $10^5$ /cm<sup>2</sup>), during which time the cells were estimated to have undergone 3 to 4 cell doublings. Those cells, which retained the cobblestone morphology of endothelium on reaching confluence and were free of contamination of other cells were expanded to 25 cm<sup>2</sup> flasks and grown up for experiment or stored in liquid nitrogen for future use.

## 2.2.6. Endothelial Cell Identification

Most of endothelial cell cultures were identified on morphological criteria. Bovine aortic endothelial cells tend to be polygonal in shape and form a tight cobblestone appearance when confluent. Pure endothelial cells grow in a single uniform monolayer with cells showing contact inhibition. Some batches of BAEC may slightly overlap each other in confluence.

The most common contaminants of endothelial cultures from large vessels are fibroblasts and smooth muscle cells. They are elongated and less well defined in shape. Fibroblasts grow in multiple layers and very quickly overgrow any endothelial cells present in a culture. The striking difference in morphology between these cells helps to eliminate contaminated cultures early on.

Human umbilical vein endothelium are less easily discriminated from contaminating cells in early primary cultures. Human cells in non confluent cultures have a tendency to grow in an elongated manner until the cells are confluent and then they assume a more typical polygonal-cobblestone appearance. They do exhibit contact inhibition and monolayer growth. But the cell turnover in confluent HUVEC is much higher than that of BAEC.

Sometimes the von Willebrand factor had also been used to identify endothelial cells (Jaffe, 1984). Endothelial cells cultured on glass slides were fixed with 4% formalin for 30 min. After washing three times with PBS the cells were incubated with rabbit anti-vWF antibody (from Sigma, 10 µl antibody in 50 µl PBS with 0.5% Tween 20 for each slide) for 60 min at room temperature. The samples were again washed twice and labelled with FITC conjugated anti-rabbit antibody (from Sigma, 10 µl antibody in 50 µl PBS with 0.5% Tween 20 and 0.5% rabbit serum for each slide) for 30 min. After washing the samples were counter stained with propidium iodide (PI) and vWF staining was identified by fluorescent microscope. A negative control was processed the same way, only 10 µl rabbit serum was used to replace rabbit anti-vWF antibody. Usually more than 80% cells were vWF positive in BAEC and more than 95% were positive in HUVEC.

## 2.2.7. Liquid nitrogen storage

Confluent cloned cells in their second passage (in 24 well plates) were trypsinised and washed in PBS. The cells were finally resuspended in 90% FCS : 10% DMSO at a density of about 2 x  $10^5$  cell/ml (approximately 1 ml/well) and then dispensed into sterile cryotubes in 1 ml aliquots. The cells were cooled at -70 °C for 12 to 24 hours in well insulated containers to allow a slow drop in temperature, before being transferred to vapour phase in liquid nitrogen.

Immediately before use the vials were removed from the liquid nitrogen and thawed quickly, but not completely in a 37 °C water bath. The cell suspension of each tube was transferred to a sterile plastic tube and 10 ml of ice-cold growth medium was added drop by drop. Slow addition of cold medium at this stage prevents rupture of the cells through heat or osmotic shock.

The cells were recovered by gentle centrifugation at 500 g for 10 minutes and resuspended in 10 ml growth medium at room temperature and centrifuged again. Cells were finally resuspended in 20 ml of growth medium at 37 °C and plated in 80 cm<sup>2</sup> flasks. The average time for the cells to reach confluence was about 3 to 5 days depending on their viability after storage and recovery.

# 2.3. General Assay

# 2.3.1 Measurement of Hydrogen Peroxide

Hydrogen peroxide concentration was measured in cell-free or protein-free system by its absorbance at 240 nm. The concentration was calculated using the molar extinction coefficient of 43.6 M<sup>-1</sup>cm<sup>-1</sup>. All hydrogen peroxide stock and standards were diluted with pure water from a 30% solution and quantified by absorbance at 240 nm.

In biological samples the concentration of hydrogen peroxide was measured by a Xylenol orange method (PeroXOquant quantitative peroxide assay from Pierce). In this assay, hydroperoxides convert ferrous to ferric ions at acidic pH. The hydrogen peroxide first reacts with sorbitol, converting it to a peroxyl radical. The peroxyl radical then initiates ferrous oxidation to ferric. In sulphuric acid solution,

the Fe<sup>3+</sup> complexes with the xylenol orange dye to yield a purple product with an absorbance between 540 to 580 nm. The molar extinction coefficient of the xylenol orange-Fe<sup>3+</sup> complex is  $1.5 \times 10^4 \text{ M}^{-1}\text{cm}^{-1}$  in 25 mM H<sub>2</sub>SO<sub>4</sub> at room temperature. When using microtitre plates, the best wavelength to read in terms of signal-to-noise ratio is 595 nm due to the background of plate. The maximum absorbance of the assay components prior to addition of peroxide is at 430 nm.

The standard solution was made from diluting hydrogen peroxide stock with pure water within the range of concentrations from 1  $\mu$ M to 1 mM. The sensitivity of the assay was at  $\mu$ M. The working range of this assay is from 1  $\mu$ M to 1 mM, but the standard curve is not linear over that entire range. For most experiments presented in this thesis the concentrations of hydrogen peroxide were at or below 100  $\mu$ M, at which the assay was linear.

To measure hydrogen peroxide concentration in culture supernatants, 20  $\mu$ l of samples were added into 200  $\mu$ l freshly prepared assay solution (250  $\mu$ M ammonium ferrous sulphate, 25 mM H<sub>2</sub>SO<sub>4</sub>, 100 mM sorbitol, 125  $\mu$ M xylenol orange in water) in a 96 well plate. The samples were incubated at room temperature for 20 min before absorbance was measured at 595 nm using a microtitre plate reader. The wavelength of 595 nm was chosen because it gave largest absorbance after subtracting background. All samples and standards were kept away from light to prevent hydrogen peroxide decaying.

#### **2.3.2. Protein Assay**

#### 2.3.2.1. Bio-Rad Protein Assay

This assay is an improved Bradford assay based on the observation that the absorbance maximum for an acidic solution of Coomassie Brilliant Blue G-250 shifts from 465 nm to 595 nm when binding to protein occurs. This assay is

sufficiently sensitive (1 to 25  $\mu$ g/ml) and accurate for most of the experiments if the concentrations of detergents is less than 0.1%. But the measuring range of this assay is not wide (only 25 times). The advantage of this assay is that it will not be interfered by oxidative or reductive substances, which are usually present in samples obtained during experiments in this thesis.

The Dye Reagent Concentrate was from Bio-Rad. Two hundred  $\mu$ l of sample and 50  $\mu$ l of Dye Reagent were transferred into each well of 96 well plates, mixed well, stand at room temperature for 10 minutes, and the absorbance at 595 nm was measured by a microplate reader. The concentrations of protein in samples were calculated by a standard curve generated from known concentrations of bovine serum albumin.

#### 2.3.2.2. BCA Protein Assay

This assay is carried out using a BCA Protein Assay kit from Pierce. It is based on the principle that protein peptide bonds react with  $Cu^{2+}$  for form tetradentate- $Cu^{+1}$  complex, which then reacts with bicinchoninic acid (BCA) for form purple colored BCA- $Cu^{+1}$  complex with absorption peak at 562 nm.

This assay has a very big range  $(20 - 2000 \ \mu g/ml, 100 \ times)$  and is compatible with 5% detergents. The major disadvantage of this assay is that it is not compatible with oxidative or reductive substances, so it was only used in samples without adding with reducing reagents or being exposed to reactive oxygen species.

## **2.3.3.** $\beta$ -galactosidase activity

 $\beta$ -galactosidase activity was measured by method of Dimri et al (Dimri et al, 1995). BACE cultured in 25 cm<sup>2</sup> or 75 cm<sup>2</sup> flasks were fixed with 4% formalin

for 10 min at room temperature, washed twice with PBS. The cells were then stained for 24 hours at 37 °C in fresh  $\beta$ -gal solution with 1 mg/ml X-Gal in 40 mM citric acid/sodium phosphate, pH 6, with 5 mM potassium ferrocyanide, 5 mM potassium ferricyanid, 150 mM NaCl and 2 mM MgCl<sub>2</sub>.

# 2.4. Assessment of Cell Damage

#### 2.4.1. Oxidative Exposure

Endothelial cells were cultured in 96, 24 or 6 well plates and 25  $\text{cm}^2$  or 80  $\text{cm}^2$  flasks depending on the experimental need. Usually the cells were used when they reached confluence, unless otherwise stated in the text. There are many antioxidants in serum so the culture medium was thoroughly removed and the cells were washed carefully before oxidative stress. In all experiments the reactive oxygen species exposure was done in Hanks solution without phenol red, which will be bleached by hydrogen peroxide, leading to the loss of oxidant.

Preliminary experiment confirmed that endothelial cells did not show any damage in Hanks solution for at least 4 hours as measured by mitochondrial activity and Tritiated thymidine uptake. Oxidative stress was introduced to cells either by xanthine/xanthine oxidase system or by a single dose of hydrogen peroxide.

Xanthine/xanthine oxidase: Xanthine oxidase catalyses the reaction of xanthine and oxygen to produce superoxide anion. Superoxide anion will dismutate to hydrogen peroxide and the latter may react with ferrous iron or copper to produce hydroxyl radicals. So in this system the cells were exposed to a mixture of superoxide anion, hydrogen peroxide and hydroxyl radicals.

After washing endothelial cells in 24 well plates were incubated with 0.8 mmol/L xanthine solution in Hanks at 0.5 ml/well. Other reagents like SOD, catalase and myoglobin were added before xanthine oxidase. The final concentration of

xanthine was 0.4 mmol/L, xanthine oxidase 0.04 U/ml in a volume of 1 ml/well. All other reagents were all expressed as final concentration. The cells were then left in a 37 °C incubator. Although the substrate would have been exhausted in less than 1 hour, the incubation time was 4 hours in order to obtain maximum damage. During this time the total superoxide anion production measured by cytochrome C assay was about 0.7 mmol/L (average of 2 experiments), which might have been under estimated because of the nature of the assay.

For hydrogen peroxide exposure a single dose of stock reagent grade hydrogen peroxide was added in Hanks solution at 37 °C. In early experiments the exposure time was 4 hours, and in later experiments the exposure time was usually 60 min, and occasionally 30 min. The cells were then returned to normal culture medium and the culture continued in incubator for given times.

In earlier experiments the hydrogen peroxide added to cells was normalised by the concentration and expressed as µmol/L or mmol/L, as commonly used in the During the experiments it has been noticed that while different literature. concentrations of hydrogen peroxide will cause different degrees of damage, the absolute amount of hydrogen peroxide available for each cell is more important than concentration. For this reason, in an attempt to normalise the experimental condition, hydrogen peroxide were calculated both at concentration (mmol/L or  $\mu$ mol/L) and at the amount for each cell (micromoles per million cells,  $\mu$ mol/10<sup>6</sup> cells, or picomoles per cell, pmol/cell). Briefly, before exposing cells to hydrogen peroxide, one flask or well of cells was trypsinised and counted, then the concentration and amount of hydrogen peroxide and the total volume of Hanks solution calculated. For example, if there are 5 x  $10^6$  cells in a flask and the hydrogen peroxide concentration of 100  $\mu$ mol/L and the amount of 0.5  $\mu$ mol/10<sup>6</sup> cells (0.5 picomoles per cell) is to be used. First it can be worked out that total 2.5  $\mu$ mol amount of hydrogen peroxide is needed [(5 x 10<sup>6</sup> cells) x (0.5  $\mu$ mol/10<sup>6</sup> cells)]. Then it will be calculated that to make a concentration of 100  $\mu$ mol/L, a volume of 25 ml Hanks solution is needed [ $(2.5\mu mol/25 ml) = (100\mu mol/1000 ml)$ ].

# 2.4.2. Cell Proliferation

#### 2.4.2.1. Tritiated Thymidine Uptake

Following hydrogen peroxide exposure, endothelial cells were incubated with culture medium containing 0.037MBq/ml [<sup>3</sup>H] thymidine (adding 1 µl of 37 MBq/ml stock into 1 ml of medium) for 3 to 24 hours, dependent on experimental need, at 37 °C. For cells in 96 well plates the amount of tritiated thymidine containing culture medium was 0.1 ml/well, and 1 ml/well in 24 well plates.

At given times the isotope containing medium was removed, cells washed with fresh medium twice, and chased with fresh isotope free medium for 60 min at 37 °C to reduce the un-incorporated tritiated thymidine in cytoplasm. Samples were then washed again with PBS, and treated with 5% ice-cold trichloracetic acid for 60 min to precipitate DNA and incorporated tritiated thymidine (0.1 ml/well in 96 well plate and 1 ml/well in 24 well plate). After removal of trichloracetic acid the cells were washed with ethanol once, added with a small amount of fresh ethanol and left to dry overnight to remove any remaining free isotope. The washed precipitate was re-dissolved in 2N NaOH for 60 min, 50 µl/well in 96 well plate and 100  $\mu$ l/well in 24 well plate. The cell lysate was transferred to a counting vial containing 10 ml of Biofluor scintillant. After good mixing the samples were left still for at least 4 hours to eliminate chemiluescence. The radioactivity in the samples was counted by Tri-Carb 1500 liquid scintillation analyser (Packard) with counting window set at tritium and a counting efficiency of about 65%. Each sample was counted for 5 min and all the samples were counted for 2 rounds to check variation.

#### 2.4.2.2. BrdU Labelling

DNA incorporation of the thymidine analogue 5-bromo- 2'-deoxyuridine (BrdU) was also used to measure cell proliferation. Endothelial cells were cultured on glass slides and incubated with 10  $\mu$ mol BrdU in culturing medium for continued labelling (4 to 24 hours) or 50  $\mu$ mol for pulse labelling (30 to 60 min). Experiments were performed in two ways: in the first, cells were incubated with BrdU for varying periods up to 24 hours, in the second cells were pulse-labelled with BrdU for 30 or 60 minutes, washed and replaced in fresh medium for further incubation. At the end of the experiment cells were fixed, and BrdU detected by anti-BrdU antibody.

Cells actively growing on glass slides were loaded with BrdU at 10 or 50  $\mu$ mol for given times in culture medium at 37 °C. The cells were then washed twice with ice-cold Hanks solution and fixed with ice-cold 4% formalin for 15 min on ice. After washed twice with PBS the samples were incubated with cold 70% ethanol for 60 min on ice, or stored at -20 °C for up to a week.

The slides were washed twice with PBS at room temperature, and incubated with 2 M HCl for 30 min at room temperature to unwind DNA. After two washes with PBS 60  $\mu$ l of Anti-BrdU solution (6  $\mu$ l rabbit anti-BrdU antibody + 54  $\mu$ l PBS with 0.5% Tween 20) were applied to each slide. The samples were covered with coverslips and incubated for 60 min at room temperature. The cells were washed twice with PBS again, and incubated with 60  $\mu$ l of FITC-antibody solution (6  $\mu$ l FITC-mouse anti-rabbit IgG antibody + 54  $\mu$ l PBS with 0.5% rabbit serum and 0.5% Tween 20) for 30 min at room temperature, covered with coverslips. The slides were washed twice with PBS, and added with 60  $\mu$ l PBS with 10  $\mu$ g/ml PI and 0.1 mg/ml RNase, covered with coverslips and sealed off. The samples were then examined under a fluorescent microscope.

#### 2.4.2.3. DNA Quantification

In some experiments the cell number was estimated by a CyQUANT cell proliferation assay kit. It uses a proprietary green fluorescent CyQUANT GR dye, which exhibits strong fluorescence enhancement when bound to cellular nucleic acids. It has a large linear range from 50 to 50,000 cells in 200  $\mu$ l volume.

Medium was removed from cells in 96 well plates. The cells were then frozen at - 80 °C for more than 2 hours. The samples were then thawed up for 30 min at room temperature, and 200 µl of lysis buffer containing CyQUANT dye was added into each well. The fluorescence was then measured by a fluorescence micro plate reader with excitation at 485 nm and emission detection at 550 nm. The number of cells in each well were calculated against standard curve obtained from known numbers of cells.

## 2.4.3. Membrane Damage

#### 2.4.3.1. Tritiated Adenine retention

Live endothelial cells need to uptake adenine to synthesise ATP and DNA. These large molecules can not pass through intact cell membrane. Therefore their release indicates the integrity of cellular membrane has been compromised.

Endothelial cells were preloaded with [ ${}^{3}$ H] adenine for 24 hours in culture medium at 0.037 MBq/ml/well (adding 1 µl of 37 MBq/ml stock into 1 ml of medium) at 37 °C, washed 3 times with Hanks solution and then treated with oxidants. Cells were again washed 3 times with Hanks solution, lysed in 2 N NaOH at 0.5 ml/well for 2 hours. Cell lysate was transferred to counting vials with 10 ml Biofluor scintillant. Samples were mixed well, left standing for 4 hours to eliminate chemiluescence. The radioactivity in the samples was counted by a Tri-Carb 1500 liquid scintillation analyser (Packard) with counting window

set at tritium and a counting efficiency of about 65%. Each sample was counted for 5 min and all the samples were counted for 2 rounds to check variation.

#### 2.4.3.2. Tritiated Adenine Uptake

Endothelial cells were treated with oxidants before loading with [<sup>3</sup>H] adenine for 60 min in culture medium at 0.037 MBq/ml/well at 37 °C. The cells were washed 3 times and radioactivity counted as described above.

# 2.4.4. Cell Function

#### 2.4.4.1.Mitochondrial Dehydrogenase Activity Assay

Dehydrogenase activity was determined by a commercial colorimetric kit-CellTiter 96 Aq<sub>ueous</sub> Non-Radioactive Cell Proliferation Assay-from Promega. The kit is composed of solutions of a tetrazolium compound (3-(4,5-dimethylthiazol-2yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulphophenyl)-2H-tetrazolium inner salt (MTS), and an electron coupling reagent (phenazine methosulfate; PMS). MTS (Owen's reagent) is bioreduced by cells into a formazan that is soluble in tissue culture medium. The absorbance of the formazan at 490 nm can be measured directly from 96 well assay plates without additional processing. The conversion of MTS into the aqueous soluble formazan is accomplished by dehydrogenase enzymes found in metabolically active cells. The quantity of formazan product, and thus the amount of 490 nm absorbance, is directly proportional to the number of living cells in culture.

This assay was originally developed to assess the number of viable tumour cells undergoing proliferation, after exposure to cytotoxic agents. However this assay actually is based on the cellular dehydrogenase activity rather than cell proliferation, so the term "cell metabolic activity" was used, and this assay was used to test whether brief exposure to low concentrations reactive oxygen species causes a fall in oxidative activity in the cells, rather than cell death.

Confluent endothelial cells cultured in 24 well plates were subjected to different concentrations hydrogen peroxide or other treatment. At given time points the cells were incubated with MTS solution in culture medium (with FCS but without phenol red, 100  $\mu$ l MTS + 400  $\mu$ l medium for each well) for 2 to 6 hours at 37 °C. The absorbance at 490 nm was monitored continuously throughout the experiment. Cell damage was expressed as percent loss of dehydrogenase activity compared to untreated control cells.

#### 2.4.4.2. Prostacyclin Measurement

Prostacyclin is unstable and has a half-life of approximately 3 minutes *in vitro* at 37 °C (Cho and Allen, 1978). It undergoes a spontaneous hydrolysis to 6-keto-prostaglandin  $F_{1\alpha}$ , which is relatively stable. Due to this spontaneous hydrolysis of prostacyclin, the quantification of 6-keto-prostaglandin  $F_{1\alpha}$  is accepted as a measure of prostacyclin formation.

Prostacyclin was measured using a commercial kit 6-keto-prostaglandin  $F_{1\alpha}$ enzyme immunoassay system. The assay is competitive ELISA based on the competition between unlabelled 6-keto-prostaglandin  $F_{1\alpha}$  and a fixed quantity of peroxidase labelled 6-keto-prostaglandin  $F_{1\alpha}$  for a limited number of binding sites on a 6-keto-prostaglandin  $F_{1\alpha}$  specific antibody immobilized on a microtitre plate. With fixed amounts of antibody and peroxidase labelled 6-keto-prostaglandin  $F_{1\alpha}$ the amount of peroxidase labelled ligand bound by the antibody will be inversely proportional to the concentration of added unlabelled ligand. The peroxidase ligand bound to the rabbit anti-6-keto-prostaglandin  $F_{1\alpha}$  antibody is immobilised on to polystyrene microtitre wells precoated with second donkey anti-rabbit antibody. Any unbound ligand is removed from the well by washing.

The amount of peroxidase labelled 6-keto-prostaglandin  $F_{1\alpha}$  bound to the antibody is determined by addition of a tetramethylbenzidine (TMB)/hydrogen peroxide single pot substrate. The reaction is stopped by addition of an acid solution, and the resultant colour read at 450 nm in a microtitre plate photometer. The concentration of unlabelled 6-keto-prostaglandin  $F_{1\alpha}$  in samples is determined by interpolation from a standard curve.

Endothelial cells cultured in 24 well or 96 well plates were incubated with Hanks for 10 min after oxidative stress. The supernatant (50  $\mu$ l/well) was transferred into each well precoated with Donkey anti-rabbit IgG antibody, two wells for each sample. The standard was prepared at range of 0.5 to 64 pg/well with double concentration at every step, and 50  $\mu l$  of 6-keto-prostaglandin  $F_{1\alpha}$  standard was transferred into precoated well as samples. Fifty micro litre rabbit anti-6-ketoprostaglandin  $F_{1\alpha}$  antibody was added into each well and the plate was covered and incubated for 30 min at room temperature with shaking. Then 50 µl of 6keto-prostaglandin  $F_{1\alpha}$ -peroxidase conjugate was added into each well, the plate was covered and incubated for 60 min at room temperature with shaking. The wells were washed 4 times with PBS at 300 µl/well. The plate was then incubated for 15 min at room temperature with 150 µl substrate under shaking. The absorbance at 630 nm was read immediately or the absorbance at 450 nm was read within 30 min after adding 100  $\mu$ l of 1 mol/L sulphuric acid/well. The percent bound for each standard and sample was calculated as following:

$$\%B/B_{o} = \frac{\text{Standard or Samples - Non specific binding}}{\text{Blank - Non specific binding}} \times 100$$

The standard curve was plotted using  $B/B_0$  as linear y axis against pg 6-ketoprostaglandin  $F_{1\alpha}$  as log x axis and the sample 6-keto-prostaglandin  $F_{1\alpha}$ concentration was derived from standard curve.

# 2.4.5. Intracellular peroxidation

Intracellular peroxide was measured by flow cytometer using 2'7' dichlorofluorescein diacetate (DCF-DA), modified from the technique of Carter *et al* (Carter *et al.*, 1994). DCF-DA is incorporated into lipid-rich regions of cells and hydrolysed to 2'7' dichlorofluorescein (DCF) by cellular esterases. In the presence of peroxide DCF is oxidised to fluorescein, which emits green fluorescence with maximum emission at 514nm and maximum excitation at 490nm, with near maximum excitation at 488nm.

Endothelial cells cultured in flasks were resuspended as single cells suspension using trypsin-EDTA and washed with Hanks solution. Cell suspensions at 10<sup>6</sup>/ml in Hanks' saline were preincubated with 2 to 10 µmol/L DCF-DA for 30 minutes at 37 °C and propidium iodide was added at final concentration of  $2 \mu g/ml$  at 20 min, giving 10 minutes incubation at 37 °C. Propidium iodide cannot penetrate intact membrane and will only stain the nuclei of damaged cells. In this way the damaged cells can be distinguished from those with intact cell membranes. Hydrogen peroxide was added at different aliquots to produce ratio of 5 to 50 femtomol/cell (final concentrations from 5 to 100  $\mu$ M). At times from 1 to 30 minutes after the addition of hydrogen peroxide samples were analysed in a Becton-Dickinson FACScan flow cytometer. An argon laser operating at 488nm was used for excitation, fluorescein fluorescence was detected using a  $530 \pm 30$ nm band-pass filter, and propidium iodide fluorescence using a 630 nm long band pass filter. Ten thousand cells were analysed per sample, and dead cells were gated out using propidium iodide fluorescence. Only cells with negative PI staining were used to measure intracellular peroxidation. Data were presented as

mean fluorescence per cell (standard deviation), expressed as a percentage of mean fluorescence.

## 2.4.6. Apoptosis

#### 2.4.6.1. Morphology

Apoptosis usually occurs in scattered single cells and has characteristic morphology, such as cell shrinkage, chromatin condensation, and the formation of apoptotic bodies. The morphological changes were constantly examined under phase contrast microscope. In some experiments the cells in the same fields were monitored for more than 2 weeks. The fields were marked with scratches on the cells side and marker pen on the outside of the culture flasks. When searching for the field first the maker on the outside was positioned and then the culture flask was fine adjusted according to the scratches on the culture surface.

Some cells were treated with hydrogen peroxide and the morphology examined after haematoxylin staining. Endothelial cells cultured on 24 or 6 well plates were exposed to hydrogen peroxide and then returned to culture medium and incubator to continue the culture. At given times the cells were washed and fixed with 4% formalin or 0.5% glutaraldehyde for 30 min. Haematoxylin staining solution A was prepared by dissolving 1 gram of haematoxylin in 100 ml of absolute alcohol, and solution B was prepared by dissolving 1 g ferric chloride in 100 ml of water and 1 ml of 37% HCl. The cells were covered with staining solution for 20 min, which was made from mixing equal volume of solution A and solution B. The cells were then rinsed in tap water and the staining was checked under the microscope.

An important character of apoptosis is intact membrane with condensed chromatin and apoptotic bodies. This was shown by the use of a cell membrane permeant fluorescent DNA dye SYTO in the combination of PI. SYTO will stain DNA in all cells, both with intact membrane and with interrupted membrane. PI will only stain DNA in cells with compromised membrane. In cells with intact membrane SYTO staining will mark DNA green under fluorescence microscope, and in cells with compromised membrane the relative weak green signal will be overlapped by strong PI signal and showing red.

For SYTO staining, cells cultured on glass slides were exposed to oxidative stress and then incubated with SYTO containing medium (DMEM without phosphate) in an incubator. SYTO dye was diluted at 1:100 in phosphate free medium, and the cells were usually loaded for 2 to 4 hours. PI of 10  $\mu$ g/ml was added in for 10 min and the cells were examined under fluorescence microscope.

In some experiments hydrogen peroxide treated cells were fixed with 4% formalin, stained with 10 or 20 µg/ml PI and the DNA condensation and apoptotic bodies were checked under fluorescence microscope.

#### 2.4.6.2. Gel Electrophoresis (DNA Ladder)

To extract DNA for electrophoresis 2 x 80 cm<sup>2</sup> flasks of cells were washed and lysed with 5 ml lysis buffer, which was made of 150 mM NaCl, 10 mM Tris, 25 mM EDTA and 0.5% SDS. The samples were stored at -80  $^{\circ}$ C for further process.

The cell lysates were thawed up at 37 °C, 50  $\mu$ l of stock proteinase K solution (20mg/ml) was added into each sample with final concentration of 0.2 mg/ml, and incubated at 37 °C for 60 minutes. The protease-treated cell lysate was transferred to a Corex tube containing 5 ml of 1:1 Tris-buffered phenol: chloroform (pH 8), mixed very gently, and then cooled on ice for 15 minutes. The homogenate was centrifuged for 10 min at 12,000g in the SS34 rotor on the Sorvall RC5 at 4 °C. The upper (aqueous) phase was transferred to a fresh centrifuge tube, and

centrifuged again for 10 min at 12,000g at 4 °C. The upper (aqueous) phase was transferred to a fresh centrifuge tube and added with 5 ml chloroform. The samples were centrifuged for 10 minutes at 4 °C at 12,000g. Again the upper (aqueous) phase was transferred to a fresh centrifuge tube and 500 µl 3 M sodium acetate and 5 ml isopropanol were added into the tube. After mixing very gently, the DNA was precipitated by cooling on ice for 15 minutes. The samples were centrifuge for 15 minutes at 4 °C at 12,000g, the supernatants removed and the pellets washed with 5 ml 75% ethanol. After centrifugation for 10 minutes at 4 °C at 12,000g, all the supernatant was removed and the DNA pellet was left to dry for 10 minutes. The DNA pellet was resuspended in 400 µl tris-EDTA (TE) buffer, transferred to a 1.5 ml Eppendorf tube and added with 100 µl stock RNase A (1 mg per ml). The RNA contamination was removed by 15 minutes incubation at 37 °C. The purified DNA was extracted once with 1 ml of phenol: chloroform and once with 1 ml of chloroform. The DNA was precipitated with 50 µl of 3 mol/L sodium acetate and 1 ml of ethanol on ice for 20 min. The samples were centrifuged at 12,000g for 20 min on a micro centrifuge, washed with 75% ethanol and centrifuged for 5 min at 12,000g. The DNA was resuspended in 10 µl TE buffer, and microfuged for 30 sec at 3000g to spin down any samples on the wall of vials. The concentration of DNA was measured at 260 nm and the concentration in samples were adjusted to  $1 \mu g/\mu l$  with TE buffer. Ten micro gram of DNA sample or 2  $\mu$ g of 1 kb DNA ladder standard was mixed with 2  $\mu$ l of loading buffer (0.25% bromophenol blue, 0.25% xylene cyanol FF and 15% glycerol in water) and loaded onto each well of a 1.8% agarose gel. The gel was freshly made with 0.9 gram of agarose and 5  $\mu$ l of 10 mg/ml ethidium bromide in 50 ml of TE buffer, and a voltage of 100 V was applied. The gel was checked under UV light when bromophenol blue run near the bottom.

#### 2.4.6.3. DNA End labelling

DNA fragmentation was visualized by a commercial labelling kit ApopTag from ONCOR. The labelling target of the ApopTag kit is the multitude of new 3'-OH

DNA ends generated by DNA fragmentation and typically localised in morphologically identifiable nuclei and apoptotic bodies. In contrast, normal or proliferating nuclei, which have insignificant numbers of DNA 3'-OH ends, do not stain with the kit.

This method utilises digoxigenin–labelled reagents for non-isotopic DNA endextension *in situ*, and antibodies to digoxigenin for immunohistochemical staining of the extended DNA. Residues of digoxigenin-nucleotide are catalytically added to the DNA by terminal deoxynucleotidyl transferase (TdT), an enzyme which catalyses a template independent addition of deoxyribonucleotide triphosphate to the 3'-OH ends of double- or single-stranded DNA. The incorporated nucleotides form a random heteropolymer of digoxigenin-11-dUTP and dATP, in a ratio that has been optimised for anti-digoxigenin antibody binding. The anti-digoxigenin antibody fragment carries a fluorophore (FITC) to the reaction site. When excited by light of around 494 nm wavelength, the fluorescein generates an intense signal at 523 nm. This mixed molecular biological-cytochemical system allows for sensitive and specific staining of the very high concentrations of 3'-OH ends that are localised in apoptotic bodies.

Endothelial cells were cultured on 76 x 26 mm glass slides in square dishes and exposed to hydrogen peroxide as described before. The cells were then returned to culture medium and the culture continued. At given times the cells were fixed with 4% formalin for 10 min at 4 °C, washed twice with PBS. After gently taping off excess liquid and carefully blotting around the section, two drops of equilibration buffer were immediately applied directly on the cells. The cells were covered by a plastic coverslip and placed in a humidified chamber for 5 minutes at room temperature. After removing the coverslip, gently tapping off excess liquid, and blotting around section, 54  $\mu$ l of TdT Enzyme was applied on cells. Covered with a plastic coverslip the sample was incubated in a humidified chamber at 37 °C for 1 hour. The coverslip was removed and the cells were put in a coplin jar

containing pre-warmed Stop/Wash buffer and incubate for 30 minutes at 37 °C, and agitate slide by dipping in and out of buffer once every 10 minutes. After gently tapping off extra liquid and blotting around section, the cells were washed in 3 changes of PBS for 3 minutes each. Fifty two micro litre anti-digoxigenin-fluorescein was applied to the cells, and covered with a plastic coverslip the samples were incubated in a humidified chamber for 30 minutes at room temperature. The coverslip was then removed and the cells were washed in 3 changes of PBS in a coplin jar for 5 minutes per wash at room temperature. After adding 50  $\mu$ l PI/Antifade solution the samples were mounted under glass coverslip and examined under fluorescence microscope. In some experiment in order to avoid strong PI signal interfering with relatively weak FITC signal, the PI was omitted. Cells treated with 45 Kunitz units/ml micrococcal DNase for 60 min at room temperature after fixation were used as positive controls.

#### 2.4.6.4. Flow Cytometry

Apoptosis was also measured by flow cytometry after selective DNA extraction from apoptotic cells. The method is based on the fact that during apoptosis the cells will lose some DNA due to DNA fragmentation, and the observation that extraction of ethanol pre-fixed cells with a phosphate-citric acid buffer at pH 7.8 quite selectively removes a portion of DNA from apoptotic cells. When stained with DNA dye those apoptotic cells will form a distinct sub  $G_0/G_1$  peak on flow cytometer (Gong *et al.*, 1994).

Endothelial cells cultured in 80 cm<sup>2</sup> flasks were subjected to different amounts of hydrogen peroxide for 60 min and then returned to normal culturing conditions. The cells were trypsined at given times and washed with PBS. After counting number the cells were suspended at  $10^7$  cells/ml in PBS and 1 ml suspension was injected into 9 ml of 80% ethanol, or 0.5 ml cell suspension was injected into 4.5 ml of 80% ethanol. The samples were stored in -20 °C for 2-7 days, but it could

be left for as long as 4 weeks. Ethanol was removed after centrifugation at 1000g for 10 min at room temperature. The fragmented DNA in cells was extracted by adding 192  $\mu$ l of 0.2 mol/L Na<sub>2</sub>HPO<sub>4</sub> and 8  $\mu$ l of 0.1 mol/L citric acid at pH 7.8 for 60 min. The samples were centrifuged at 1000g for 10 min, and the cells were resuspended at 10<sup>6</sup> cells/ml in PBS with 50  $\mu$ g/ml PI and 0.1 mg/ml RNase (type I-A from sigma). The DNA contents in cells were analysed by FACScan flow cytometer.

# 2.5. Preparation of Myoglobin

# 2.5.1 Metmyoglobin

Metmyoglobin (type III, from horse heart) was purchased from Sigma. Commercial myoglobin may contain superoxide dismutase and catalase activity, so it is necessary to purify all myoglobin chromatographically. Metmyoglobin powder was dissolved in PBS at a concentration of approximately 10 mmol/L and purified on Sephadex G50 before use. Three ml of metmyoglobin solution was applied to the bottom end of a Sephadex G50 column (25 cm x 1.5 cm) equilibrated with PBS. The sample was run from bottom to top of the column by a pump. High molecular weight contaminants like SOD or catalase will not enter G50 beads and will be flushed out of the column before myoglobin, while low molecular weight contamination will be trapped inside the G50 beads and come out of the column after myoglobin. Only the highly concentrated major myoglobin peak from the column was collected and used for experiments. The metmyoglobin solution was then filtered through a 0.22 µm filter, the concentration was determined by Drabkin's reagent (see below), and stored at -80 <sup>o</sup>C. Metmyoglobin solution was stable in such conditions.

## 2.5.2. Oxymyoglobin

Oxymyoglobin was prepared by adding an excess of sodium dithionite (1.2 times of myoglobin in molar basis) into un-purified 10 mmol/L metmyoglobin solution in the presence of oxygen. Sodium dithionite transforms metmyoglobin into deoxymyoglobin, which combines with oxygen and forms oxymyoglobin. This solution was purified on a Sephadex G50 column the same way as metmyoglobin solution, to remove both possible original contamination and the sodium dithionite. The Sephadex G50 column chromatograph also oxygenates myoglobin. The oxymyoglobin solution was then filtered through a 0.22  $\mu$ m filter, the concentration of total heme was determined by Drabkin's reagent (see below), and the concentration of oxymyoglobin and metmyoglobin was estimated by absorbance at 490 and 560nm as described below. A small percentage of metmyoglobin (<2%) usually existed in the solutions of oxymyoglobin was quite stable at -80 °C, so the stock solution was usually stored at -80 °C.

# 2.5.3. Apomyoglobin

Apomyoglobin purchased from Sigma was used after overnight dialysis against Hanks solution at 4 °C. Apomyoglobin was also prepared by acid-acetone precipitation of metmyoglobin, further washing with acid acetone, and Sephadex G50 column filtration of the re-dissolved apomyoglobin (Ascoli *et al.*, 1981). Acid-acetone was prepared by adding 2.5 ml of 2 M HCI to 1 litre of acetone. Five millilitres of ice-cold 1 mmol/L metmyoglobin solution was added drop-wise into 200 ml of acid-acetone pre-cooled at -20 °C under stirring, and stirred vigorously for another 2 min to remove heme. The suspension was centrifuged at 5000 rpm at -20 °C for 20 min, and the globin pellet was washed with 50 ml of acid-acetone. The precipitate of globin was resuspended in 4 ml of cold water and dialysed at 4 °C against 500 ml of 0.1% sodium bicarbonate, then against 500 ml of 0.05 mol/L phosphate buffer at pH 7.4. The globin preparation was then centrifuged at 5000 rpm at 4 °C to remove un-dissolved precipitate, and dialysed against PBS overnight. The globin solution was then centrifuged to remove un-dissolved particles and concentrated by freeze-drier. After filtered through a 0.22  $\mu$ M filter the protein concentration was determined by Bradford method and the molar concentration of the apomyoglobin was calculated by dividing the mass of protein against molecular weight of apomyoglobin. Apomyoglobin solution stored at -80 °C, and was stable in this condition.

## 2.5.4. Ferrylmyoglobin

Ferrylmyoglobin was made by mixing equal amount of 2 mmol/L purified oxymyoglobin solution with 20 mmol/L hydrogen peroxide for 10 min at room temperature. The remaining hydrogen peroxide was removed by addition of catalase at 1000 U/ml. Ferrylmyoglobin was not stable, so fresh preparation was always necessary for every experiment.

## 2.5.5. Quantification of Myoglobin

Total myoglobin concentrations were determined using Drabkin's reagent with 100 mg/L NaCN and 300 mg/L  $K_3$ FeCN<sub>6</sub> in water. Myoglobin solution was diluted with Drabkin's reagent to 1 mg/ml for 5 min and the absorbance was measured with a spectrophotometer at 450 nm. The total myoglobin concentration was calculated using a molar coefficient of 11.5/mM heme (Whitburn, 1987a).

Oxy-, met- and ferryl-myoglobin have different characteristic absorption spectra between 450 and 700 nm examined by spectrophotometer (Figure 2-1). The oxymyoglobin and metmyoglobin were prepared as described above in 50  $\mu$ mol/L concentration; the ferrylmyoglobin was prepared by reaction of 50  $\mu$ mol/L oxymyoglobin with hydrogen peroxide at final concentration of 500  $\mu$ mol/L at 4 °C for 30 min. Oxymyoglobin (red) has a absorption valley at 515 nm, double peaks at 540 and 580 nm, with 560 nm as the lowest point in between and the peak at 580 nm as the higher one. On the contrary metmyoglobin (black) has an absorption peak at 510 nm, no peak at 540 nm, and a low plateau at over 560 to 610 nm, with a shoulder at 630 nm. Ferryl myoglobin (pink) has absorption valley at 480 nm, a peak at 545 nm and a shoulder over 570 to 580 nm. Although those derivatives of myoglobin all have characteristic spectrum of their own in relatively pure solution, the spectrum of myoglobin reacting with hydrogen peroxide cannot be easily recognized and quantified. First because the mixed solution of oxy-, metand ferry-myoglobin will produce a mixed absorption spectrum; second the scanning will take at least 1 minute at which time the constitution has changed and does the spectrum. A common approach is to measure absorption SO simultaneously at 3 different wavelengths and then calculate the concentrations of different myoglobin derivatives. There are a few similar formulae which have been proposed to calculate their respective concentration. In my experience the formulae by Whitburn and Halliwell gave the most accurate results. So in this thesis the concentrations of oxymyoglobin, metmyoglobin and ferrylmyoglobin were calculated from absorbance at 490, 560 and 580 nm using formulae of Whitburn and Puppo and Halliwell (Whitburn, 1987a; Puppo and Halliwell, 1988c):

> $[Oxymyoglobin] = 2.8 A_{490} - 127 A_{560} + 153 A_{580}$ [Metmyoglobin] = 146 A\_{490} - 108 A\_{560} + 2.1 A\_{580} [Ferrylmyoglobin] = -62 A\_{490} + 242 A\_{560} + 123 A\_{580}

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# 1.6.1. Preparation of Spin Traps



nitric acid thoroughly before ase and washed again during changing of samples to

**Figure 2-1** Spectral characterisation of myoglobin derivatives. Red: oxymyoglobin; Black: metmyoglobin; Pink: ferrylmyoglobin.

without letting in any air, since even a single tiny hubble in the quartz cell would produce false ESR signal. The quartz cell needed to be correctly positioned in the

# **2.6. ESR Experiment**

## **2.6.1. Preparation of Spin Traps**

5,5'dimethyl-l-pyrroline-N-Oxide (DMPO), 3,3,5,5 tetramethyl-l-pyrroline-Noxide (TMPO) and 1-alpha-phenyl-tert-butyl nitrone (PBN) were purified by active charcoal. The spin traps were transferred onto a thin layer of active charcoal supported by whatman filter paper on a vacuum funnel. The reagents were forced to pass active charcoal where any auto-adducts would be removed. Purified spin traps were diluted to 1 M in concentration with pure water, aliquoted under nitrogen gas and stored at -20 °C before use. The stable radical 2,2,6,6 tetramethylpiperidine-N-oxyl (TEMPO) was used as a standard.

In all experiments the initial concentration of DMPO or TMPO was 100 mM in PBS, with PBN at 300 mM in the reaction mixture.

#### **Experimental Procedure**

ESR spectra were recorded using a JEOL JES-RE1X spectrometer with a flat quartz cell. The JEOL JES-RE1X spectrometer is very sensitive and stable, with a very flat quartz cell to greatly reduce energy absorption by  $H_2O$ . It is an ideal tool to study free radicals in aqueous solution at room temperature. The cell has a volume about 100 µl, so 200 µl reaction mixture was prepared for all experiments. The order of adding chemicals was PBS, spin trap, ferrous iron, and the reaction was started by adding hydrogen peroxide. The quartz cell was washed with 20% nitric acid thoroughly before use and washed again during changing of samples to obtain a very clean surface and even capillary effect. When sucking the reaction mixture into quartz cell extreme care was taken to transfer the samples steadily without letting in any air, since even a single tiny bubble in the quartz cell would produce false ESR signal. The quartz cell needed to be correctly positioned in the

cavity to optimise signal detection. This study used microwave frequency at 9.42 GHz, time constant of 0.1 second, modulation of 1 Gauss. This gives a centre field of 3340 Gauss. With scan range of 100 Gauss and 1 min of scanning time, the signal was very satisfactory. The microwave power was from 1 to 20 mW and the gain was from 50 to 500, depending on the intensity of signal. The minimum time needed to transfer samples into quartz cell, fix cell in detection cavity and adjust ESR parameters is about 30 second. In most of the experiments the adducts were monitored from 1 min after adding hydrogen peroxide and continually monitored for 60 min or longer. For some experiments the whole range of magnetic field (3290 Gauss to 3390 Gauss) was scanned and the signal spectra were recorded at given times. In experiments of monitoring signal dynamic change, the magnetic field was fixed on the peak of the signal and the signal intensity was monitored continually for up to 60 min.

For the purposes of the present study, the signal amplitude or intensity measured 1 minute after mixing was defined as the peak amplitude, and the apparent half-life of the signal as the time for it to decay to half the peak value. All water was purified by the Milli-Q system; no DMPO adducts were detected on mixing hydrogen peroxide and DMPO (without added iron). The ESR spectra of other spin traps were monitored in a similar way to that of DMPO-OH.

# 2.7. Statistical analysis

Statistical comparisons were made using one way or two way analysis of variance followed where appropriate by the Student-Newman Keuls test or Student's t test. The actual analysis was performed with Excel, Minitab or SPSS.

# Chapter 3: Lethal Oxidative Damage and Protection by Myoglobin

# **3.1. Introduction**

It has been suggested that during ischaemia/reperfusion large amounts of reactive oxygen species may be produced, the concentration of hydrogen peroxide *in vivo* could reach as high as 1 to 2 mmol/L if the area represents a limited diffusion space (Doan *et al.*, 1994; Boyer *et al.*, 1995; Bychkov, 1999). Such high concentration of reactive oxygen species may cause lethal endothelial damage.

Myoglobin is a heme protein, it may interact with reactive oxygen species including hydrogen peroxide in a variety of ways (Whitburn and Hoffman, 1985; Whitburn, 1987b; Harel *et al.*, 1988b). It is unclear whether these interactions are protective, irrelevant or actually harmful. It has even been suggested that myoglobin might react with hydrogen peroxide producing hydroxyl radical to cause cell damage.

# **3.2. Experimental Protocol**

Confluent bovine aorta endothelial cells cultured in 24 well plates with 16 mm diameter wells (approximately 1.8 x  $10^5$  cells/well) were washed with Hanks solution at 2 ml/well. Reagents were added to the cells in the order: Hanks solution; ascorbic acid (0.01 – 1 mM); or catalase ( $10^3$  U/ml) or superoxide dismutase (100 U/ml); myoglobin ( $10 - 100 \mu$ M); xanthine/xanthine oxidase or hydrogen peroxide. All concentrations were final concentrations in a reaction volume of 1 ml. Eight replicates were used per experiment. Unless otherwise stated, incubation with hydrogen peroxide or xanthine/xanthine oxidase was for 4 hours at 37 °C.

Endothelial cell damage was measured by tritiated adenine retention assay and terminal adenine uptake assay as described in Chapter 2 General Methods.

# **3.3. Oxidative Cell Damage and Protection**

## 3.3.1. Myoglobin Protecting Cell Membrane

Incubation of endothelial cells with xanthine/xanthine oxidase for 4 hours caused damage to cell membrane, as measured by retention of tritiated adenine assay (Column B of Figure 3-1). Cells exposed to oxidative stress lost 70% of their tritiated adenine content compared to control cells (Column A) without being exposed to oxidative stress.

Oxymyoglobin gave protection against oxidative damage, with increased protection between 10 and 20  $\mu$ mol/L (Column C and D), but after this point increased concentrations of oxymyoglobin did not show increased protection (Columns E and F). Apomyoglobin did not show any protection (Column G).

Superoxide dismutase did not have any effect on cell damage in xanthine/xanthine oxidase system on its own (Column H). Catalase, on the other hand, reduced the loss of tritiated adenine by oxidative stress from 70% to less than 20% (Column I). SOD failed to affect the protective effect of catalase (Column J).

## **3.3.2. Effect of Ascorbate**

Four hours exposure to 1 mmol/L hydrogen peroxide also severely disrupted endothelial cell integrity as assessed by tritiated adenine retention (Column B of Figure 3-2). With 1 mM hydrogen peroxide about 90% cells died or lost their membrane integrity compared to control cells without hydrogen peroxide exposure (Column A).


Figure 3-1 Endothelial Integrity Damage by Xan/Xan Oxidase. Confluent BAEC in 24 well plates were pre-loaded with 0.037 MBq/ml/well <sup>3</sup>H adenine for 24 hours and washed before incubated with Xan/Xan oxidase in Hanks for 4 hours. The cells were then lysed and radioactivity counted, with control cells as 100%. Result was representive of 3 separate experiments with 8 wells in each group, expressed as mean  $\pm$  sd.



Figure 3-2 Effect of ascorbate on myoglobin protection of oxidative stress. Confluent BAEC in 24 well plates were pre-loaded with 0.037 MBq/ml/well <sup>3</sup>H adenine for 24 hours and washed before incubated with Xan/Xan oxidase in Hanks for 4 hours. The cells were then lysed and radioactivity counted, with control cells as 100%. Result was representive of 3 separate experiments with 8 wells in each group, expressed as mean  $\pm$  sd.

While the damage could be partially reversed by oxymyoglobin (Column C), ascorbate at the range of 10 to 1000  $\mu$ mol/L showed a dose dependent enhancement of the protection by oxymyoglobin against oxidative damage (Column D, E and F). However ascorbic acid on its own was ineffective in the absence of myoglobin (Column G) and ascorbate did not potentiate the protective effect of catalase (data not shown).

#### 3.3.3. Adenine uptake

Those studies were replicated using a terminal adenine uptake assay (Fig. 3-3). Cells exposed to 1 mmol/L hydrogen peroxide for 1 hour lost 95% of their ability to uptake tritiated adenine (Column B) compared to control cells (Column A). Oxymyoglobin in the range of 10 to 100  $\mu$ mol/L showed dose dependent protection of cell function against oxidative damage, to a maximum protection of about 55% in this particular model (Columns C, D, E and F), compared to about 90% protection offered by catalase (Column J). Oxymyoglobin at concentrations of above 100  $\mu$ mol/L did not show increased protection (Column G). Metmyoglobin (Column H) and myoglobin reconstituted from apomyoglobin and hemin had similar effects (Column I).

## 3.3.4. Change of Myoglobin by Oxidants

During the course of the incubation with xanthine/xanthine oxidase system, oxymyoglobin was converted to metmyoglobin with the intermediate formation of ferrylmyoglobin. Photo 3-1 shows the change of myoglobin spectrum during the reaction. At 10 seconds the oxymyoglobin was the dominant form with low absorption at 490 nm and 2 absorption peaks at 540 and 580 nm with high valley at 560 nm, and the 580 nm peak was the highest. At 30 min the spectrum was characteristic of metmyoglobin, with elevated absorption at 490 nm, decreased



Figure 3-3 Effect of OxyMb on Endothelial Adenine Uptake. Confluent BAEC in 24 well plates were exposed to 1 mM  $H_2O_2$  for 4 hours before loading 0.037 MBq/ml/well <sup>3</sup>H adenine for 24 hours in culture medium. The cells were then washed and radioactivity counted, with control cells as 100%. Result was representive of 3 separate experiments, 8 wells/group, expressed as mean  $\pm$  sd.



**Photo 3-1** Absorption spectrum change of oxymyoglobin. Oxymyoglobin (50  $\mu$ mol/L) incubated with 500  $\mu$ mol/L hydrogen peroxide . Spectra recorded at 10 seconds and 2, 5, 10, 20 and 30 minutes after adding of hydrogen peroxide, showing progressive conversion of oxymyoglobin to ferryl and metmyoglobin, representive of 4 separate experiments.

peak at 540 and the peak at 580 nm reduced to a shoulder, much lower than that of 560 nm. Between those two spectra there were spectra of variably mixed oxymyoglobin, ferrylmyoglobin and metmyoglobin. Panel A of Figure 3-4 shows the change of oxymyoglobin in xanthin/xanthin oxidase system against time. The concentrations of oxy-, ferryl- and met-myoglobin were calculated by the absorbance at 490, 560 and 580 nm, using formulae described in Chapter 2 General Methods. Near 10% oxymyoglobin transferred into ferrylmyoglobin and metmyoglobin in one minute. Within 60 minutes more than 95% oxymyoglobin became ferryl or metmyoglobin. Within 20 minutes oxymyoglobin reduced to less than 8% of total myoglobin and remained at this concentration there after.

Both ferryl and metmyoglobin seemed to appear immediately after the reaction started. Within the first 10 minutes ferryl and metmyoglobin increased at similar rate at the expense of oxymyoglobin. Ferrylmyoglobin reached its peak of 45% total heme at 10 minutes. When the reaction was carried on, the amount of ferrylmyoglobin started to decrease and at 30 minutes it only accounted for less than 5% of total heme.

The amount of metmyoglobin was increasing all the time, at the expense of both oxy and ferrylmyoglobin. About 90% of total heme was in metmyoglobin form within 30 minutes of the start of the reaction, closing to the plateau there after. There was no detectable loss of heme under conditions used, as adding all the calculated myoglobin in different forms together and measuring total heme by Drabkin's method.

Xanthine oxidase acting aerobically upon xanthine generates superoxide anion as well as hydrogen peroxide (McCord and Fridovich, 1968), which had also been confirmed later in this chapter. Superoxide anion would immediately dismutate to produce hydrogen peroxide. Adding SOD to scavenge superoxide anion had no



Figure 3-4 Change of Oxymoglobin by Reactive Oxygen Species. Hydrogen peroxide was added into 22  $\mu$ M OxyMb solution at final concentration of 200  $\mu$ M. The final concentration of SOD or catalase was 100 U/ml when added. The concentrations of Oxy, Met and Ferryl-myoglobin were calculated from absorbances at 490, 560 and 580 nm using formulae described in Chapter 2.

effect on the transformation of metmyoglobin and ferrylmyoglobin from oxymyoglobin by xanthine/xanthine oxidase system (Panel B of Figure 3-4). By contrast, adding catalase not only conserved oxymyoglobin from 5.7% to 45.8% in 60 minutes, but completely prevented the production of ferrylmyoglobin and reduced the formation of metmyoglobin from 91.9% of total heme to 54.2% (Panel C of Figure 3-4).

## 3.4. ESR Study of Myoglobin Reaction with H<sub>2</sub>O<sub>2</sub>

The possibility of myoglobin to generate hydroxyl radical or superoxide anion during reaction with hydrogen peroxide was studied in detail, using electron spin resonance

## **3.4.1. ESR Spectra of Oxygen Free Radical Adducts**

ESR spectra of hydroxyl radical adduct of DMPO, TMPO and PBN formed in the presence of hydrogen peroxide and ferrous iron in PBS (pH 7.4) are shown in Photo 3-2. All spectra were recorded at 1 minute after adding hydrogen peroxide at room temperature, with centre field at 3340 G, scan range of 100 G, time constant of 0.1 second, modulation of 1 G, frequency 9.42 GHz, and scan time of 1 minute. Panel A was obtained from reaction of 1 mmol/L hydrogen peroxide and ferrous iron in the presence of 100 mmol/L DMPO in phosphate buffered saline at pH 7.4, with a gain of 100 and power of 1 mW. It is a 1:2:2:1 4 line spectrum with splitting constants of  $A_N = A_H = 15.0$  G, representing DMPO-OH adduct ESR signal. Panel B was obtained from reaction of 1 mmol/L hydrogen peroxide and ferrous iron in the presence of 100 mmol/L TMPO in phosphate buffered saline at pH 7.4, with gain of 500 and power of 1 mW. The splitting constants of TMPO-OH adduct were  $A_N = 15.3$  and  $A_H = 16.9$  G. Panel C was the spectrum from reaction of 10 mM hydrogen peroxide and ferrous iron in the presence of 100 mmol/L TMPO in phosphate buffered saline at pH 7.4, with gain of 500 and power of 1 mW. The splitting constants of TMPO-OH adduct were  $A_N = 15.3$  and  $A_H = 16.9$  G. Panel C was the spectrum from reaction of 10 mM hydrogen peroxide and ferrous iron in the presence of 300 mM



**Photo 3-2** Typical ESR spectra of hydroxyl radical adducts of DMPO (**A**), TMPO (**B**) and PBN (**C**). **A**: DMPO 100 mM,  $H_2O_2 \ 1$  mM, Fe<sup>2+</sup> 1 mM in PBS, pH 7.4.  $A_N = A_H = 14.7$  G. Gain 100, power 1 mW. **B**: TMPO 100 mM,  $H_2O_2 \ 1$  mM, Fe<sup>2+</sup> 1 mM in PBS, pH 7.4.  $A_N = 15.3$  G,  $A_H = 16.8$ G. Gain 500, power 1 mW. **C**: PBN 300 mM,  $H_2O_2 \ 10$  mM, Fe<sup>2+</sup> 10 mM in PBS, pH 7.4.  $A_N = 15.5$  G,  $A_H = 2.7$ G Gain 500, power 20 mW. All spectra were recorded at RT 1 minute after adding hydrogen peroxide. Power 1 mW, centre field 3340 G, scan range 100 G, time constant 0.1 second, modulation 1 G, frequency 9.42 GHz, scan time 1 minute.

20 mW. PBN adduct showed 6 line spectrum with splitting constants of  $A_N = 16$  G and  $A_H = 3.2$  G. The ESR spectra and their parameters were identical with those described in the literature for the respective free radicals (Buettner, 1987).

The amplitude of the ESR signal was corresponding to the concentration of ferrous ion and hydrogen peroxide (Photo 3-3 and Figure 3-5). Spectra A, B and C of Photo 3 were obtained from the reaction of 0.5, 1 and 2 mmol/L hydrogen peroxide and ferrous iron in the presence of 100 mmol/L DMPO, respectively, with ESR parameters similar to that of Panel A in Photo 3-2. DMPO-OH adduct generated by mixing equal amount of ferrous iron and hydrogen peroxide in the presence of DMPO was linear at concentrations from 0.1 to 5 mmol/L (Figure 3-5), with  $R^2 = 0.998$ .

Photo 3-4 demonstrates the production of superoxide anion in the system of xanthine/xanthine oxidase. Xanthine 0.4 mmol/L was mixed with xanthine oxidase 40 mU/ml in phosphate buffered saline, pH 7.4, in the presence of 100 mmol/L DMPO. Spectra were recorded at room temperature 1 minute after adding xanthine oxidase, with centre field of 3340 G, scan range of 100 G, power of 10 mW, time constant of 1 second, modulation of 1 G, frequency of 9.42 GHz, and scan time 8 minute. The spectrum had splitting constants of  $A_N = 14.2$  G,  $A_{H\beta} = 11.3$  G and  $A_{H\gamma} = 1.25$  G, which is typical of DMPO superoxide anion adduct. Because of the auto dismutation of superoxide anion into hydrogen peroxide and subsequent production of hydroxyl radical, this spectrum actually is a mixture of DMPO-OO and DMPO-OH.



**Photo 3-3** DMPO-OH signal intensity and concentrations of  $H_2O_2$  and  $Fe^{2+}$ : A 0.5 mmol/L; **B** 1 mmol/L, and **C** 2 mmol/L. All spectra were recorded at RT, 1 minute after adding  $H_2O_2$ . Power 1 mW, centre field 3340 G, scan range 100 G, time constant 0.1 second, modulation 1 G, frequency 9.42 GHz, scan time 1 minute. Signal intensity (relative units) was measured by height of peak at 3334 G.



**Figure 3-5** Linear relationship of DMPO-OH signal amplitude and hydroxyl radical concentration. Spectra were recorded at RT, 1 minute after adding hydrogen peroxide. Power 1 mW, centre field 3340 G, scan range 100 G, time constant 0.1 second, modulation 1 G, frequency 9.42 GHz, scan time 1 minute. Signal intensity (relative unit) was measured by the height of signal at 3334 G.



**Photo 3-4** ESR spectrum of DMPO-OO adduct. DMPO 100 mM + xanthine 0.4 mM + xanthine oxidase 40 mU/ml in phosphate buffered saline pH 7.4.  $A_N = 14.2$  G,  $A_{H\beta} = 11.3$  G and  $A_{H\gamma} = 1.25$  G. Spectra were recorded at RT 1 minute after adding xanthine oxidase. Centre Field 3340 G, Scan range 100 G, power 10 mW, time constant 1 second, modulation 1 G, frequency 9.42 GHz, scan time 8 minute.

## 3.4.2. ESR Spectra of Myoglobin

No DMPO-OO or DMPO-OH signal was detected by ESR in the reaction of oxymyoglobin or metmyoglobin in the range of 10  $\mu$ mol/L to 300  $\mu$ mol/L with hydrogen peroxide in the range of 10  $\mu$ mol/L to 1 mmol/L (data not shown). At concentrations less than 100  $\mu$ mol/L there was no detectable ESR signal from the reaction of oxymyoglobin or metmyoglobin with hydrogen peroxide in the presence of DMPO (data not shown).

At high concentrations (for example 500  $\mu$ mol/L) oxymyoglobin or metmyoglobin produced a DMPO adduct with characteristic "slow tumbling" ESR spectrum in the reaction with 5 mmol/L hydrogen peroxide (Spectrum A of Photo 3-5). Adding 1 mmol/L ferrous iron led to the decrease of signal (Spectrum B), and increasing ferrous iron to 2 mmol/L led to further decrease of the signal (C of Photo 3-5).

#### 3.4.3. Myoglobin and DMPO

Because of the failure to detect any hydroxyl radical or superoxide anion signal by ESR from the reaction of myoglobin and hydrogen peroxide, extreme care was taken to make sure there was no error in the experiment. In addition, myoglobin was added into a hydroxyl radical production system to check for any possible effect of myoglobin on hydroxyl radical and/or spin adduct.

As shown on Photo-3-6, adding 20  $\mu$ M oxymyoglobin abolished the DMPO-OH signal. When added into a superoxide anion generating system (xanthine/xanthine oxidase) 10  $\mu$ M oxymyoglobin also abolished DMPO-OO signal (Photo 3-7).

By fixing the magnetic field at the peak of the DMPO-OH signal (3332 Gauss in this experiment) the dynamic change of signal was plotted against time. At 20

µmol/L oxymyoglobin totally abolished DMPO-OH signal in less than one and half minutes (Photo 3-8 and Table 3-1). Oxymyoglobin both diminished the peak signal intensity and shortened the apparent half-life in a concentration dependent manner. The effect of metmyoglobin was similar to oxymyoglobin (data not shown).

Apomyoglobin reduced peak DMPO-OH signal, but did not affect the rate of signal decay (Photo 3-9). The effect of apomyoglobin depended on the initial hydrogen peroxide concentration. At low initial hydrogen peroxide concentrations (1 mM), 5  $\mu$ M apomyoglobin produced about 50% of the inhibition of the DMPO-OH signal produced by the same concentration of oxymyoglobin. Increasing apomyoglobin concentration however led to a plateau, with some DMPO-OH remaining detectable at high apomyoglobin concentration. Bovine serum albumin behaved in a similar way to apomyoglobin. With high concentrations of hydrogen peroxide, the relative inhibition of the DMPO-OH signal by apomyoglobin or albumin compared with oxymyoglobin was much less.

Increasing hydrogen peroxide concentrations from 1 to 10 mM in the presence of sufficient oxymyoglobin (20  $\mu$ mol/L) completely to suppress the DMPO-OH signal at the original hydrogen peroxide concentration led to reappearance of the DMPO-OH signal. Decreasing ferrous iron concentration from 1 mM to 0.1 mM diminished the size of the DMPO-OH signal and also diminished the quantity of myoglobin required to suppress it from 20  $\mu$ M to 5  $\mu$ M. Oxy- or met-myoglobin also suppressed the DMPO-OH signal produced by photolysing hydrogen peroxide in the presence of DMPO but the absence of ferrous iron.

During the course of the reaction with  $H_2O_2$  in the presence of Fe<sup>2+</sup> and DMPO, oxymyoglobin was converted initially to ferrylmyoglobin, and subsequently to metmyoglobin over a time course of several minutes, similar to the reaction of oxymyoglobin with hydrogen peroxide without ferrous ion and DMPO.



**Photo 3-5** ESR spectra of DMPO-myoglobin radical adduct. A DMPO (100 mM) + oxymyoglobin (500  $\mu$ M) + hydrogen peroxide (5 mM); **B** as for (A) + Fe<sup>2+</sup> 1 mM; **C** as for (A) + Fe<sup>2+</sup> 2 mM. Spectra were recorded at RT 1 minute after adding H<sub>2</sub>O<sub>2</sub>. Power 1 mW, centre field 3340 G, scan range 60 G, time constant 1 second, modulation 1 G, frequency 9.42 GHz.



**Photo 3-6** Effect of myoglobin on DMPO-OH signal. A: DMPO 100 mM + 1 mmol/L hydrogen peroxide and ferrous iron; B: as A but with 10  $\mu$ M oxymyoglobin. Spectra were recorded at RT, 1 minute after adding hydrogen peroxide. Power 1 mW, centre field 3340 G, scan range 100 G, time constant 0.1 second, modulation 1 G, frequency 9.42 GHz, scan time 1 minute, representative of 4 separate experiments.



**Photo 3-7** Effect of myoglobin on DMPO-OO adduct. A DMPO + xanthine + xanthine oxidase; **B** as A but with 10  $\mu$ M oxymyoglobin. Spectra were recorded at RT 1 minute after adding xanthine oxidase. Centre field 3340 G, scan range 100 G, power 10 mW, time constant 1 second, modulation 1 G, frequency 9.42 GHz, scan time 8 minute, representative of 4 separate experiments.



**Photo 3-8** Dose dependent change of the DMPO-OH signal by oxymyoglobin. A 5 mM hydrogen peroxide and ferrous iron; **B**, **C** and **D** with 5, 10 and 20  $\mu$ M oxymyoglobin, respectively, in addition to 5 mM hydrogen peroxide and ferrous iron. Changes of DMPO-OH signal intensity were monitored by fixing magnetic field at 3334 G (peak of DMPO-OH). Recording started at 1 minute after adding hydrogen peroxide. Power 1 mW, time constant 0.1 second, modulation 1 G, frequency 9.42 GHz, representative of 4 separate experiments.

Concentration	H <sub>2</sub> C	O <sub>2</sub> 1 mM	H <sub>2</sub> O	<sub>2</sub> 5 mM
(μ <b>M</b> )	Peak <sup>a</sup>	Half-life <sup>b</sup>	Peak	Half-life
0	20.4	16.2	100.0	12.1
5	11.6	3.0	58.0	3.5
10	6.1	0.5	29.6	0.2
20	1.4	0.2	8.2	0.2
50	0	0	0	0
5	11.4	13.7	93.8	11.6
10	9.2	12.2	90.8	11.4
20	8.6	11.0	80.6	11.3
50	7.6	7.1	68.4	11.2
100	6.9	5.0	56.1	10.5
	Concentration (μM) 0 5 10 20 50 50 50 50 10 20 50 100	Concentration $H_2$ ( $\mu$ M) $(\mu$ M)Peaka020.4511.6106.1201.4500511.4109.2208.6507.61006.9	Concentration $H_2O_2 1 \text{ mM}$ ( $\mu$ M)PeakaHalf-lifeb020.416.2511.63.0106.10.5201.40.25000511.413.7109.212.2208.611.0507.67.11006.95.0	Concentration $H_2O_2 1 \text{ mM}$ $H_2O_2 (\mu M)$ ( $\mu M$ )PeakaHalf-lifebPeak020.416.2100.0511.63.058.0106.10.529.6201.40.28.250000511.413.793.8109.212.290.8208.611.080.6507.67.168.41006.95.056.1

 Table 3-1

 Effects of oxymyoglobin and apomyoglobin on the ESR signal of DMPO-OH

All results average of 4 experiments.

<sup>a</sup> Peak amplitude measured 1 min after mixing, expressed as % of signal amplitude for  $5 \text{ mM H}_2O_2$  control.

<sup>b</sup> Apparent half life defined as time for signal amplitude to decline to half its amplitude at 1 min.



**Photo 3-9** Effect of apomyoglobin on DMPO-OH signal. A: 5 mM hydrogen peroxide and ferrous iron; **B**, **C** and **D** with 10, 20 and 100  $\mu$ M apomyoglobin, respectively, in addition to 5 mM hydrogen peroxide and ferrous iron. Changes of DMPO-OH signal intensity were monitored by fixing magnetic field at 3334 G (peak of DMPO-OH). Recording started at 1 minute after adding hydrogen peroxide. Power 1 mW, time constant 0.1 second, modulation 1 G, frequency 9.42 GHz, representative of 4 separate experiments. ESR signal of 5 mM hydrogen peroxide and ferrous iron as 100%.

# **Chapter 4 Sublethal Oxidative Damage**

# **4.1. Introduction**

In the past the studies of endothelial damage by reactive oxygen species, including Chapter 3 of this thesis, concentrated on observing the effects of relatively high concentrations of reactive oxygen species, which cause increased endothelial membrane permeability to calcium ions and cell death (Hiraishi *et al.*, 1994; Volk *et al.*, 1997; Hu *et al.*, 1998)

However, briefly exposing cultured endothelial cells to concentrations or amounts of hydrogen peroxide lower than those that cause immediate cell lysis leads to a range of cellular function damage. This chapter describes the effects of sublethal amount of hydrogen peroxide on endothelial cells, including effects on morphology, mitochondrial activity, cell migration, prostacyclin production and cell proliferation. When exposing endothelial cells to hydrogen peroxide, on one hand the cells will be damaged by the oxidant, on the other hand the hydrogen peroxide is also eliminated by the cells. It is an interactive reaction; any cellular effect on the change of hydrogen peroxide will contribute to the ultimate fate of cells themselves. So it is very important also to study the effect of endothelial cells on the changes of hydrogen peroxide. It will also show that the concentration of hydrogen peroxide is a very important consideration in comparing oxidative damage, but the most important factor is the absolute amount of hydrogen peroxide per cell.

## 4.2. Morphology

The morphological effects of exposing a confluent BAEC monolayer to 0.25  $\mu$ mol/10<sup>6</sup> cells (0.25 picomol/cell, equivalent of 100  $\mu$ mol/L) hydrogen peroxide for 30 minutes, followed by washing and replacing medium, are shown in Photo 4-1 (representative of 3 experiments).

There was little morphological change at 1 hour after the starting of hydrogen peroxide exposure (Panel A of Photo 4-1), but by 3 hours the cells started to contract and detach from the substratum (Panel B and C of Photo 4-1). This process continued until few cells remained attached after 24 hours (Panel D of Photo 4-1). The monolayer was then gradually reconstituted by spreading of the remaining cells and migration from surrounding regions (Panel E of Photo 4-1). The cells adhered to the culture surface were counted under phase contrast microscope and represented as cells/mm<sup>2</sup>. The change of cell number was given in Figure 4-1, expressed as a function of time. The cell loss was peaked at 24 hours after oxidative stress, after that the cell number started to increase, either from migration of surrounding cells or proliferation of surviving cells.



Figure 4-1 Change of BAEC number after oxidative stress. Confluent BAEC in 80 cm<sup>2</sup> tissue culture flask were exposed to 0.25  $\mu$ mol/10<sup>6</sup> cells hydrogen peroxide for 60 min in Hanks before returned to normal culture medium. Cell numbers were counted under microscope at 5 randomly selected fields for 48 hours. Result is representative of 3 experiments.



**Photo 4-1 AB** Phase contrast micrographs of BAEC briefly exposed to 0.25 picomol/cell  $H_2O_2$  for 30 min. A: one hour and B: three hours after oxidative stress. Original magnification x 10, representative of 2 experiments with 4 samples each.



**Photo 4-1 CDE** Phase contrast micrographs of BAEC briefly exposed to 0.25 picomol/cell  $H_2O_2$  for 30 min. C: 7 hours; D: 24 hours and E 48 hours after oxidative stress, respectively. Original magnification x 10, representative of 2 experiments with 4 samples each.

Photos 4-2 shows the morphological change of confluent BAEC subjected to 60 min exposure to different amounts (or concentrations) of hydrogen peroxide. There was no visible change in control cells for up to 48 hours (Photos 4-2-C). When exposed to 20  $\mu$ mol/L hydrogen peroxide (0.05  $\mu$ mol/10<sup>6</sup> cells, 0.05 pmol/cell) relatively few cells rounded up and died off within 10 hours after oxidative stress (panels 1 to 6 of Photo 4-2-HA). Twenty-four hours after oxidative stress no area was left uncovered, but the cell size seemed to become larger compared to that of control cells or the same cells before oxidative stress (panels 7 and 8 of Photo 4-2-HA). When hydrogen peroxide was increased to 50  $\mu$ mol/L in concentration (0.1  $\mu$ mol/10<sup>6</sup> cells, 0.1 pmol/cell) endothelial cells began to round up at 1 hour after oxidative stress (panel 2 of Photo 4-2-HB), which increased by 2 hours and the cells were seen to detach (panel 3 of Photo 4-2-HB). This process seemed to continue for up to 24 hours (panels 4 to 6 of Photo 4-2-HB), then the cells remaining alive began to stretch out and grow in size (panels 7 and 8 of Photo 4-2-HB). Cells treated with 100  $\mu$ mol/L (0.2  $\mu$ mol/10<sup>6</sup> cells, 0.2 pmol/cell) hydrogen peroxide showed similar damage to that seen with 50  $\mu$ mol/L (0.1  $\mu$ mol/10<sup>6</sup> cells, 0.1 pmol/cell) hydrogen peroxide, but more cells died off and less cells were left to form giant cells at 48 hours after oxidative assault (Photo 4-2-HC). More hydrogen peroxide caused different pattern of damage. At 500  $\mu$ mol/L (1  $\mu$ mol/10<sup>6</sup> cells, 1 pmol/cell) hydrogen peroxide mainly caused cell lysis, with few cells rounded up and came off, most of the dead cells remained on the culture surface (Photo 4-2-HD). At concentration of 1 mmol/L (2  $\mu$ mol/10<sup>6</sup> cells, 2 pmol/cell) hydrogen peroxide killed all the cells instantly without any cells rounding up or detaching (Photos 4-2-HE).



**Photo 4-2 C** Phase contrast micrographs of control BAEC. From C1 to C8 photos were taken before and at 1, 2, 3, 5, 10, 24 and 48 hours. Representative of 2 experiments with 2 samples each, 100 x magnification.



**Photo 4-2 HA** Phase contrast micrographs of BAEC exposed to 0.05 pmol/cell  $H_2O_2$  for 60 min before returning to culture medium. From HA1 to HA8 photos were taken before and at 1, 2, 3, 5, 10, 24 and 48 hours after oxidant stress. Representative of 2 experiments with 2 samples each, 100 x magnification.



**Photo 4-2 HB** Phase contrast micrographs of BAEC exposed to 0.1 pmol/cell  $H_2O_2$  for 60 min before returning to culture medium. From HB1 to HB8 photos were taken before and at 1, 2, 3, 5, 10, 24 and 48 hours after oxidant stress. Representative of 2 experiments with 2 samples each, 100 x magnification.



**Photo 4-2 HC** Phase contrast micrographs of BAEC exposed to 0.2 pmol/cell  $H_2O_2$  for 60 min before returning to culture medium. From HC1 to HC8 photos were taken before and at 1, 2, 3, 5, 10, 24 and 48 hours after oxidant stress. Representative of 2 experiments with 2 samples each, 100 x magnification.



**Photo 4-2 HD** Phase contrast micrographs of BAEC exposed to 1 pmol/cell  $H_2O_2$  for 60 min before returning to culture medium. From HD1 to HD8 photos were taken before and at 1, 2, 3, 5, 10, 24 and 48 hours after oxidant stress. Representative of 2 experiments with 2 samples each, 100 x magnification.



**Photo 4-2 HE** Phase contrast micrographs of BAEC exposed to 2 pmol/cell  $H_2O_2$  for 60 min before returning to culture medium. From HD1 to HD8 photos were taken before and at 1, 2, 3, 5, 10, 24 and 48 hours after oxidant stress. Representative of 2 experiments with 2 samples each, 100 x magnification.

## 4.3. Mitochondrial Dehydrogenase Activity

The effects of 60 min exposure to different concentrations of hydrogen peroxide on endothelial mitochondrial dehydrogenase activity are shown in Figure 4-2 to 4-5. Bovine endothelial cells were cultured in 96 well plates at densities from 5 to 40 x  $10^3$ /well, exposed to hydrogen peroxide at concentrations of 5 to 100  $\mu$ mol/L, 200 µl/well, 8 wells for each group. Cellular dehydrogenase activity was measured from 2 to 24 hours after oxidative stress using a CellTiter proliferation assay kit, with that of control cells without being exposed to hydrogen peroxide as 100%. The cell number was quantified by a CyQUANT proliferation assay kit. Mitochondrial dehydrogenase activity was partially impaired initially (within 6 hours after oxidative stress) in all concentrations of hydrogen peroxide and in different cell density. This decrease of dehydrogenase activity was time and hydrogen peroxide concentration dependent, with fastest drop within 2 hours after oxidative stress, which slowed down there after, and after 6 hours some of the cells started to recover their lost dehydrogenase activity. At the same concentration, hydrogen peroxide caused more damage in relatively sparse cells than that in dense cells. For example, the dehydrogenase activity of cells treated with 20  $\mu$ mol/L hydrogen peroxide in density of 5 x 10<sup>3</sup>/well did not recover after 24 hours (Figure 4-2), while that of cells treated with 100 µmol/L hydrogen peroxide in density of 40 x  $10^3$ /well did (Figure 4-5).

Detailed examination of the results revealed that endothelial damage depended on the amount of hydrogen peroxide available to each cell, rather than the concentration. When cellular dehydrogenase activity was expressed against the amount of hydrogen peroxide available to each cell but not the concentration, the apparent effect of cell density on damage by hydrogen peroxide was removed (Table 4-1). For example, 6 hours after being exposed to 0.1 picomol/cell hydrogen peroxide the cellular dehydrogenase activities were all between 63 to 69% of control cells, irrespective of whether the cell density was 5 x  $10^3$ /well



**Figure 4-2** Reduction of endothelial dehydrogenase activity by  $H_2O_2$  at cell density of 5 x 10<sup>3</sup>/well. BAEC in 96 well plate were exposed to  $H_2O_2$  for 60 min before returning to culture medium. Dehydrogenase activity was measured by MTS assay at given times with control cells as 100%. From A to F the  $H_2O_2$  concentration was 0, 5, 10, 20, 50 and 100  $\mu$ M, respectively. Graph is representative of 4 sets of separate experiments.



Figure 4-3 The same as in Figure 4-2, but with cell density of  $10 \times 10^3$ /well.



**Figure 4-4** Reduction of endothelial dehydrogenase activity by  $H_2O_2$  at cell density of 20 x  $10^3$ /well. BAEC in 96 well plate were exposed to  $H_2O_2$  for 60 min before returning to culture medium. Dehydrogenase activity was measured by MTS assay at given times with control cells as 100%. From A to F the  $H_2O_2$  concentration was 0, 5, 10, 20, 50 and 100  $\mu$ M, respectively. Graph is representative of 4 sets of separate experiments.



Figure 4-5 The same as in Figure 4-4, but with cell density of  $40 \times 10^3$ /well.

Time	Control	0.0125	0.025	0.05	0.1	0.2	0.5	1	2	
2 Hours	100	-	-	-	65	54	45	32	26	
6 Hours	100	-	-	-	69	57	37	23	14	
12 Hours	100	-	-	-	73	62	31	19	11	
24 Hours	100	-	-	-	78	68	19	8	6	

# Table 4-1 Damage of BAEC Dehydrogenase Activity by Different Amounts of H<sub>2</sub>O<sub>2</sub> (pmol/cell) at Different Cell Density

 $5 \times 10^3$  cells/well

1	A		11	3	aalla	1	- 11
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Time	Control	0.0125	0.025	0.05	0.1	0.2	0.5	1	2	
2 Hours	100	-	-	74	61	49	43	26	-	
6 Hours	100	-	-	81	66	53	37	21	-	
12 Hours	100	-	-	88	70	58	28	16	-	
24 Hours	100	-	-	92	74	64	17	8	-	

 $20 \times 10^3$  cells/well

Time	Control	0.0125	0.025	0.05	0.1	0.2	0.5	1	2	
2 Hours	100	-	83	71	65	47	35	-	-	
6 Hours	100	-	86	77	69	52	31	-	-	
12 Hours	100	-	91	86	73	62	28	-	-	
24 Hours	100	-	96	90	78	69	16	-	-	

Time	Control	0.0125	0.025	0.05	0.1	0.2	0.5	1	2
2 Hours	100	94	86	74	61	44	-	-	-
6 Hours	100	97	91	80	63	47	-	-	-
12 Hours	100	100	93	84	69	56	-	-	-
24 Hours	100	102	96	92	74	64	-	-	-

 $40 \ge 10^3$  cells/well

BAEC cultured in 96 well plates with densities of 5 - 40 x  $10^3$ /well were exposed to different concentrations of H<sub>2</sub>O<sub>2</sub> for 60 min before returning to culture medium. Dehydrogenase activity was measured by MTS assay at given time with control cells as 100%. Data are representative of 4 sets of separate experiments.
or 40 x  $10^3$ /well (Table 4-1). When the amount of hydrogen peroxide was no more than 0.2 pmol/cell, it seemed the mitochondrial oxidative activity could gradually recover 2 hours after oxidative stress, indicating cell survival. When the amount of hydrogen peroxide was at 0.5 pmol/cell or higher, cell mitochondrial activity would continue to decline, indicating cell death.

# 4.4. Tritiated Thymidine Uptake

Hydrogen peroxide in the amount of 0.0125 to 0.5 picomol/cell (12.5 to 500 femtomol/cell) inhibited endothelial cell thymidine uptake in a dose dependent manner (Figure 4-6). This reduced thymidine uptake could be caused by cell loss,



**Figure 4-6** Effect of hydrogen peroxide on BAEC dehydrogenase activity and tritiated thymidine uptake. Confluent BAEC in 96 well plates were subjected to 60 min exposure to different concentrations of hydrogen peroxide before returning to culture medium with or without 0.0037 MBq/ml <sup>3</sup>H thymidine. Cellular dehydrogenases activity and <sup>3</sup>H thymidine uptake was measured 24 hours after oxidative stress, with control cells without exposing to hydrogen peroxide as 100%. Result was representative of 3 separate experiments, 8 wells in each group.



**Figure 4-7.** Semilogarithmic plot of tritiated thymidine uptake by BAEC. BAEC in 96 well plates were subjected to 60 min exposure to hydrogen peroxide before returning to culture medium with 0.0037 MBq/ml [<sup>3</sup>H] thymidine. Cellular [<sup>3</sup>H] thymidine uptake was measured at given time after oxidative stress. A and B: control cells plated at  $1.8 \times 10^3$  and  $3.75 \times 10^3$  per well respectively (densities chosen to bracket thymidine uptake of hydrogen peroxide treated cells after exposure); C: endothelial cells with initial density 24 x 10<sup>3</sup> per well treated with 0.25 pmol/cell hydrogen peroxide. Result was representative of 3 separate experiments. or by reduced DNA synthesis in damaged but surviving cells indicating inhibition of cell proliferation. To confirm the inhibition of DNA synthesis in damaged, but surviving cells, parallel experiment was carried out using mitochondrial dehydrogenase activity to demonstrate the existence of living cells. Figure 4-6 shows the cells surviving by mitochondrial dehydrogenase activity, but with impaired DNA synthesis after sublethal (causing cell damage but not cell death) hydrogen peroxide treatment. The results showed that hydrogen peroxide had a dramatic damage effect on endothelial cell proliferation at even very low dose (for example, more than 90% inhibition at 0.1 pmol/cell for 60 min exposure).

Endothelial cells have a tendency to grow a bit faster at relatively high density and a little bit slower at relatively low density. To make sure the reduced DNA synthesis caused by hydrogen peroxide was not mainly due to lower cell density through cell death, experiment was designed to bracket untreated control cells with comparable thymidine uptake in 24 hours close to the expected uptake by hydrogen peroxide treated cells. For example if the expected thymidine uptake after hydrogen peroxide treatment is around 50 counts/min, the control cells will be plated at different densities to obtain 30 to 100 counts/min thymidine uptake. Figure 4-7 shows marked long term inhibition of  $[{}^{3}H]$  thymidine uptake by H<sub>2</sub>O<sub>2</sub> treated cells compared to control cells plated at lower density but with comparable initial thymidine uptake in first 24 hours. The cells were treated with 0.25 picomol/cell hydrogen peroxide for 60 min and the cells were returned to culturing medium containing tritiated thymidine. Eight replicates were used for each sample and the result is representative of 2 experiments. Control cells, though at very low density, showed logarithmic growth measured by thymidine uptake. Hydrogen peroxide treated cells showed continued decline of thymidine uptake for as long as 3 days after oxidative exposure.

# 4.5. Incorporation of BrdU

Proliferating BAEC incorporate BrdU from the medium into DNA, which can be visualised by FITC conjugated antibody, as illustrated in Panel A of Photo 4-3. In parallel with the [ $^{3}$ H] thymidine uptake experiments, hydrogen peroxide treated cells were incubated with BrdU. Very little nuclear uptake could be seen for 24 hours incubation after oxidative stress (Panel B of Photo 4-3 and Figure 4-8), while control cells had 100% positive labelling. The inhibition of BrdU incorporation lasted for at least 4 days after 60 min exposure to hydrogen peroxide at 0.125 picomol/cell (figure 4-8).



Figure 4-8 Effect of Hydrogen Peroxide on BAEC BrdU Labelling. Same experiment as in Photo 4-3. Representative of 2 separate experiments of 2 samples each.



**Photo 4-3** BrdU staining of BAEC. Confluent BAEC were exposed to  $H_2O_2$  for 60 min in Hanks before loaded with 10  $\mu$ M BrdU for 24 hours in culture medium. Incorporated BrdU was labelled by FITC conjugated antibody and visualised by fluorescence microscope. A: Control cells; B: Cells exposed to 0.125 pmol/cell  $H_2O_2$  for 60 min before BrdU labelling, 100 x magnification.

## **4.6. Re-Endothelialisation**

When confluent endothelium is denuded, surrounding cells will move in to cover the area. Using a glass rod to scratch confluent BAEC monolayer to create a denuded area of about 60 to 100 cells (Panel C1 of Photo 4-4), neighbouring cells started to migrate in less than an hour (Panel C2 of Photo 4-4). The whole area was completely recovered by migrating cells in less than 3 hours (Panel C3 of Photo 4-4). In contrast, exposing cells to very small amount of hydrogen peroxide (0.05 pmol/cell), too little to cause cell death, remarkably prolonged the reendothelialisation process. Although there was some cell migration after 1 hour of denudation (Panel H2), most of the denuded area was still uncovered by endothelial cells at 6 hours after mechanical scratch (Panel H4 of Photo 4-4).



**Photo 4-4** Effect of hydrogen peroxide on re-endothelialisation. Confluent BAEC were scratched with a glass rod. Photos C1 to C4 were taken from control cells at 0, 1, 3 and 6 hours from same field; Photos H1 to H4 were taken from cells treated with 0.05 pmol/cell (20  $\mu$ mol/L) for 30 min before scratch at the same time frame. Representative of 2 experiments, 100 times magnification.

# **4.7. Intracellular Peroxidation**

The staining pattern of DCF-DA in endothelial cells was first examined under fluorescence microscope in cells cultured on glass slides (Photo 4-12). Propidium iodide was used to identify cells with membrane damage, which stains the DNA and RNA of damaged cells so that they can be distinguished from the cells with intact cell membranes. Normal endothelial cells stained with DCF-DA showed very weak background green fluorescence, visible under fluorescence microscope, but it could not be photographed because the signal was too weak and it would be bleached in seconds. Photo 4-5 was taken from cells treated with hydrogen peroxide at 50 fmol/cell. The signal was much brighter than that from control cells, but again it was bleached by light very quickly. This rapid bleaching of the signal makes it impractical to quantify the difference in intracellular peroxidation by fluorescence microscopy or imaging analysis. Nevertheless it did show that cells stained by green fluorescence were not stained with red PI did not stain with DCF, indicating DCF-DA would and only stained undamaged cells.

To examine the intracellular peroxidation of DCF-DA by flow cytometry endothelial cells were suspended in Hanks solution at  $10^6$ /ml and loaded with 20 µmol/L DCF-DA for 30 min at 37 °C as described in Chapter 2. There were 3 groups of cells according to their DCF and PI staining measured by flow cytometer (Photo 4-6). Cells in Region A (R1) had weak DCF staining and negative PI staining, representing normal cells with background DCF and intact cell membrane. Cells in Region B (R2) had strong PI signal and negative DCF signal, representing dead cells with broken membranes. Cells neither in R1 nor in R2 had variable degree of both positive PI staining and DCF staining, representing partially damaged cells with compromised membranes. Only cells in R1 with positive DCF staining and negative PI staining, indicating they were viable cells with intact membrane, were used to measure intracellular peroxidation.



**Photo 4-5** DCF staining of endothelial cells. BAEC on glass slides were loaded with 10  $\mu$ M DCF-DA for 30 min at 37 °C. Cells were then added with hydrogen peroxide at final concentration of 10  $\mu$ M and the photo was immediately taken under fluorescence microscope. Live cells with intact membrane were stained green in whole cells and without red staining in nuclei; membrane damaged cells had strong PI staining in nuclei but did not show green staining in cytoplasm. Photo is representative of 3 experiments with magnification of 200.



DCF (Green)

**Photo 4-6** Flow cytometry detection of endothelial cells DCF staining. Endothelial cells were suspended in Hanks solution and loaded with 10  $\mu$ M DCF-DA for 30 min at 37 °C. PI was added at 2  $\mu$ g/ml before running samples on FACScan. Cells in R1 with background DCF staining (Green) and negative PI staining (red) represented undamaged cells and were used to measure intracellular peroxidation in all experiments.

Adding hydrogen peroxide into endothelial cell suspensions caused a dose and time dependent increase in intracellular peroxidation measured by DCF and flow cytometry (Figure 4-9). As low as 5 femtomol/cell extracellular hydrogen peroxide led to detectable increase in intracellular peroxidation within 5 minutes. The increase in intracellular peroxidation was instant, measurable within 1 minute, indicating the ability of hydrogen peroxide to rapidly travel through cell membrane. The signal reached a peak at about 10 min, after which its intensity gradually reduced, possibly due to the leaking of DCF out of cells and/or the consumption of hydrogen peroxide by cells. In the range of 5 to 50 fmol/cell, intracellular peroxidation increased with increased amount of hydrogen peroxide. Above 50 fmol/cell, increased hydrogen peroxide only caused much limited increase in intracellular peroxidation (data not shown).

Exposing cells to extracellular t-butyl hydroperoxide instead of hydrogen peroxide also increased intracellular peroxidation in a dose and time dependent manner, but with much greater effects (Figure 4-10). t-Butyl hydroperoxide increased cellular peroxidation to as much as three times of that by equivalent amount of hydrogen peroxide. It seemed t-butyl hydroperoxide also slowed down the reduction of signal and apparently shifted the peak time from 10 min after treatment of hydrogen peroxide to 15 min after treatment of hydroperoxide.



Figure 4-9 Endothelial intracellular peroxidation by hydrogen peroxide measured by DCF and flow cytometry. Endothelial cells were suspended in Hanks solution and loaded with 10  $\mu$ M DCF-DA for 30 min at 37 °C. PI was added at 2  $\mu$ g/ml to mark and gate out damaged cells. Different amounts of hydrogen peroxide was added into cells and cellular fluorescence was measured by FACScan. Figure is representative of 6 experiments.



**Figure 4-10** Endothelial intracellular peroxidation by t-butyl hydroperoxide measured by DCF and flow cytometry. Endothelial cells were suspended in Hanks solution and loaded with 10  $\mu$ M DCF-DA for 30 min at 37 °C. PI was added at 2  $\mu$ g/ml to mark and gate out damaged cells. Different amounts of t-butyl hydroperoxide was added into cells and cellular fluorescence was measured by FACScan. Figure is representative of 2 experiments.

# 4.8. Elimination of Hydrogen Peroxide by Endothelial Cells

Endothelial cells have systematic antioxidant defences. Extracellular hydrogen peroxide not only has to penetrate into cells but also has to overcome cellular defence before it can cause intracellular damage. The elimination of extracellular hydrogen peroxide was used to monitor the dynamic change of hydrogen peroxide and estimate the total antioxidant potential in endothelial cells.

Hydrogen peroxide at initial amounts of 0.25 to 16 picomol/cell was consumed by endothelial cells in a way dependent both on time and on the initial amount available to each cell (Figure 4-11, 4-12 and Table 4-2). The consumption was relatively high over the first 10 to 20 minutes and slowed down later.

The elimination of hydrogen peroxide was dependent on cell number. The higher the cell number the faster and more reduction of hydrogen peroxide. Not only the amount of hydrogen peroxide removed was dependent on the number of endothelial cells, the removal rate seemed dependent on the amount of hydrogen peroxide for each cell and had no relationship with initial concentration (Figure 4-13 and Table 4-3). For example at initial hydrogen peroxide amount of 1 fmol/cell, endothelial cells would remove about 0.8 femtomol in 60 min for each cell, regardless of whether the initial concentration was 50, 100 or 200  $\mu$ mol/L. When the amount of hydrogen peroxide no more than 2 picomol per cell, endothelial cells were able to increase the removal rate with increased amount of hydrogen peroxide. At amounts above 2 pmol/cell, the increase in hydrogen peroxide removal was getting less and less, indicating most of the cellular antioxidant ability was being exhausted.



Figure 4-11 Decrease of hydrogen peroxide by different numbers of endothelial cells. BAEC were cultured in 96 well plates at densities of A:2500; B: 5000; C: 10000; D: 20000 and E: 40000 cells/well. Cells were incubated with hydrogen peroxide for 60 minutes in Hanks at initial concentration of 50  $\mu$ mol/L, 200  $\mu$ l/well and total amount of 10 nano mol/well, 8 wells in each group. Hydrogen peroxide concentrations were measured by Xylenol orange method. Figure is representive of 3 separated experiments.



Figure 4-12 hydrogen peroxide removal by BAEC. Cells were cultured in 96 well plates at densities of 2500 to 40000/well. Different numbers of cells were incubated with different concentrations of  $H_2O_2$  for 60 minutes in Hanks: A: 50  $\mu$ mol/L; B: 100  $\mu$ mol/L and C: 200  $\mu$ mol/L, 8 wells in each group. The amounts of hydrogen peroxide removed were expressed as picomol/cell in 60 min. Hydrogen peroxide concentrations were measured by Xylenol orange method. Figure is representive of 3 separated experiments.



**Figure 4-13** Reduction of hydrogen peroxide concentration by endothelial cells. Data are from the same experiment as presented in Figure 4-12, but the removal of hydrogen peroxide is expressed as the decrease in concentration rather than amount/cell.

5 Min									
H <sub>2</sub> O <sub>2</sub>	0.25	0.50	1.00	2.00	4.00	8.00	16.00		
50 µM	0.06	0.09	0.13	0.21	0.31				
100 µM		0.17	0.23	0.28	0.40	0.64			
200 µM			0.24	0.35	0.44	0.56	0.73		
10 Min									
H <sub>2</sub> O <sub>2</sub>	0.25	0.50	1.00	2.00	4.00	8.00	16.00		
50 µM	0.10	0.16	0.25	0.39	0.58				
100 µM		0.23	0.36	0.52	0.76	1.16			
200 μΜ			0.36	0.56	0.73	1.05	0.91		
			20 N	lin					
HaOa	0.25	0.50	1.00	2.00	4.00	8.00	16.00		
11202							10.00		
50 µM	0.14	0.25	0.40	0.64	1.01	4 60			
100 μΜ		0.30	0.51	0.81	1.28	1.68			
200 µM		. <u></u>	0.53	0.87	1.28	1.76	1.44		
			<b>40</b> M	lin					
H <sub>2</sub> O <sub>2</sub>	0.25	0.50	1.00	2.00	4.00	8.00	16.00		
50 µM	0.20	0.36	0.60	1.04	1.59				
100 µM		0.39	0.70	1.22	1.86	2.32			
200 μΜ			0.71	1.22	1.88	2.24	2.00		
	``````````````````````````````````````		60 M	lin					
H <sub>2</sub> O <sub>2</sub>	0.25	0.50	1.00	2.00	4.00	8.00	16.00		
50 µM	0.23	0.44	0.76	1.30	2.03				
100 µM		0.44	0.79	1.37	2.06	2.44			
200 μΜ			0.84	1.43	2.20	2.55	2.71		

### Table 4-2 Removal of H<sub>2</sub>O<sub>2</sub> by Endothelial Cells (pmol/cell)

 $H_2O_2$  (200 µl/well) at concentrations of 50, 100 or 200 µmol/L was added to different numbers of cells (2500 to 40000 cells/well) for 60 min and initial amounts were calculated as from 0.25 to 16 picomol/cell. The amount of hydrogen peroxide removed was also calculated as picomol/cell. Data are representative of 3 separated experiments.

e man meubación with Different fumber of Endomenar cons							
H <sub>2</sub> O <sub>2</sub>	No Cell	2500	5000	10000	20000	40000	
50 µM	0.5	3.4	4.8	6.2	8.3	11.7	
100 µM	0.9	6.9	9.2	13.0	22.2	33.5	
200 µM	2.2	6.9	11.9	19.9	32.5	45.9	

# Table 4-3 Reduction of Hydrogen Peroxide Concentration by Endothelial Cells

# 5 Min Incubation with Different Number of Endothelial Cells

10 Min Incubation with Different Number of Endothelial Cells

H <sub>2</sub> O <sub>2</sub>	No Cell	2500	5000	10000	20000	40000
50 µM	0.2	6.8	9.3	12.0	15.3	19.4
100 μM	1.8	12.6	17.2	24.4	34.0	44.5
200 µM	3.6	9.7	24.6	34.9	54.7	71.2

20 Min Incubation with Different Number of Endothelial Cells

H <sub>2</sub> O <sub>2</sub>	No Cell	2500	5000	10000	20000	40000
50 μM	0.2	12.1	15.6	19.5	24.1	28.3
100 μM	1.8	19.3	30.1	38.8	48.9	58.0
200 µM	3.6	15.4	41.4	61.6	84.7	103.3

#### 40 Min Incubation with Different Number of Endothelial Cells

H <sub>2</sub> O <sub>2</sub>	No Cell	2500	5000	10000	20000	40000
50 µM	0.4	19.5	25.5	29.8	35.6	38.9
100 µM	2.2	26.7	44.2	59.0	68.0	76.5
200 µM	2.9	22.1	52.9	90.9	118.9	139.2

#### 60 Min Incubation with Different Number of Endothelial Cells

H <sub>2</sub> O <sub>2</sub>	No Cell	2500	5000	10000	20000	40000
50 µM	0.3	25.2	32.4	38.0	43.4	45.6
100 μM	1.7	28.9	49.7	67.0	76.8	86.0
200 µM	4.2	25.2	58.6	104.9	137.3	162.8

 $H_2O_2$  (200 µl/well) at concentrations of 50, 100 or 200 µmol/L was added to different numbers of cells (2500 to 40000 cells/well) for 60 min. Data are from the same experiment as presented in Table 4-2, only the reduction of hydrogen peroxide was expressed as change in concentration without reference to number of cells.

# **4.9. Prostacyclin Production**

Production of prostacyclin is one of the most important endothelial cell functions. Cultured BAEC released about 30 pg PGI<sub>2</sub> into the medium for every mg of protein in one minute (Figure 4-14). Stimulation by bradykinin, or arachidonic acid or calcium ionophore A23187 increased endothelial cell PGI<sub>2</sub> production. However, exposure to 0.25 pmol/cell (100  $\mu$ mol) hydrogen peroxide for 60 min inhibited about 90% endothelial cell PGI<sub>2</sub> production immediately after oxidative stress. Stimulating cells with bradykinin, or arachidonic acid or calcium ionophore A23187 failed to restore endothelial cell PGI<sub>2</sub> release after oxidative stress.



**Figure 4-14** Effect of hydrogen peroxide on endothelial cell basal and stimulated PGI<sub>2</sub> production. Control: Cells not exposed to hydrogen peroxide; HOOH: Cells exposed to 0.5 pmol/cell (100  $\mu$ mol) hydrogen peroxide for 60 min. Basal: no stimulation; A23187, BK and AA: Cells stimulated by 10  $\mu$ M calcium ionophore 23187, bradykinin or arachidonic acid, respectively. Results are expressed as mean  $\pm$  SD, representative of 2 experiments.

# **Chapter 5 Apoptosis**

Results in Chapters 3 and 4 have shown that high concentrations (>1 mmol/l) or large amount (>1  $\mu$ mol/10<sup>6</sup> cells) hydrogen peroxide will kill endothelial cells instantly by cell necrosis. Briefly exposing cultured endothelial cells to concentrations or amounts of hydrogen peroxide lower than those causing immediate cell lysis leads to a range of cellular function damage as described in Chapter 4. In addition it has been noticed in previous experiments that some cell shrinkage and fragmentation appeared at several hours after low dose and brief hydrogen peroxide exposure similar to that of apoptosis. The following experiments were designed to study endothelial cell apoptosis by hydrogen peroxide.

# 5.1. Microscopy Study

### 5.1.1. Live Cells

Confluent bovine endothelial cells cultured in 80 cm<sup>2</sup> flasks were exposed to 0.25 picomol/cell hydrogen peroxide for 60 min in Hanks. The cells were then returned to normal culturing medium to continue culture. Endothelial cell apoptosis were examined under phase contrast microscope with magnification of 10 x 10 and photos were taken at given times (Photo 5-1).

At 3 hours after hydrogen peroxide exposure, some cells had rounded up and some other cells fragmented into apoptotic bodies (panel B of Photo 5-1). Similar morphology was seen at 4 hours after oxidative stress (panel C). From 5 hours to 24 hours after the treatment there were variable number of rounded cells and apoptotic bodies (panels D to H). The rounded cells usually came off the culturing surface or advanced into necrosis. Because the apoptotic cells usually



**Photo 5-1** Endothelial morphological change after 60 min exposure to 0.5 pmol/cell hydrogen peroxide. From A to H, the photos were taken at 0, 3, 4, 5, 6, 8, 10 and 24 hours after oxidative stress, respectively. Arrow in Panel B points to apoptotic bodies, 100 x magnification.

came off the surface at different stages, it is difficult to calculate the percentage or occurrence of apoptosis by counting rounded cells and/or apoptotic bodies.

Continued monitoring of endothelial cells by time lapse video microscopy revealed that mitotic cells were among the first to undergo apoptosis when treated with hydrogen peroxide (photos not shown because the low quality of video image).

### 5.1.2. Haematoxylin Staining

In another experiment the morphology of endothelial apoptosis was examined after haematoxylin staining. Photo 5-2 and 5-3 show morphology of apoptosis in HE stained endothelial cells. Confluent endothelial cells in 6 well plates were exposed to 0.25 picomol/cell hydrogen peroxide for 60 min in Hanks. The cells were then returned to normal culturing medium to continue culture. At given times cells were washed with PBS and fixed with 1% paraformaldehyde for 10 min at room temperature. The samples were then stained with haematoxylin as described in Chapter 2 General Methods.

Photos in Photo 5-2 were taken under phase contrast microscopy and photos in Photo 5-3 were taken from bright field microscopy, all with magnification of  $10 \times 40$ . These photos show that cell shrinkage and apoptotic bodies appeared as early as 2 hours after oxidative stress. Morphological changes seemed to reach the peak at 8 hours, but were still visible even at 24 hours.

#### 5.1.3. Propidium Iodide Staining

Confluent endothelial cells cultured on glass slides were exposed to 0.25 or 0.5 picomol/cell hydrogen peroxide for 60 min in Hanks. The cells were then returned to normal culturing medium to continue culture. At given



**Photo 5-2.** Apoptotic morphology of haematoxylin stained BAEC under phase contrast microscope. A: Control; from B to E: Cells at 2, 4, 8 and 24 hours after oxidative stress. Arrow points to an apoptotic cell, 400 times magnification.



**Photo 5-3.** Apoptotic morphology of haematoxylin stained BAEC under bright field microscope. A: Control; from B to E: Cells at 2, 4, 8 and 24 hours after oxidative stress. Arrow points to an apoptotic cell, 400 times magnification.

times cells were washed with PBS and fixed with 1% paraformaldehyde for 10 min at room temperature. Cellular DNA was stained by 10  $\mu$ g/ml PI in the presence of RNAse to avoid RNA staining. Photographs were taken under the fluorescence microscope.

Photo 5-4 shows typical apoptotic endothelial cells. The cells rounded up with condensed and marginalized DNA, a nuclear appearance typical of apoptotic cell death.

At 4 hours after oxidative stress, some nuclei became condensed and fragmented (panels B and C of Photo 5-5). In control cells there was no DNA fragmentation, but some mitotic cells also had condensed nuclei (panel A of Photo 5-5). It was difficult to count exactly how many cells were undergoing apoptosis using this criterion, because endothelial cells entered apoptosis at different times, dying cells continuing to come off and not all cells with condensed nucleus are undergoing apoptosis. Nevertheless, cells exposed to 0.5 picomol/cell hydrogen peroxide did give the impression of having more apoptosis than that of cells exposed to 0.25 picomolar hydrogen peroxide. Similar results were seen from cells at 6 and 12 hours after oxidative stress, with varying number of apoptotic cells.

Treating HUVEC with colcemid for 2 hours in control samples obtained about 10% metaphase cells. But in HUVEC treated with 0.5 picomol/cell hydrogen peroxide no metaphase cells could be seen with or without colcemid (photos not show).

To investigate if actively growing cells were more susceptible to apoptosis induced by hydrogen peroxide than cells in other phase, BrdU was used to mark those cells before or after hydrogen peroxide exposure.



**Photo 5-4.** BAEC DNA fragmentation. The cells were exposed to 0.5 picomol/cell hydrogen peroxide for 60 min, fixed at 4 hours after treatment and stained with 10  $\mu$ g/ml PI for 30 min before taking photographs under fluorescence microscope with 400 times magnification. Arrow points to DNA fragmentation in cell.

Enoto 5-5. PART deprese in 4 hourd ofter solidate the Ar Control, B and C cells express which is 125 or 0.5 picomol/cell 100, for 60 min. Photos were from cells fixed as a toward other treatment. Acrows point to apoptitic cells, 100 times magnification.



**Photo 5-5.** BAEC apoptosis at 4 hours after oxidative stress. A: Control; B and C: cells exposed to 0.25 or 0.5 picomol/cell  $H_2O_2$  for 60 min. Photos were from cells fixed at 4 hours after treatment. Arrows point to apoptotic cells, 100 times magnification.

### 5.1.4. BrdU staining

The relationship of hydrogen peroxide induced apoptosis and cell phase are shown in Photo 5-6 and Photo 5-7. In Photo 5-6 about 70% confluent HUVEC cultured on glass slides were loaded with 50 µM BrdU in culture medium before exposed to 0.5 picomol/cell hydrogen peroxide for 60 min in Hanks solution. The cells were then returned to normal culture medium for 4 hours, fixed and processed as described in Chapter 2 General Methods. BrdU would only be incorporated into growing cells, labelled by FITC conjugated antibody showing green under fluorescence microscope. PI would stain DNA in all cells red. In the case of nuclei were stained both by green FITC and red PI the nuclei would show yellow as a result. There were about 50% of yellow BrdU positive cells in control, but no DNA condensation or fragmentation. In contrast in the hydrogen peroxide treated sample all BrdU positive cells showed DNA condensation and/or fragmentation while all BrdU negative cells did not. It implies active growing cells are more susceptible to hydrogen peroxide induced apoptosis than resting cells.

Similar results were shown with HUVEC treated with 0.5 picomol/cell hydrogen peroxide for 60 min before loaded with 50  $\mu$ M BrdU for 30 min (Photo 5-7). Again, there was no DNA condensation or fragmentation in control cells and all BrdU positive cells showed some DNA condensation or fragmentation in hydrogen peroxide treated sample. This further confirmed that it is the actively growing cells that will first undergo apoptosis when induced by hydrogen peroxide. It seemed hydrogen peroxide would not stop endothelial cells DNA synthesis immediately after 60 minute exposure.

### 5.1.5. SYTO Staining

An important feature of apoptosis is that cells still possess intact membranes in the early stage, even when the DNA condensation and fragmentation have started.



**Photo 5-6.** Apoptosis in BrdU loaded cells. HUVECs were loaded with BrdU for 30 min before exposed to 0.25 picomol/cell  $H_2O_2$  for 60 min and fixed at 4 hours after oxidative stress. A: Control, B:  $H_2O_2$  treated cells. Arrow points to a BrdU positive apoptotic cells, 200 times magnification.



**Photo 5-7.** Apoptosis in BrdU loaded cells. HUVECs were exposed to 0.25 picomol/cell  $H_2O_2$  for 60 min before loaded with BrdU for 30 min and fixed at 4 hours after oxidative stress. A: Control, B:  $H_2O_2$  treated cells. Arrow points to a BrdU positive apoptotic cell, 200 times magnification.

SYTO 16 is a fluorophore capable of penetrating intact live cells with extremely low intrinsic fluorescence. When bound to nucleic acids it has excitation and emission spectra similar to fluorescein. SYTO 16 would also stain DNA in dead cells, but usually the relatively weak green staining would be overlapped by strong red staining from PI. Endothelial cells cultured on glass slides were exposed to 0.5 picomol/cell hydrogen peroxide for 60 min in Hanks. The cells were incubated for another 3 hours in medium, and then with SYTO 16 for 60 min to stain DNA in live cells and 2  $\mu$ g/ml PI for 10 min to counter stain DNA in dead cells before photos were taken under the fluorescence microscope.

Most of the control cells were alive, with bright staining of DNA in nuclei, weak green staining of RNA in cytoplasm and negative PI staining (panel A of Photo 5-8). Similar staining pattern was seen in samples subjected to oxidative stress. Notably there were some cells showed fragmented nuclei or DNA, typical of apoptotic morphology. These fragmented DNA had intense green staining but no PI staining, indicating the cell membrane was still intact (panel B of Photo 5-8).

# **5.2. DNA Fragmentation**

DNA fragmentation is characteristic of apoptosis (Wyllie, 1992). Three different methods were used to show endothelial cell DNA fragmentation by hydrogen peroxide.

### **5.2.1. Gel Electrophoresis (DNA Ladder)**

Confluent BAEC cultured in 80 cm<sup>2</sup> flasks were treated with 0.5 picomol/cell hydrogen peroxide for 60 min and then returned to normal culture medium. Cells were washed and harvested with 5 ml/flask lysis buffer at given times after oxidative stress. The samples were stored at -80  $^{\circ}$ C before DNA was extracted and run on 1.8% agarose gel as described in General Methods.



**Photo 5-8.** Photomicrograph of apoptosis in SYTO stained cells. BAEC were treated with 0.5 pmol/cell  $H_2O_2$  for 60 min and returned to culture medium for 3 hours. Cells were then loaded with SYTO 16 for 60 min and PI for 10 min before taking photos. A: Control cells; B: Oxidative stressed cells. Arrow points to a cell with condensed and fragmented DNA but intact membrane, 200 times magnification

The majority of DNA in control samples was intact and did not run very far from wells in 1.8% agarose gel. There was a little smear in control samples from background apoptosis or necrosis in normal culture. Some of it could also come from DNA degradation during sample processing (Lane A, B and in Photo 5-9). DNA extracted from cells 6 hours after hydrogen peroxide exposure shows a characteristic nucleosomal "ladder" pattern on gel electrophoresis (Lane E of Photo 5-9). All DNA had run away from sample well, indicating there was no intact DNA left. At 2 hours most of DNA did not run away from well, indicating they were still intact or in very large fragments (Lane D of Photo 5-9). There was just visible nucleosomal "ladder" pattern, indicating in this batch of endothelial cells apoptosis started before 2 hours after oxidative stress. At 24 hours after hydrogen peroxide exposure all DNA had run out of well, but mainly stayed close to the well with relative weak distribution of 'ladder' pattern (Lane F of Photo 5-9). The process of apoptosis continued by time, probably reached peak at 6 hours, and then gradually became necrosis.

### 5.2.2. DNA End Labelling

For the DNA end labelling assay confluent endothelial cells cultured on glass slides were exposed to 0.5 picomol/cell hydrogen peroxide for 60 min. The cells were returned to normal culturing medium for 4 hours, washed with ice cold PBS, fixed with 4% formalin for 10 min at 4 °C and stored at -20 °C in 75% ethanol. The samples were then processed as described in Chapter 2 General Methods.

DNA end labelling showed bright fluorescence of morphologically apoptotic nuclei or nuclear fragments (Photo 5-10), but cells did not exhibit positive fluorescence until morphological changes began to appear. At the time when this work was done we did not have access to a high quality fluorescence microscope. PI counter staining of DNA was not used in this experiment because the fluorescence of DNA end labelling was not strong enough. PI would stain all cells



**Photo 5-9**. Gel electrophoresis of DNA 'ladder'. Lanes A, B and C were DNA from control cells at 2, 6 and 24 hours after treatment. Lanes D, E and F were DNA from cells at 2, 6 and 24 hours after treated with 0.5 picomol/cell hydrogen peroxide for 60 minutes. Lane G is molecular weight markers. There was no 180 bp or multiples nucleosomal fragmentation in control samples (Lanes A, B and C). There was just visible nucleosomal fragmentation from cells at 2 hours after oxidative stress (Lane D). Cellular DNA showed maxim DNA fragmentation at 6 hours (E). At 24 hours there was more DNA smear than nucleosomal fragmentation (F).



**Photo 5-10** Fluorescence photomicrograph of apoptosis *in situ* end labelling. Photos were taken from BAEC monolayer 3 hours after treatment with 0.5 picomol/cell hydrogen peroxide for 60 minutes, 3'OH DNA ends labelled with dUTP-digoxigenin, and incubated with fluorescein-labelled anti-digoxigenin antibody, showing fluorescence of apoptotic nuclei.
red and make it very difficult to recognise the green FITC positive cells. So the samples were only stained with FITC. Control samples were almost totally dark, as a result no photos were shown.

#### 5.2.3. Flow Cytometry

Confluent endothelial cells cultured in 80 cm<sup>2</sup> flasks were exposed to 0.5 picomol/cell hydrogen peroxide for 60 min. Cells were then returned to normal culture medium and trypsinized at given times. Suspended cells were washed and fixed, fragmented DNA extracted by sodium citrate buffer and the samples run on flow cytometer as described in Chapter 2 General Methods.

Under the conditions used, DNA content of control endothelial cells had a single peak at region 1. Most of the cells were in G0/G1 phase with very few cells in S and M phase, no cells in sub G1 region (panels A of Photo 5-11). At 4 hours after oxidative stress a large proportion of cells had DNA content in the sub G1 region, indicating the loss of DNA through apoptosis (panel B). At 6 hours there were more cells in the sub G1 region (panel C).



**Photo 5-11.** Histograms of DNA contents of apoptotic BAEC. BAEC were exposed to 0.5 picomol/cell hydrogen peroxide for 60 min. The cells were then returned to culture and fixed at 4 and 6 hours after oxidative stress. The fragmented DNA was extracted by sodium citrate, cells were stained with PI, and DNA contents measured by FACScan. Panel A: Control cells, M1 98%, M2 2%; B:  $H_2O_2$  treated cells at 4 hours after oxidative stress, M1 82% and M2 18%; C:  $H_2O_2$  treated cells at 6 hours after oxidative stress, M1 27% and M2 73%.

## **Chapter 6 Oxidative Senescence**

Chapters 3, 4 and 5 investigated the relative short time damage of endothelial cells by oxidative stress (no more than 2 days after 60 minutes expose). High amounts of hydrogen peroxide (more than 1  $\mu$ mol/cell) resulted in cell necrosis within hours. Relative low amounts of hydrogen peroxide (0.25 to 0.5  $\mu$ mol/cell) will induce apoptosis from a few hours up to 48 hours. Damaged cell functions take much longer to recover. Physiologically relevant concentrations (for example at about 50  $\mu$ mol/L) of hydrogen peroxide will induce apoptosis in a proportion of cultured endothelial cells within 2 days. After that time a large number of surviving cells started to show enlarged cell size with great variability, increased cytoplasm to nucleus ratios and bizarre nuclear morphology. Those cells are similar in morphology with senescent cells seen in multiply passaged endothelial culture. This chapter is to investigate whether low dose hydrogen peroxide will induce endothelial cell senescence.

## **6.1. Introduction**

Endothelial cells have limited number of population doublings in culture. The exact dividing number that each individual cells can achieve in culture varies from cell to cell, even in cell populations derived from a single parent cell. But generally bovine aorta endothelial cells will be senescent at about 70 to 100 population doublings in culture (Photo 6-1). HUVEC will be senescent much earlier, probably at 10 to 20 population doubling times (Augustin-Voss, 1993).

## 6.2. Change of Morphology

#### 6.2.1. Phase Contrast Microscopy

Sparsely plated actively growing bovine aorta endothelial cells at a density of  $1200/\text{cm}^2$  were exposed to hydrogen peroxide at 0.5 picomol/cell for 60 min in



**Photo 6-1** Replicative senescence of cultured BAEC. A: endothelial cells at 10<sup>th</sup> population doubling time; B: same batch of cells at 100<sup>th</sup> population doubling time. Phase contrast microscope, 100 times magnification.

Hanks solution. The cells were then returned to normal culturing medium to continue culture. Photos were taken before and at 1, 2, 3, 4, 5, 6, 8, 10, 12 and 14 days after oxidative stress. All the photos were taken from the same field for each sample under phase contrast microscope with 100 times magnification. The cells were plated at very low density before the start of experiment to leave room for the growing of control cells and the expanding of senescent cells for uninterrupted monitoring of 14 days without subculturing.

In the control sample, the cells appeared normal with typical cobble stone morphology and reached confluence in less than 3 days (C1 to C4 in Photo 6-2 and C1 to C4 in Photo 6-3). After 3 days the cells were becoming more and more dense, and retained their monolayer morphology for the remaining of experiment. Even when the cells reached very high density they were not completely growth arrested by contact inhibition. Some cells grew on top or even underneath the monolayer. The overgrowing cells had a spindle shape.

In contrast to the control, cells exposed to hydrogen peroxide for 60 min produced a large number of apoptotic cells by 24 hours, with decreased cell density (H2 of Photo 6-2). From 2 days after oxidative stress the surviving cells started to show a completely different phenotype (H3 and H4 of Photo 6-2 and H1 to H4 of Photo 6-3, and A, B and C of Photo 6-4). The cells increased in size, and were of irregular shape, eventually occupying all area without proportional increase in cell number. For example, there were about 1500/mm<sup>2</sup> cells in control at 14 days after the treatment, while there was only about 12 cells covered the same sized area in hydrogen peroxide treated cells. There could be one or more nuclei within a cell surrounded by different numbers of hollow bodies. Those giant cells were very much long lived, which could survive more than 40 days if left in normal culture conditions. The giant cells were not dying cells because subculture showed they were perfectly capable of attaching to fresh surfaces after being detached from original culture vessels and staying in culture for at least 2 weeks.



**Photo 6-2** Oxidative senescence by hydrogen peroxide. C1 to C4: control cells before and at 1, 2 and 3 days after treatment; H1 to H4: BAEC exposed to 0.5 picomol/cell  $H_2O_2$  for 60 min at before and 1, 2 and 3 days after oxidative stress. Photos were taken under phase contrast microscope with 100 times magnification.



**Photo 6-3** Oxidative senescence by hydrogen peroxide. C1 to C4: control cells at 4, 5, 6 and 8 days after treatment; H1 to H4: BAEC exposed to 0.5 picomol/cell  $H_2O_2$  for 60 min at 4, 5, 6 and 8 days after oxidative stress. Photos were taken under phase contrast microscope with 100 times magnification.



**Photo 6-4** Oxidative senescence by hydrogen peroxide. **A**, **B** and **C** were taken from BAEC at 10, 12 and 14 days after exposure to 0.5 picomol/cell hydrogen peroxide for 60 min. Photos were taken under phase contrast microscope with 100 times magnification.

The number of giant senescent cells in hydrogen peroxide treated culture is very difficult to be accurately estimated because of the great variety of cell sizes after oxidative stress. Non-senescent cells may start to proliferate at 2 days after oxidative stress. If the senescent cells were evenly mixed with non-senescent cells, as in B of Photo 6, its number could be fairly counted. However, usually the senescent cells did not evenly mix with non-senescent cells. Instead the senescent and non-senescent cells tended to form some kind of islands of their own. In this case, the number of non-senescent cells was not determined by their ability to proliferate, but by the accessible space, which varied greatly from one sample to another.

#### 6.2.2. Haematoxylin Staining

Confluent BAEC were exposed to 0.25 picomol/cell hydrogen peroxide for 60 min in Hanks solution and then returned to normal culture medium. The cells were fixed at 5 days after oxidative stress and stained with haematoxylin. Control samples showed a uniform endothelial cell monolayer with normal nuclei staining (panel A of Photo 6-5). In hydrogen peroxide treated samples a larger proportion of cells showed increased cell and nuclei sizes with bizarre morphology (panel B of Photo 6-5). There were many hollow bodies of variable sizes surrounding nuclei, some times even within the nuclei.

#### 6.2.3. Time Lapse Video Microscopy

Continued monitoring of endothelial cells by time lapse video microscope showed that giant senescent cells formed from cells that increased in size but failed to divide (Photos not shown because of the low quality of video image). While most of the giant senescent cells had lost the ability to divide, a few giant cells were still capable of dividing, although at a much slower rate.

#### Chapter 6: Oxidative Senescence



**Photo 6-5** Haematoxylin staining of BAEC oxidative senescence. Photos were taken at 5 days after exposing cells to 0.25 picomol/cell  $H_2O_2$  for 60 min. A: control; B: hydrogen peroxide treated cells, 100 times magnification.

## **6.3.** β-Galactosidase Expression

Most cells express lysosomal  $\beta$ -galactosidase activity, which is optimally detectable at pH 4 (Morreau *et al.*, 1989). Dimri *et al* discovered that senescent human endothelial cells and other cells expressed  $\beta$ -galactosidase activity detectable at pH 6 (Dimri *et al.*, 1995). They termed this  $\beta$ -galactosidase activity as SA- $\beta$ -Gal and suggested to use it as a biomarker for senescence.  $\beta$ -Galactosidase activity was detected at pH 6 in hydrogen peroxide treated bovine aorta endothelial cells.

#### 6.3.1. Short Term

Bovine aorta endothelial cells were cultured in 25 cm<sup>2</sup> or 80 cm<sup>2</sup> tissue culture flasks. Active growing or confluent cells were exposed to hydrogen peroxide for 60 min and then returned to normal culturing medium to continue culture. At given time the cells were fixed with 4% formaldehyde and stained for  $\beta$ -galactosidase activity as described in Chapter 2 General Methods.

There were always some senescent cells in endothelial culture; very few at the first few subcultures but the number of giant cells increases with the number of subculture, until after 70 to 100 population doubling times most of the cells will be senescent. Cultured young endothelial cells (at  $10^{th}$  population doubling) did not express any SA- $\beta$ -gal (panel A of Photo 6-6), but the occasional giant cells with senescent morphology in culture did. When the cells had undergone 100 population doublings all of the cells in culture showed senescent morphology and most of them were SA- $\beta$ -Gal positive (panel B of Photo 6-6). Some of the senescent looking cells were SA- $\beta$ -Gal negative.



**Photo 6-6** SA  $\beta$ -Gal staining of young and senescent BAEC. A: young endothelial cells stained at 10<sup>th</sup> population doubling; **B**: the same batch of cells but stained at 100<sup>th</sup> population doubling time. The arrows point to SA- $\beta$ -Gal positive cells, 100 times magnification.

When exposed to 0.25 picomol/cell hydrogen peroxide for 60 min, confluent bovine aorta endothelial cells at 10<sup>th</sup> population doubling did not show positive SA- $\beta$ -Gal staining in first 2 days after oxidative stress except very few giant cells already in culture before hydrogen peroxide exposure (panels A and B of Photo 6-7). From 3 days after hydrogen peroxide treatment some cells started to show relatively weak positive SA- $\beta$ -Gal staining (panel C of Photo 6-7). Thereafter the intensity of the staining became progressively stronger with more and more cells were stained positively (Photo 6-8). It is interesting to notice that most of the SA- $\beta$ -Gal negative cells appeared normal, while most of the positive cells showed senescent morphology with increased and variable body size. The result was consistent with that of Dimri (Dimri *et al.*, 1995)

#### 6.3.2. Longer Term

To investigate long-term effects of hydrogen peroxide on endothelial cell senescence and the effect of different amounts of hydrogen peroxide, early passage (at 10<sup>th</sup> population doubling) BAEC were briefly exposed to 0.2 or 0.5 picomol/cell hydrogen peroxide and the cells were monitored for 18 days.

At the sixth day after the treatment, there were very few SA- $\beta$ -Gal positive cells in control sample (A of Photo 6-9). In samples exposed to 0.2 picomol/cell hydrogen peroxide most of cells seemed slightly enlarged, but not many showed typical senescent morphology (B of Photo 6-9), about 10% of cells were SA- $\beta$ -Gal positive. In samples treated with 0.5 picomol/cell hydrogen peroxide nearly all of the cells had typical senescent morphology. But surprisingly very few were SA- $\beta$ -Gal positive (C of Photo 6-9).

By 12 days there were increased number of SA- $\beta$ -Gal positive cells in the control, although most of the staining was relatively weak (A of Photo 6-10). At the same time cultures treated with 0.2 picomol/cell hydrogen peroxide contained two

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**Photo 6-7** SA  $\beta$ -Gal staining of oxidatively stressed cells. BAEC at 10<sup>th</sup> population doubling subjected to 0.25 picomol/cell H<sub>2</sub>O<sub>2</sub> for 60 min. A, B and C were cells fixed and stained for SA- $\beta$ -gal at 1, 2 and 3 days after oxidative stress. Photos are representatives of 4 experiments, arrows point to SA- $\beta$ -Gal positive cells, 100 times magnification.

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**Photo 6-8** SA  $\beta$ -Gal staining of oxidatively stressed cells. BAEC at 10<sup>th</sup> population doubling subjected to 0.25 picomol/cell H<sub>2</sub>O<sub>2</sub> for 60 min. A, B and C were cells fixed and stained for SA- $\beta$ -gal at 4, 5 and 6 days after oxidative stress. Photos are representatives of 4 experiments, arrows point to SA- $\beta$ -Gal positive cells, 100 times magnification.



**Photo 6-9** SA  $\beta$ -Gal staining of oxidatively stressed cells at day 6. BAEC at 10<sup>th</sup> population doubling were exposed to H<sub>2</sub>O<sub>2</sub> for 60 min and cells were stained for SA- $\beta$ -gal at 6 days after oxidative stress. A: Control; **B** and **C**: cells treated with 0.2 and 0.5 picomol/cell hydrogen peroxide, respectively. Photos are representatives of 4 experiments, arrows point to SA- $\beta$ -Gal positive cells, 100 times magnification.



**Photo 6-10** SA  $\beta$ -Gal staining of oxidatively stressed cells at day 12. BAEC at 10<sup>th</sup> population doubling were exposed to H<sub>2</sub>O<sub>2</sub> for 60 min and cells were stained for SA- $\beta$ -gal at 12 days after oxidative stress. A: Control; B and C: cells treated with 0.2 and 0.5 picomol/cell hydrogen peroxide, respectively. Photos are representatives of 4 experiments, arrows point to SA- $\beta$ -Gal positive cells, 100 times magnification.

totally different groups of cells: SA- $\beta$ -Gal positive senescent giant cells and SA- $\beta$ -Gal negative normal looking cells (B of Photo 6-10). The number of senescent cells was reduced but the size of the cells increased. The higher dose hydrogen peroxide (0.5 pmol/cell) had quite a different effect on endothelium morphology and SA- $\beta$ -Gal staining (C of Photo 6-10). The number of senescent cells were reduced compared to the day 6 culture and replaced with normal looking endothelial cells. The remaining senescent giant cells started to show positive SA- $\beta$ -Gal staining, with some cells quite strongly stained, some quite weak and some were still negative. All the newly grown cells, assuming from the proliferation of non-senescent cells, were SA- $\beta$ -Gal negative.

When the cells were left in confluence for 18 days without subculture, control endothelial cell monolayer kept their cobblestone morphology but nearly all cells became SA- $\beta$ -Gal positive (A of Photo 6-11). The staining was stronger than that at day 12, but not as strong as that in senescent cells. The number of positive cells in 0.2 picomol/cell hydrogen peroxide treated samples was also increased. Compared to day 12, in addition to positive senescent giant cells there were now many normal looking cells expressing SA- $\beta$ -Gal (B of Photo 6-11). In contrast the staining pattern of higher dose hydrogen peroxide treated samples were similar to that of samples treated with lower dose hydrogen peroxide at day 12, with all the senescent looking cells positively stained and all the normal looking cells negative (C of Photo 6-11), though there were many more cells had senescent morphology in the sample exposed to higher dose of hydrogen peroxide.

Those results imply that SA- $\beta$ -Gal is probably a biomarker of cell quiescence rather than senescence in cultured bovine aorta endothelial cells. More experiments were carried out to test the meaning of SA- $\beta$ -Gal expression.



**Photo 6-11** SA  $\beta$ -Gal staining of oxidatively stressed cells at day 18. BAEC at 10<sup>th</sup> population doubling were exposed to H<sub>2</sub>O<sub>2</sub> for 60 min and cells were stained for SA- $\beta$ -gal at 18 days after oxidative stress. A: Control; **B** and **C**: cells treated with 0.2 and 0.5 picomol/cell hydrogen peroxide, respectively. Photos are representatives of 4 experiments, arrows point to SA- $\beta$ -Gal positive cells, 100 times magnification.

#### 6.3.3. Subculture

BAEC at  $10^{\text{th}}$  population doubling were exposed to 0.5 picomol/cell hydrogen peroxide for 60 min. The cells were then maintained in culture for another 20 days with medium changed at every 2 or 3 days. Both the control and hydrogen peroxide treated cells were then resuspended and plated at the ratio of 1:5. One flask of cells were fixed before, and at 4 hours and 1, 2 and 3 days after subculture. All samples were stained for SA- $\beta$ -Gal immediately following fixation.

The control cells exhibited normal culture morphology, but SA- $\beta$ -Gal was seen to a greater or lesser degree in almost all cells after they had been confluent for 20 days (A of Photo 6-12), consistent with previous experiments. Those SA- $\beta$ -Gal positive cells had the appearance of normal endothelial cells capable of reattaching to culture surface after they had been detached. At 4 hours after subculture they expressed similar level of SA- $\beta$ -Gal as that of cells prior to subculture (B of Photo 6-12).

By 24 hours after subculture there were less and less cells stained for SA- $\beta$ -Gal and the staining decreased in intensity (Photo 6-13). By 3 days after subculture, except for occasional senescent giant cells, which were still positive for SA- $\beta$ -Gal, all cells with normal endothelial morphology were SA- $\beta$ -Gal negative. The cells were doubling at every 22 to 24 hours and there was no apparent cell death. This suggests that not only were the newly grown cells SA- $\beta$ -Gal negative but also the original SA- $\beta$ -Gal positive cells had gradually lost the expression of this isoform.

At 20 days after brief oxidative stress, the hydrogen peroxide treated sample had 2 distinctive populations: the senescent giant cells with strong SA- $\beta$ -Gal staining and the normal looking cells with relatively weak staining (A of Photo 6-14).



**Photo 6-12** Change of SA- $\beta$ -Gal staining in normal BAEC. A: endothelial cells at 10<sup>th</sup> population doubling were kept in culture for 20 days; B: cells were subcultured for 4 hours before SA- $\beta$ -Gal staining. Photos are representative of 4 experiments, arrows point to SA- $\beta$ -Gal positive cells, 100 times magnification.

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**Photo 6-13** Change of SA- $\beta$ -Gal staining in normal BAEC. Endothelial cells at 10<sup>th</sup> population doubling were kept in culture for 20 days. **A**, **B** and **C** were taken from cells subcultured and stained for SA- $\beta$ -Gal at 1, 2 and 3 days after subculture. Photos are representative of 4 experiments, arrows point to SA- $\beta$ -Gal positive cells, 100 times magnification.



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**Photo 6-14** Change of SA- $\beta$ -Gal staining in oxidatively stressed BAEC. Endothelial cells treated with 0.5 picomol/cell H<sub>2</sub>O<sub>2</sub> for 60 min at 10<sup>th</sup> population doubling were kept in culture for 20 days. Cells were then stained for SA- $\beta$ -Gal (A) or subcultured for 4 hours before SA- $\beta$ -Gal staining (B). Photos are representative of 4 experiments, arrows point to SA- $\beta$ -Gal positive cells, 100 times magnification. At 4 hours after subculture the senescent cells kept their SA- $\beta$ -Gal staining but the cells with normal morphology had all became SA- $\beta$ -Gal negative (B of Photo 6-14). In the following 3 days the normal SA- $\beta$ -Gal negative cells quickly over grew senescent cells, which remained positive until they eventually died (Photo 6-15).

## 6.4. BrdU Incorporation and Lipofuscin Accumulation

Cells treated with 0.5 picomol/cell hydrogen peroxide for 60 min were left in culture for a further 4 days before loaded with 10  $\mu$ mol/L BrdU for 24 hours. The samples were then stained with anti-BrdU and FITC-conjugated antibodies as described in Chapter 2 General Methods. Most of the newly grown endothelial cells with normal morphology showed positive BrdU staining. By contrast, senescent giant cells showed no positive BrdU staining in the nuclei (arrow in Cell A of Photo 6-16). In another cell there were many small auto fluorescent bodies surrounding nuclei (Cell B of Photo 6-16). To investigate whether those auto fluorescent bodies were products of oxidative stress flow cytometry was used to monitor the change after brief exposing of endothelial cells to hydrogen peroxide. The green-yellow auto fluorescence could be detected in both the FITC and PI channels. The signal in the FITC channel was stronger so it was chosen to measure cellular auto fluorescence.

There was a large amount of variation in auto fluorescence between individual cells, with CV at about 80% for most of the samples. Photo 6-17 showed a typical flow cytometer histogram of auto-fluorescence from cells at 9 days after 60 min exposure to different amounts of hydrogen peroxide. The result showed that brief hydrogen peroxide exposure led to a time and dose dependent increase in cellular auto fluorescence, the signal could be as much as 8 times higher in 9 days (Figure 6-1).



**Photo 6-15** Change of SA- $\beta$ -Gal staining in oxidatively stressed BAEC. Endothelial cells treated with 0.5 picomol/cell H<sub>2</sub>O<sub>2</sub> for 60 min at 10<sup>th</sup> population doubling were kept in culture for 20 days. Cells were then subcultured and stained for SA- $\beta$ -Gal at 1 (A), 2 (B) and 3 (C) days after subculture. Photos are representative of 4 experiments, arrows point to SA- $\beta$ -Gal positive cells, 100 times magnification.



**Photo 6-16 Auto fluorescence and BrdU staining of oxidative senescent cells.** BAEC were exposed to 0.5 picomol/cell  $H_2O_2$  for 60 min and then returned to culture for 6 days before loading 10 µmol/L BrdU for 24 hours and stained with anti-BrdU antibody. Arrow in Cell A points BrdU negative nucleus and arrow in Cell B points to lipofuscin body. Photo is representative of 3 experiments, 400 times magnification.



**Photo 6-17 Auto fluorescence of oxidatively stressed cells.** BAEC were exposed to  $H_2O_2$  for 60 min and then returned to culture for 9 days before auto fluorescence was measured by flow cytometer. A: control; from B to E cells were treated with 0.125, 0.25, 0.5 and 1 picomol/cell  $H_2O_2$ , respectively.



Figure 6-1 Auto fluorescence of BAEC after oxidative stress. Endothelial cells were exposed to 0.25 (B), 0.5 (C) and 1 (D) picomol/cell hydrogen peroxide for 60 min and then continued in culture. The cells were suspended and auto fluorescence measured by FACScan from 1 to 9 days after oxidative stress. A represents control cells. Results are shown as average relative units per cell with the value of first day's control as 100 units. It is representative of 6 experiments.

## **Chapter 7** Discussion

Endothelial cells are frequently under oxidative stress. The exact amount or concentration of hydrogen peroxide present in vivo is unclear. In normal situations the concentration of reactive oxygen species in blood is very low. Measuring in plasma without leukocytes but with sodium azide to inhibit catalase Lacy et al reported a production of hydrogen peroxide at 36.1 +/- 7.6 µmol/L (Lacy, 1998). However in the case of atherosclerosis and particularly in the case of ischaemia/reperfusion, the situation will be totally different. It has been suggested that the concentration of hydrogen peroxide in vivo could reach as high as 1 to 2 mmol/L if such a site represents a limited diffusion space (Doan et al., 1994; Boyer et al., 1995; Bychkov, 1999). It is possible for reactive oxygen species to reach such a high concentration at a very localised area in a very short time, during the reperfusion injury, when large number of neutrophils accumulate in the vessels and infiltrate into myocardium. Hu et al have suggested an in vivo oxidant production in the micromolar range (Hu et al., 1998) in the postischemic/anoxic endothelium. In atherosclerosis endothelial cells are more likely to be exposed to reactive oxygen species at concentrations less than 100 micromolar, when a limited number of leukocytes adhere to endothelium in localised areas.

The results of this thesis have shown that endothelial cells are sensitive to reactive oxygen species attack. Depending on the amount available to each cell (or the concentration, as reported in most of the literature) the damage may range from increased intracellular peroxidation to temporal inhibition of cell function or to cell senescence and cell death, including apoptosis and necrosis. Figure 7-1 illustrates the simplified relationship between the amount of hydrogen peroxide and endothelial damage.



#### Figure 7-1 Amounts of Hydrogen Peroxide and Endothelial Damage

## 7.1. Advantage and Disadvantage of Endothelial Culture

Cell culture was used in this thesis as a model system. The development of endothelial cell culture has provided the impetus for the current wealth of information on those cells. Cells in culture provide a means of dissecting out the contributions of individual cell types, for assaying the effects of naturally occurring and synthetic products and for examining cell-cell and cell-molecule interrelationships. In many instances such studies have revealed fundamental properties of the cells themselves, their enzymes, receptors, transduction mechanisms, and gene regulation.

The technique of tissue culture also provides sufficient experimental material; partially substitutes animal experiment, greatly reduces the cost of research, and in some cases it is the only experimental material practically available.

Limitations of this approach have become apparent, however, with the realisation that cell culture perturbs endothelial cells from their quiescent *in vivo* state (0.1% replications per day) to an activated phenotype (1% to 10% replications per day) with some loss of specialised functions associated with some vessels and organ systems. *In situ* studies of endothelium behaviour indicate that the constitutive phenotype of endothelial cells may be unstable and their behaviour may change once explanted. Commonly used culture conditions may activate or otherwise alter the endothelial phenotype (Grant *et al.*, 1992). There is, as yet, no model for generating the resting endothelium *in vitro*. Thus, all the experiments carried out and results obtained should be considered in the context of the cell source as well as the *ex vivo* culture conditions, including passaging, the omission of shear forces, and factors released into blood that alter the behaviour of the endothelium *in vivo*.

Cultured bovine aorta endothelial cells were used in the majority of experiments in this thesis. Ideally human artery endothelial cells should have been used, as they represent a model that is closer to the *in vivo* situation. Whilst it was possible to obtain small sections of small arteries from surgery, this was insufficient to obtain usable numbers of endothelial cells and supply was restricted. Although ex vivo expansion can produce large number of cells, multiple passages will lead to cellular changes before the cells reached the number needed for experiment. There are a few commercial sources for human artery endothelial cells, but the cost is almost prohibitive, and the cells are estimated to have undergone about 10 population doublings, and not enough growth potential is left to obtain enough cells for experiment. HUVECs were used in some of the experiments. Although they are cultured from human vessels, they have different morphology, protein synthesis and other characteristics compared to endothelial cells from arteries. Bovine aorta endothelial cells, although from different species and certainly have different characteristics with human cells, at least come from artery, and hopefully have similar characteristics to that of human artery endothelial cells. Bovine aorta endothelial cells are very robust in culture; it can undergo about 70 to 100 population doublings before showing signs of senescence, compared to about 10 to 15 population doublings for HUVEC. This makes it possible to obtain pure endothelial cell culture by cloning. The cost of BAEC culture is also much lower, only about 1/10 of that of HUVEC culture.

# 7.2. Endothelial Anti-Oxidant Defence and Oxidative Damage

The most common anti-oxidant enzymes are superoxide dismutase, catalase and glutathione-peroxidase. Superoxide anion poorly pass through cell membranes and superoxide dismutase is not effective against the intrusion of extracellular reactive oxygen species. Catalase is a very efficient enzyme that is able to reduce hydrogen peroxide to water at a very fast rate. The effect of catalase against hydrogen peroxide-induced intracellular damage is very limited, as in most mammalian cell types, including endothelial cells, catalase is exclusively found within peroxisomes (del Rio et al., 1992; del Rio et al., 1996). Since extracellular hydrogen peroxide will enter into cytoplasm first, where there is no catalase present, glutathione peroxidase is the major defence against hydrogen peroxide in cytoplasm. Glutathione peroxidase rapidly and efficiently catalyses the reaction of glutathione and hydrogen peroxide to produce oxidised glutathione and water. Glutathione reductase re-reduces oxidised glutathione into glutathione in the presence of NADPH to continue the action of glutathione peroxidase. Other small molecules such as ascorbate, vitamin E, selenium, etc are also contributing to the anti-oxidant defence, but themselves will be oxidised after reacting with oxidants.

After those defence lines any oxidants left within a cell will attack protein, DNA and lipids to cause oxidative damage. At the same time those large molecules will also act as a 'sink' to scavenge oxidants. Lipid peroxidation is a set of chain reactions, which are initiated by the abstraction of a hydrogen atom (from carbon) in an unsaturated fatty acid chain by reactive oxygen species to form lipid peroxyl radical. Once initiated, lipid peroxyl radical can further propagate the peroxidation chain reaction by abstracting a hydrogen atom from other vicinal unsaturated fatty acids. The resulting lipid hydroperoxide can decompose into several reactive species including lipid alkoxyl radicals, aldehydes (malonyldialdehyde), alkanes, lipid epoxides and alcohols (Halliwell, 1989).

Peroxidised membranes become rigid, lose selective permeability and can lose their integrity. Water-soluble lipid peroxidation products (the aldehydes) have been shown to diffuse from membranes into other sub cellular compartments (Halliwell and Gutteridge, 1984). Dialdehydes can act as cross-linking reagents, and are thought to play a role in protein aggregation and formation of lipofuscin (Terman and Brunk, 1998).

Reactive oxygen species induce protein damage in a number of ways. These include oxidation of sulphydryl groups, reduction of disulphides, oxidative adduction of amino acid residues close to metal-binding sites via metal-catalysed oxidation, reactions with aldehydes, protein-protein cross-linking, and peptide fragmentation. A number of enzymes possessing active-site iron-sulphur clusters are acutely sensitive to inactivation by reactive oxygen species. Oxidative modifications of protein amino acid side chains by reactive oxygen species or by the lipid peroxidation products can all lead to the formation of protein carbonyl derivatives. (Uchida and Stadtman, 1993).

Oxidised proteins are recognized by proteases and completely degraded to amino acids (Grune *et al.*, 1995). New proteins will be synthesized to replace the damaged one. Oxidised amino acids within oxidatively modified proteins may be eliminated, or used as carbon sources for ATP synthesis. Since an oxidatively modified protein may contain only two or three oxidized amino acids probably most of the amino acids from an oxidized and degraded protein are re-utilised for protein synthesis. If oxidative damage is overwhelming over cellular proteolytic capacity there will be accumulation of oxidised proteins, and if the replacement of damaged proteins is not fast enough the cells will lose functions involving those proteins. Accumulation of damaged proteins is also detrimental to cell functions.

DNA is a biologically important target for reactive oxygen species. Hydrogen peroxide reacts with ferrous iron to form hydroxyl radical and causes DNA damage. Luo *et al* found that in the presence of the very complex ligand, DNA, there are three kinetically distinguishable oxidants formed that cause DNA strand breakage (Luo *et al.*, 1994a). One of these is easily scavengeable, consistent with it being hydroxyl radicals, whereas the other two vary in their scavenging susceptibilities and one or both of these might be an iron (IV) species.

DNA damage occurs by the attack of a radical such as the hydroxyl radical either at a sugar or a base residue (Imlay *et al.*, 1988). Attack at a base produces several general classes of damage: simple base modification such as 5-hydroxymethyluracil, oxidised pyrimidine residues such as thymine glycol and its breakdown products, oxidised purines such as 4,6-diamino-5-formamidopyrimidine, and 8-oxoguanine, or in cases where the damage is unstable, a baseless (AP) site. In all, attack at DNA bases leads to as many as 50 base alterations (Henle *et al.*, 1996; Luo *et al.*, 1994b).

Attack can also occur at a sugar residue to produce strand breaks (Imlay and Linn, 1988). These are not generally simple hydrolytic breaks such as those produced by DNase, but instead usually contain 3'-phosphate residues and possibly sugar fragments such as a glycolate residue, or even gaps at the site of cleavage. More complex products formed by radicals include cross-links and double-strand

breaks. DNA-bound iron may interact with nascent DNA radicals and thereby qualitatively and quantitatively alter the products (Henle and Linn, 1997).

Once DNA-nucleoside damage is manifested, enzymatic mechanisms are necessary to correct the alteration. The damage must be recognized, removed, and replaced with normal nucleotides, and DNA ligase must seal all strand breaks (Demple and Harrison, 1994).

## 7.3. Lethal Oxidative Damage and Role of Myoglobin

There are generally two ways to expose endothelial cells to oxidative stress. The first is to expose cells to a single dose of oxidants; the second is to subject cells to a continuing reactive oxygen species system. In this thesis the method of single dose has been used to mimic the situation of leukocyte activation in atherosclerosis, and of ischaemia/reperfusion injury.

Exposure of endothelial cells to hydrogen peroxide at concentrations of more than 1 mmol/L causes immediate death by cell lysis (necrosis) (Ager and Gordon, 1984; Whorton *et al.*, 1985) (Martin, 1984). In Chapter 3 it was shown that endothelial cells lost the membrane integrity after oxidative stress by 1 mmol/L hydrogen peroxide or 40 mU/ml xanthine oxidase, as evidenced by their failure to retain tritiated adenine. Most of the cells were positive for Trypan blue staining 4 hours after exposure to reactive oxygen species, and LDH was released (data not shown). Similar results were obtained with both the superoxide anion generating system and hydrogen peroxide. Hiraishi *et al* have reported dose dependent increase of  ${}^{51}$ Cr release in endothelial cells by hydrogen peroxide with concentrations comparable to that used in this thesis (Hiraishi *et al.*, 1994).

The most likely mechanism for membrane damage by reactive oxygen species is lipid peroxidation. Hydrogen peroxide, either directly generated or dismuted from superoxide anion, may react with ferrous iron to produce hydroxyl radical. The latter then abstracts a hydrogen atom in an unsaturated fatty acid to initiate a chain reaction. The subsequent production of lipid peroxyl radical further propagates to cause magnified lipid peroxidation and produce more reactive species including lipid alkoxyl radicals, aldehydes, alkanes, lipid eposides and alcohols. Membrane peroxidation makes cell membranes rigid and causes them to lose integrity, eventually leading to cell lysis (Halliwell, 1994).

Myoglobin is a ubiquitous protein in cardiac and skeletal muscle, occurring physiologically at concentrations of up to 0.3 mM. It is known that myoglobin reacts, via its heme group, with hydrogen peroxide (Whitburn, 1987c). There have been several claims that myoglobin catalyses the Fenton reaction (Puppo and Halliwell, 1988a; Harel and Kanner, 1988a). However, such claims had been based on nonspecific assays for •OH formation. It is known that mixtures of  $H_2O_2$ with oxy-haemoglobin or methaemoglobin can oxidize many molecules. The oxidising action of methemoglobin-H<sub>2</sub>O<sub>2</sub> or metmyoglobin-H<sub>2</sub>O<sub>2</sub> mixtures might be attributed to a heme-associated ferryl species resembling that present in horseradish peroxidase compounds I and II. Reaction of oxyhaemoglobin with equimolar concentrations of H<sub>2</sub>O<sub>2</sub> produces a reactive species, not identical to hydroxyl radical, that can degrade deoxyribose and stimulate lipid peroxidation. Reaction of myoglobin with H<sub>2</sub>O<sub>2</sub> produces a ferryl-type species that can Although reaction of intact haemoglobin and accelerate lipid peroxidation. myoglobin molecules with H<sub>2</sub>O<sub>2</sub> does produce some reactive higher oxidation states of the heme iron, there is no clear evidence that these proteins react with  $H_2O_2$  to form hydroxyl radicals that can be detected outside the protein.

The reaction between myoglobin and hydrogen peroxide leads to the initial formation of ferrylmyoglobin containing two oxidising equivalents: the oxoferryl
complex and an amino acid radical. Ferrylmyoglobin may subsequently react with oxymyoglobin to give metmyoglobin, or break down in other possible ways, including sacrificial autoxidation with the eventual loss of iron. Davies (Davies, 1990; Davies, 1991) described the formation of an adduct between DMPO and myoglobin in the presence of relatively high concentrations of hydrogen peroxide which was attributed to the formation of an amino-acid based globin radical following the breakdown of ferrylmyoglobin. Prasad and colleagues (Prasad *et al.*, 1989a) have confirmed that myoglobin will react with hydrogen peroxide with the eventual formation of metmyoglobin and the non- stoichiometric release of iron. In their experiments, iron release from myoglobin was much less than from haemoglobin under similar conditions.

Recently Giulivi and Cadenas reported a composite of three signals ESR spectrum from the oxidation of myoglobin by hydrogen peroxide (Giulivi and Cadenas, The low intensity signal had a g-value of 2.014, contributed 10-15% to 1998). the overall spectrum and was ascribed to a peroxyl radical. Of the two high intensity signals, one consisted of a six-line spectrum (g = 2.0048) that contributed approximately 17-19% to the overall signal with hyperfine splitting constants to ring protons to be identified as a tyrosyl radical. The other high intensity signal (with similar g-value and underlying that of the tyrosyl radical) was ascribed to an aromatic amino acid radical. The authors suggested that aromatic amino acid is the first site of protein radical; reduction of the oxoferryl complex by Tyr (FeIV=O + Tyr-OH + H+  $\rightarrow$  FeIII + H<sub>2</sub>O + Tyr-O.)-and alternatively by other amino acids leads to the subsequent formation of other amino acid radicals within an electron-transfer process throughout the protein. This view suggests that the protein radical(s) is highly delocalised within the globin moiety in a dynamic process encompassing electron tunnelling through the backbone chain or H-bonds and leading to the formation of secondary radicals.

Giulivi and Davies described the 'comproportionation' of ferryl-haemoglobin and oxyhaemoglobin as a potential antioxidant and free radical scavenging mechanism (Giulivi and Davies, 1990). Whitburn had earlier described a similar reaction between oxy- and ferryl-myoglobin (Whitburn, 1987c). Walters *et al* (Walters *et al.*, 1983) described the oxidation of myoglobin in isolated myocytes by 15-hydroperoxy-5, 8, 11, 13-eicosatetraenoic acid, but considered this as marker of oxidative stress rather than a protective mechanism. Galaris *et al* (Galaris *et al.*, 1989) described redox 'cycling' of myoglobin and ascorbate and speculated that this might act as defence mechanism, but did not demonstrate this effect in a biological system. Ozawa and Koreswka (Osawa and Korzekwa, 1991) reported a non-specific oxidase activity of myoglobin after exposure to hydrogen peroxide. In a related experiment it was shown that oxy- and met-myoglobin (but not apomyoglobin) markedly accelerated the decay of the ESR signal of the hydroxyl radical adduct of 5,5'dimethyl-1-pyrroline-N-oxide (DMPO) (data not shown) (de Bono *et al.*, 1994).

In this experimental system both oxy- and metmyoglobin at 'physiological' concentrations provided partial protection against endothelial membrane damage caused by external ROS. A non-specific radical scavenging effect by protein can be ruled out by the lack of efficacy of apomyoglobin. The transient generation of ferrylmyoglobin, and subsequent conversion to metmyoglobin, under the would compatible with peroxide-scavenging conditions used be a 'comproportionation' reaction as part of the mechanism in the case of oxymyoglobin, but would not explain the protective effect of metmyoglobin alone. In the presence of myoglobin, hydrogen peroxide will oxidise ascorbate (Galaris et Ascorbate probably reacts with ferrylmyoglobin as well as with al., 1989). metmyoglobin. Provided the supply of ascorbate (or equivalent reducing agent) is adequate, the net effect will be protective. The reactions may involve oxidised myoglobin (Fe<sup>4+</sup>OH) reduced by a low molecular weight electron donor rather than important structural components of the cell.

In the absence of ascorbate the mechanism of the protective effect may involve sacrificial auto-oxidation with loss of the heme group. Potential toxicity resulting from the release of iron from free heme (Puppo and Halliwell, 1988b; Prasad *et al.*, 1989b) might partially explain why, even in high concentrations, the protection offered by oxy- or met-myoglobin alone was less complete than with catalase. It is also possible, at least in part, to attribute the relatively less complete protection by myoglobin to the much slower rate of decomposing hydrogen peroxide.

Endothelial cells are capable of producing nitric oxide, and it has been suggested that peroxynitrite formed from superoxide and nitric oxide is a particularly damaging species (Hogg *et al.*, 1992). Heme proteins are good scavengers of nitric oxide, and this is another potential mechanism for protection.

Myoglobin in this experiment clearly had an overall protective effect on endothelium in the presence of oxidative stress, and was indeed necessary for the protective effect of biological antioxidants such as ascorbate. This is despite its facilitation of hydrogen peroxide mediated oxidative reactions: possibly because myoglobin selectively catalyses oxidation of 'expendable' small molecules rather than important membrane components. Myoglobin occurs physiologically in cell types that are continually exposed to extremes of ischaemia and reoxygenation, and has been highly conserved in evolution. The possibility that, exceptionally, it may act as 'double-edged sword' cannot be discounted, but it may have a physiological role in protection against ROS-mediated damage.

Myoglobin forms at least one radical adduct with DMPO through a globin centred radical (Davies, 1990b). The present study showed that the use of DMPO to estimate the formation of hydroxyl radicals is not valid in systems containing physiological concentrations of myoglobin, or by inference, haemoglobin. When

oxymyoglobin or metmyoglobin was added to solutions containing DMPO-OH adducts, the ESR signal was reduced, and its rate of decay greatly accelerated.

2,2,6,6 tetramethylpiperidine-N-oxyl (TEMPO) is a very stable free radical. Individually neither myoglobin, nor hydrogen peroxide had influence on TEMPO. However, in the presence of both myoglobin and hydrogen peroxide the ESR signal of TEMPO was reduced both myoglobin/hydrogen peroxide concentration dependent, and also time dependent. The behaviour of the ESR signal of TEMPO when exposed to both myoglobin and hydrogen peroxide suggested that DMPO-OH may be oxidised by ferrylmyoglobin to non-paramagnetic products.

There are three clear ways in which myoglobin could reduce the concentration of the DMPO-OH adduct: by scavenging hydroxyl radicals; by catalysing the breakdown of hydrogen peroxide; or by reacting directly with DMPO-OH. Nonspecific hydroxyl radical scavenging probably explains the effect of apomyoglobin, which behaves in a similar fashion to bovine serum albumin.

The observation of diminished DMPO-OH formation in the presence of myoglobin is a potential complicating factor in interpreting experiments which use spin trapping to detect free radical formation in biological systems. Myoglobin could react with both intra- and extra-cellular hydrogen peroxide, since hydrogen peroxide readily crosses cell membranes. The effect on species such as DMPO-OH however would be confined to the intracellular compartment (Pou *et al.*, 1989; Samuni *et al.*, 1986). Evidence for the formation of ferrylmyoglobin in biological models of oxidative stress in heart muscle has been provided by Arduini (Arduini *et al.*, 1990), this would suggest that hydrogen peroxide concentrations high enough to affect DMPO-OH in the presence of myoglobin may be generated *in vivo*.

In experiments carried out in this thesis superoxide dismutase had no effect either on the formation of ferrylmyoglobin or on cell damage. This accords with other reports that hydrogen peroxide, or more probably hydroxyl radicals, are the crucial species, and that the conversion of superoxide anion to hydrogen peroxide is not rate limiting (Link and Riley, 1988; Kvietys *et al.*, 1989). The initial concentration of hydrogen peroxide used here was relatively high, but it decreased rapidly under the incubation conditions used, and it was used to model the effects of sudden exposure to large hydrogen peroxide concentrations as a possible consequence of reperfusion after ischaemia. Continued production of hydrogen peroxide, whether by xanthine/xanthine oxidase or by activated leukocytes, was more damaging to cells even when steady-state concentrations were much lower (data not shown).

It is not only interesting to know which of those reactive oxygen species are most responsible for oxidative damage, but it will also shed light into finding the best way to prevent or reduce the damage. The high reactivity of hydroxyl radical makes it the number one candidate for the 'oxidative killer'. While it is capable of oxidising almost any molecules in the body, its production depends on the availability of hydrogen peroxide and ferrous iron at the same place and same time. In early years there were reports of detecting hydroxyl radical from activated neutrophils by ESR and spin trap, but later it has been proven that the hydroxyl radical signal detected actually came from the reaction of trace iron in the buffer and the hydrogen peroxide dismuted from superoxide anion. Thus the oxidative damage by hydroxyl radical is limited to the immediately vicinity of its production.

There are four main sites of reactive oxygen species generation in the body: mitochondrial electron transport, peroxisomal fatty acid metabolism, cytochrome P-450 reactions, and phagocytic cells (the "respiratory burst"). Superoxide anion is the major, or probably the only original product, from all of those sites. Superoxide anion is a weak oxidising agent, insufficiently reactive to abstract H from lipids. It cannot enter the hydrophobic interior of the membranes because of its charged nature, and is unable to cross biological membranes. Hydrogen peroxide, on the other hand, is much less reactive, and does not belong to the family of oxygen free radicals. Both superoxide anion and hydrogen peroxide will be present in a superoxide anion generating system. Eliminating hydrogen peroxide by catalase did reduce endothelial damage, while dismutating superoxide anion into hydrogen peroxide by SOD did not show any protection (Chapter 3). Recently Volk et al reported similar results (Volk et al., 1997). Using cultured human and rat endothelial cells they found that in a superoxide anion generating system hydrogen peroxide was responsible for transient changes in intracellular  $Ca^{2+}$  concentration, which could be totally blocked by catalase while superoxide dismutase had no effect. The concentration of hydrogen peroxide used to produce transient changes in their experiment was 0.5 to 1 mmol/L, comparable to that used in this thesis.

Experimental evidence from both this thesis and the literature has suggested hydrogen peroxide as the pivotal reactive oxygen species most responsible for extensive endothelial oxidative damage. The key factor is probably its mobility into and throughout the cell. Hydrogen peroxide can freely travel through membranes and in and out of many cellular components to cause substantial damage.

## 7.4. Sublethal Oxidative Damage of Cell Function

When exposing endothelial cells to sublethal amounts of hydrogen peroxide (< 1 picomol/cell, or < 100  $\mu$ mol/L in concentration) there was no large scale cell lysis, as was the case when the amount of hydrogen peroxide was higher than 10 picomol/cell or 1 mmol/L in concentration. In contrast, the cell damage is

manifested by altered cell functions and the induction of apoptosis. Apoptosis will be discussed in the next section (Section 7.5 Apoptosis). This section focuses on the changes of cell function induced by sublethal oxidative stress.

Hydrogen peroxide is believed to be able to travel through cellular membranes freely if there is no transition metal to react with it on its way. Endothelial cells have several anti-oxidant systems to protect themselves from oxidative damage. It is not clear whether extracellular produced hydrogen peroxide causes cell damage by reacting with receptors or other molecules on cell membrane, or by reacting with intracellular molecules. It is also interesting to know whether extracellular hydrogen peroxide is able to overcome cellular defence to cause intracellular peroxidation and if so what amount is needed.

The results in Chapter 4 showed that extracellular hydrogen peroxide rapidly increased endothelial intracellular peroxidation. As low as 5 femtomol/cell extracellular hydrogen peroxide managed to overcome cellular anti-oxidant defence and led to significant increase in endothelial intracellular peroxidation. The increase was seen from a few seconds after adding hydrogen peroxide into cell suspension and reached a peak at about 10 minutes. Thereafter the signal slowly decayed, but still significantly elevated even after 60 minutes of adding hydrogen peroxide. The hydrogen peroxide entered into cells must first overcome the glutathione peroxidase system. The rapid increase of intracellular peroxidation at first 10 minutes indicated that was easily done. The main reason for the reduction of intracellular peroxidation after 10 minutes is not clear. It might arise from the leaking of the DCF dye out of cells, or result from the hydrogen peroxide entering into cell components including mitochondria and peroxisomes, particularly as in the latter hydrogen peroxide may be reduced by catalase in peroxisomes, or a combination of both.

Although it is difficult to compare between experimental situations, the amount of hydrogen peroxide needed to increase intracellular peroxidation (5 femtomol/cell) is roughly 50 times less than that needed to produce cell damage measurable by other methods like tritiated thymidine uptake or dehydrogenase activity (250 femtomol/cell). This reveals endothelial anti-oxidant capacity against extracellular generated hydrogen peroxide is not sufficiently competent, which may be overcome in situations where, for example leukocytes adhere to, and are activated on the endothelium in atherosclerosis. It also suggests that in some situations where there is no measurable cell damage, this does not necessarily mean there is no oxidative stress.

Conversely, intracellular peroxidation is not necessarily an indicator of cell damage. When adding t-butyl-hydroperoxide into cell suspensions intracellular peroxidation was about 3 times higher than that caused by equal amounts of hydrogen peroxide, but about 10 times more t-butyl-hydroperoxide is needed to produce comparable oxidative damage caused by hydrogen peroxide, measured by cellular mitochondrial activity and tritiated thymidine uptake (data not shown). This suggests endothelial damage caused by hydrogen peroxide cannot wholly attribute to oxidative stress, it may involve other mechanisms such as signal transduction.

When added to endothelial cells at sublethal amounts (no more than 0.5 picomol/cell) hydrogen peroxide was removed from the medium in both a concentration-dependent and time-dependent fashion. Decrease of extracellular hydrogen peroxide was initially fast, and then slowed. The concentrations of hydrogen peroxide remaining in the buffer depended on the concentrations added in the first place, but the percentage change was not directly proportional to the reduction in concentration. For example, 5000 endothelial cells reduced hydrogen peroxide from 50  $\mu$ mol/L to 17.6  $\mu$ mol/L (difference of 32.4  $\mu$ mol/L when added

at 50  $\mu$ mol/L) in 60 min but from 200  $\mu$ mol/L to 141.4  $\mu$ mol/L (difference of 58.6  $\mu$ mol/L).

The number of cells had very significant effect on hydrogen peroxide elimination. More cells removed hydrogen peroxide more quickly, but again the removal was not directly proportional when calculated in change of concentrations. Different initial concentrations caused different rate of change in concentrations, and plotting the reduction of hydrogen peroxide concentration against cell number in 3 different initial concentrations gives 3 separate curves with similar pattern (Figure 4-13).

The above result indicates that the change of extracellular hydrogen peroxide concentration is not a reliable parameter to estimate, or standardise oxidative damage. Careful examination revealed that the removal of hydrogen peroxide was directly proportional to the amount available to each cell. As long as the ratio of hydrogen peroxide to each cell was the same, it did not matter whether the concentration was 50  $\mu$ mol/L or 200  $\mu$ mol/L, or the cell number is 2500 or 20000, in any case the same number of cells will remove the same amount hydrogen peroxide at the same time. Plotting the reduction of hydrogen peroxide on the basis of amount per cell against cell number with 3 different initial concentrations gave three well-fitted, overlapping curves forming a single extended curve. Experiments also showed that endothelial cell damage, measured by mitochondrial activity and tritiated thymidine uptake, was directly proportional to the ratio of hydrogen peroxide per cell rather than the concentration.

In the experimental conditions used, the hydrogen peroxide could only be eliminated by endothelial cells. The initial relatively fast removal of hydrogen peroxide may reflect the reduction by glutathione/glutathione reductase and other small anti-oxidant compounds, but it may also indicate that more hydrogen peroxide diffuses into cells at higher concentrations. The loss of small antioxidant compounds will slow down the elimination of hydrogen peroxide; particularly the loss of glutathione will reduce the function of glutathione/glutathione system. Later hydrogen peroxide may also be eliminated by entering cellular structures like peroxisomes and reduced by catalase.

Exposing endothelial cells to sublethal amounts of hydrogen peroxide led to a range of change in cell functions. Cell mitochondrial activity and cell proliferation were among the most sensitive parameters to measure oxidative damage.

Hydrogen peroxide caused a dose and time dependent reduction of endothelial cell mitochondrial activity. When the amount of hydrogen peroxide per cell was no more than 0.2 picomol/cell usually the damaged mitochondrial function showed a gradual recovery from 6 hours after oxidative stress. Taking into account the possible loss of cells the actual recovery could be higher than measured. Within 12 hours after oxidative stress the reduction mainly reflected the damage of function and after 24 hours the reduction mainly reflected the loss of cells.

Consistent with hydrogen peroxide removal, the damage to mitochondrial activity was also directly proportional to the ratio of hydrogen peroxide per cell, but not directly proportional to the concentration of hydrogen peroxide.

LDH and <sup>51</sup>Cr release have been used in the past as parameters for oxidative damage. These assays indicate whether the cell membrane has been compromised. When endothelial cells were treated with sublethal amounts of hydrogen peroxide in present studies, no increased LDH release was detected, indicating there was no substantial lose of cellular membrane integrity (data not shown).

Endothelium provides an active antithrombotic surface in blood vessels. Any damage to the integrity of endothelium may lead to the loss of antithrombotic surface and increase the risk of thrombosis. Normal endothelial cells are not still in culture, they are moving around all the time, even in confluence. When an endothelial monolayer was scratched in culture, the area was re-covered by endothelial cells migrated from surrounding area in less than 3 hours. However brief exposure of even very low amounts of hydrogen peroxide (0.05 picomol/cell for 30 min) inhibited cell migration and greatly increased the time needed to recover the area to more than six hours. The prolonged loss of endothelium might lead to the loss of antithrombotic surface and increase the risk of thrombosis.

The loss of endothelial cell migrating ability induced by hydrogen peroxide may result from cytoskeletal rearrangement. Hastie *et al* reported that exposure to hydrogen peroxide of cultured endothelial cells results in cytoskeletal rearrangement and second messenger activation (Hastie *et al.*, 1997). Hydrogen peroxide can induce filamin translocation from the membrane to the cytosol, which occurs after 1-min of treatment, while intercellular gap formation significantly increases after 15 min in human umbilical vein endothelial cell (Hastie *et al.*, 1997; Hastie *et al.*, 1998). Recently Zhao and Davis have reported that hydrogen peroxide caused actin filament rearrangement in bovine pulmonary artery endothelial cells with disruption of the dense peripheral bands and formation of stress fibers. They suggest that the signal transduction system may be involved (Zhao and Davis, 1998).

Proliferation is one of the most sensitive parameters to measure cell damage. Both tritiated thymidine uptake and BrdU incorporation have revealed dose dependent inhibition of endothelial proliferation by hydrogen peroxide. The inhibition was mainly due to interruption of DNA synthesis in surviving cells as mitochondrial activity experiments showed those cells were alive. This is potentially very important. *In vivo* mechanical or other forms of damage may cause temporary de-endothelialisation. The denuded area will normally be covered rapidly first by the migration of surrounding cells and later with newly grown cells. However in the case of oxidative stress, because both of the inhibition of cell migration in the early time and the inhibition of proliferation later, the area may be left for a much longer time without the endothelial covering. As a result there will be greatly increased possibility of thrombosis and neutrophil accumulation, which in turn may further exaggerate oxidative damage.

But not all endothelial function is substantially damaged by sublethal amounts of hydrogen peroxide. Gupta *et al* reported that hydrogen peroxide, at concentrations of no more than 100  $\mu$ mol/L, did not disrupt the arginine-nitric oxide pathway (Gupta *et al.*, 1998).

Hydrogen peroxide concentrations of below 0.5 picomol/cell (or 100  $\mu$ mol/L) did not have detectable effects on the endothelial intracellular calcium concentration (Yang and Albert, unpublished data). Although there are reports of increased intracellular calcium induced by hydrogen peroxide, the authors usually have used concentrations of hydrogen peroxide at 1 mmol/L or higher (Volk *et al.*, 1997; Hu *et al.*, 1998). As discussed earlier, the same concentration of hydrogen peroxide does not necessarily produce similar oxidative damage. When all the published data are based on concentrations it is very difficult to compare the results, because the actual hydrogen peroxide per cell can vary dramatically. Nevertheless the concentration of hydrogen peroxide is the only means for comparison between studies. Hu *et al* reported intracellular calcium oscillations when HUVEC were exposed to 100 to 500  $\mu$ mol/L hydrogen peroxide. Higher concentrations of hydrogen peroxide (1 and 10 mmol/L) increased intracellular calcium but rarely caused intracellular calcium oscillations (Hu *et al.*, 1998).

## 7.5. Apoptosis by Hydrogen Peroxide

Apoptosis, or programmed cell death, is a ubiquitous, evolutionally conserved, physiologic mechanism of cell death that regulates mass and architecture in many tissues (Wyllie, 1992; Wyllie, 1997). The genetic program for apoptosis may be activated at a defined time during embryogenesis or in the maturation of adult cells, by a wide range of physiologic stimuli. Apoptosis is generally seen as a protective process that allows the replacement of potentially damaged cells with new and fully competent ones. But excessive apoptosis is also very damaging.

Based on morphologic and biochemical differences, there are two ways of cell death: necrosis and apoptosis (Kerr, 1991). Necrosis usually affects a group of contiguous cells, and involves no messenger RNA or protein synthesis. Necrosis is also characterized by ATP depletion, cell swelling, and loss of cell membrane integrity, whereas apoptosis requires energy and is associated with cell shrinkage and phagocytosis without the primary loss of membrane integrity. In contrast, apoptosis takes place in cells with normal ATP levels and requires messenger RNA and protein synthesis. Apoptosis affects scattered single cells and involves cell shrinkage, chromatin condensation, and the formation of small membrane fragments, referred to as apoptotic bodies, which may or may not contain nuclear fragments. The most striking biochemical feature of apoptosis is the activation of endonuclease, which cleaves cellular DNA between regularly spaced nucleosomal units of 180 base-pairs or multiples thereof that are readily detected as a DNA ladder by electrophoresis (Wyllie, 1992). This characteristic feature is markedly different from the DNA breakdown that occurs during necrosis resulting in a smear of DNA as demonstrated by gel electrophoresis.

Exposure of endothelial cells to high concentrations of hydrogen peroxide causes immediate death by cell lysis (necrosis). Exposure to lower, non-lytic

concentrations caused apoptosis. The concentrations of hydrogen peroxide (25-100  $\mu$ mol/L) over which endothelial apoptosis was observed is comparable to those observed by Whorton and colleagues (Whorton *et al.*, 1985) to cause inhibition of prostacyclin production, but not cell disruption, in porcine aortic endothelial cells.

Membrane blebbing of endothelial cells initiated by oxidative injury was also observed by Geeraerts and colleagues (Geeraerts *et al.*, 1991) and endothelial apoptosis induced by radiation injury has been described by Fuks and colleagues (Fuks *et al.*, 1994) and Haimovitz-Friedman *et al* (Haimovitz-Friedman *et al.*, 1994).

In the case of oxidative damage, limited apoptosis will clear a way to replace damaged endothelial cells with healthy ones, to preserve normal endothelial function. But excessive apoptosis may lead to the loss of cells and damage to endothelium function.

Hydrogen peroxide will cause both apoptosis and necrosis in endothelial cells. Although there is no clear cut-off point, generally the relatively low amounts of hydrogen peroxide (less than 1 picomol/cell) induced predominantly apoptosis, whereas higher amounts of more than 5 picomol/cell predominantly causes necrosis in endothelial cells. Very recently Teramoto *et al* reported similar result (Teramoto *et al.*, 1999).

Chromatin condensation is one of the most characteristic morphological features of apoptosis. Increased intracellular calcium can activate endogenous endonuclease that cleaves host chromatin into oligo nucleosomal length DNA fragments. In cells treated with hydrogen peroxide and undergoing apoptosis, DNA fragmentation may result from attack by hydroxyl radicals produced by the Fenton reaction, and/or by nucleases activated by nuclear calcium. Experiments of pre-loading cells with ferrous iron showed that increased intracellular iron greatly increased cell death induced by low amounts of hydrogen peroxide (data not shown). Jornot *et al* have recently reported that hydrogen peroxide-induced DNA damage is independent of nuclear calcium but dependent on redox-active ions (Jornot *et al.*, 1998).

In many cell types the production of DNA strand breaks, for example by hydroxyl radical action on DNA, is a potent stimulus to apoptosis (McConkey *et al.*, 1994). The result of TUNEL staining has proved DNA double strand breaks produced by endonuclease activity (McConkey *et al.*, 1995). Hydrogen peroxide or hydroxyl radical tends to produce single strand breaks in DNA. It is possible that hydrogen peroxide will both directly damage DNA through the production of hydroxyl radicals and also by the activation of endonucleases.

Apoptosis is controlled in part by a family of cytoplasmic proteins, the Bcl-2 protein family (Cory, 1995). Bcl-2 can prevent or delay many forms of apoptosis induced by growth factor deprivation, gamma-irradiation, and glucocorticoids. Bax has opposing effects on cell survival, with Bcl-2 blocking and Bax promoting apoptosis. Over expression of Bcl-2 and the viral anti-apoptotic proteins BHRF-1 and E1B 19K counteracted hydrogen peroxide induced apoptosis.

The product of tumour suppressor gene (p53) can induce apoptosis. The expression of wild-type p53 in some p53-deficient cell lines results in spontaneous cell death (Yonish-Rouach, 1997), whereas loss of p53 function has been shown to delay apoptosis in haematopoietic cells (Gottlieb *et al.*, 1994). Recently, it has been reported that Bax expression is regulated positively by the p53 binding (Miyashita and Reed, 1995) and Bcl-2 expression is regulated by the p53-negative responsive element in the Bcl-2 gene (Miyashita *et al.*, 1994). Furthermore, activation of c-myc was found to induce apoptosis preceded by the stabilization of p53 gene product in mouse fibroblasts, whereas activation of c-myc did not induce

apoptosis in p53 null fibroblasts (Hermeking and Eick, 1994). It has been reported that p53 controls both the  $G_2/M$  and the  $G_1$  cell cycle checkpoints and mediates growth arrest, so it may be able to induce apoptosis at both (Agarwal *et al.*, 1995).

Caspases have recently been suggested to be a central component of highly conserved sequence of events leading to apoptosis process. Caspases are a family of cysteine dependent enzymes cleave a defined number of target proteins and lead to the characteristic features of apoptosis. Caspases normally exists in cytoplasm as inactive zymogens and must be activated to be effective. Caspase activity has been detected at early stages of apoptosis and specific inhibitors of caspases have been shown to be able to block the subsequent apoptotic changes (Zhivotovsky *et al.*, 1997; Cohen, 1997).

DiPietrantonio *et al* reported the activation of caspase 3 in HL-60 cells exposed to hydrogen peroxide. (DiPietrantonio *et al.*, 1999). They suggest that hydrogen peroxide mediates specific cleavage of poly (ADP-ribose) polymerase (PARP) and possibly apoptosis by activating caspase 3.

It has also been suggested that hydrogen peroxide is able to block Fas-mediated caspase activity and subsequent apoptosis (Hampton and Orrenius, 1997). Caspases blocked by low-level hydrogen peroxide is only temporary and the cells may be able to repair or replace the inhibited or damaged caspases. At higher levels of hydrogen peroxide the caspase pathway may be permanently blocked and the cells die of necrosis.

Very recently Migliaccio *et al* found the  $p66^{shc}$  gene to be responsible for hydrogen peroxide induced apoptosis (Migliaccio *et al*, 1999). The rate of apoptosis by hydrogen peroxide was reduced in  $p66^{shc}$  cells and the life span was increased in  $p66^{shc}$  mouse.

Most of the above mechanisms of hydrogen peroxide induced apoptosis have not been studied in endothelial cells, and thus whether endothelial cell apoptosis is controlled by the same mechanisms is speculation.

## 7.6. Oxidative Senescence

Paradoxically, the small proportion of surviving but growth inhibited cells may be particularly important in the context of endothelial function: persistent vessel wall coverage with surviving but dysfunctional cells may have more profound effects than cell loss followed by rapid regrowth of normal endothelium. Long term impairment of endothelial-dependent relaxation to aggregating platelets has been described after reperfusion injury in canine coronary arteries (Pearson *et al.*, 1990), and in angiographically "normal" vessels in post myocardial infarct patients (Uren *et al.*, 1993).

Morphological alterations in the chromatin of endothelial cells in the umbilical arteries of infants born of smoking mothers have been described by Asmussen (Asmussen, 1982a; Asmussen, 1982b) and smoking also causes alterations in the surface ultrastructure of arteries (Pittilo *et al.*, 1990).

The nuclei of senescent cells were morphologically abnormal, but they did not contain nucleosomal DNA fragments or large numbers of 3'OH DNA ends. It is uncertain whether the marked and persistent inhibition of thymidine uptake caused by exposure to low hydrogen peroxide concentrations is a consequence of, or independent from, the processes leading to apoptosis. A number of transcription factors, in particular NFkB (Collins, 1993; Toledano and Leonard, 1991) are known to be involved in endothelial cell division and are susceptible to direct modulation by reactive oxygen species, but their involvement in the processes described is at present only speculative.

Senescence means irreversible arrest of cell proliferation and altered cell function, a process controlled by multiple dominant genes and depends on the number of cell divisions, not time. Cellular senescence has important physiological significances. Firstly it may reflect processes that occur during organismal aging and may even constitute an underlying cause of aging. Secondly cellular senescence is a tumour suppressive mechanism. Senescence prevents cells from acquiring the multiple mutations necessary for malignant transformation. Certain oncogenes act at least in part by extending replicative senescence, indicating their ability to overcome senescence. Also, some well-recognised tumour suppressors, such as p53 and retinoblastoma susceptibility genes, are needed to establish and maintain senescence.

Oxidative senescence is cellular senescence induced by oxidative stress on young cells, or accelerated senescence. It does not depend on the number of cell divisions, but depends on the exposure to reactive oxygen species, for example, hydrogen peroxide. Very recently Caldini *et al* reported premature induction of aging in sub lethally hydrogen peroxide treated young MRC5 fibroblasts (Caldini, 1998).

Davies *et al* examined the endothelium of human coronary arteries from heart transplantation patients. In addition to the leukocyte adherence to endothelium in many areas, they discovered that endothelial cells covering atherosclerotic lesions of all types were irregularly arranged and varied in size and shape (Davies *et al.*, 1988). Burrig reported that giant endothelial cells (with a surface area of greater than or equal to 800  $\mu$ m<sup>2</sup>) were frequently found on the plaque surface of human coronary arteries and carotid endarterectomy specimens (Burrig, 1991). He attributed the accelerated endothelial senescence to sustained, non-denuding injury. Both scanning and transmission electron microscopy confirmed the presence of variant endothelial cells in aorta over intimal-thickened and advanced atherosclerotic lesions (Tokunaga *et al.*, 1989b).

Tokunaga *et al* analysed the primary endothelial cultures from human aorta and inferior venae cavae. They found a proportion of cells were large variant cells with a diameter of 100 microns or more, compared to about 50 to 70 microns in normal cells (Tokunaga *et al.*, 1989b). Those variant cells possessed endothelium-specific markers of vWF and Weibel-Palade bodies, but they did not incorporate tritiated thymidine. The ratio of variant to normal endothelial cells correlated well with the severity of atherosclerosis, but less so with the age of patients. This can be explained that those variant endothelial cells are induced by atherosclerosis, rather than multiple cell replications in old age.

In this study briefly exposing early passage  $(10^{th} PD)$  bovine aorta endothelial cells to small amounts of hydrogen peroxide (0.25 to 0.5 picomol/cell for 60 min) greatly accelerated cellular senescence, comparable to replicative senescent cells at their 70 to  $100^{th}$  population doublings. At 24 hours after hydrogen peroxide treatment a large proportion of cells started to increase in size and form single, or sometimes multiple, nucleated giant cells. Earlier report by Tokunaga *et al* suggested that giant cells were the product of cellular fusion rather than intra cytoplasmic nuclear division (Tokunaga *et al.*, 1989a). But time-lapse video microscopy in this study revealed that those giant single or multiple nucleated cells were mainly the consequence of the failure of cell division. Oxidatively senescent endothelial cells, with their bizarre morphology and depressed thymidine uptake were produced as a direct result of hydrogen peroxide exposure rather than as a product of multiple cell divisions. The evidence from BrdU uptake is that after exposure to hydrogen peroxide they had not divided at all.

Oxidatively senescent cells are not dying cells. They maintain certain cellular functions. The level of mitochondrial dehydrogenase activity was comparable between control cells and senescent cells, except during the first 3 or 4 days after oxidative stress, which greatly decreased dehydrogenases activity. Senescent cells also retained some capability to produce t-PA, although it was difficult to compare

because of the varying expression in control cells (data not shown). It has been reported that senescent endothelial cells have increased production of PA1 protein and mRNA (Comi *et al.*, 1995). They even retain the ability to express factor VIII-related antigen. In fact, oxidatively senescent cells are relative stable and could survive for at least a few weeks in culture. It has been reported that senescent cells are more resistant to apoptosis because of their increased expression of bcl 2 (Wang, 1995).

Giant senescent endothelial cells in human aorta have elevated LDL receptor expression, which is normally low in normal endothelial cells (Tokunaga *et al.*, 1998). Electron microscopy showed those cells had increased LDL-gold uptake, whereas the normal endothelial cells in the same aorta had much lower LDL uptake.

Chen *et al* very recently reported hydrogen peroxide induced senescence in human fibroblasts (Chen *et al.*, 1998). They reported transient elevation of p53, high level of p21, lack of Rb phosphorylation, G1 arrest and inability to replicate when G1 arrest is inactivated in oxidatively senescent cells.

Dimri *et al* first reported the detection of  $\beta$ -galactosidase activity in senescent cells at pH 6 (Dimri *et al.*, 1995). They named it as SA- $\beta$ -Gal and proposed it to be a biomarker of senescence. However in this thesis not all of the replicative senescent endothelial cells were SA- $\beta$ -Gal positive. The rate of apparent positive cells in cultures of 100<sup>th</sup> population doubling showing 100% senescent morphology was about 70 to 80%. In normal culturing conditions there were no false positives, but when the cells were left in confluence for more than 3 days, they gradually started to show positive staining. The staining increased in density progressively throughout the time the cells remained in confluence. By three weeks the pre-senescent cells stained SA- $\beta$ -Gal as strongly as that of senescent cells. The expression of SA- $\beta$ -Gal could be repeatedly switched on and off in the same cells by subjecting them to, or relieving them from, contact inhibition. This suggested that SA- $\beta$ -Gal is probably a biomarker of non-proliferation instead of senescence. Indeed Dimri *et al* noticed that in confluent culture some presenescent cells were positive if they were dense and overlapped. Unfortunately the experiment was terminated at this stage. If they had left cells for another week or so they would probably have found out that all pre-senescent cells had been SA- $\beta$ -Gal positive and comparable to that of senescent cells. It is true that SA- $\beta$ -Gal positive pre-senescent cells will become negative in two days after plating and that SA- $\beta$ -Gal positive senescent cells will remain positive after plating. But this actually indicates that SA- $\beta$ -Gal activity is accumulated in cells when they are not proliferating and lost during proliferation.

Chapter 6 showed a time, and hydrogen peroxide dose, dependent increase of endothelial cell autofluorescence, which was attributed to the production of lipofuscin. Lipofuscin is an electron dense and autofluorescent inclusion in cells characteristic of aging (Terman and Brunk, 1998). It has been found progressively accumulated in lysosomes of postmitotic cells over time. Lipofuscin is a mixture of different chemical substances composed mainly of proteins and lipids, but also exhibits the activity of some lysosomal enzymes and the presence of metals (Miyagishi *et al.*, 1967). The fluorophores responsible for lipofuscin autofluorescence may be formed as a result of reactions between carbonyls (mainly aldehydes produced by lipid peroxidation) and amino compounds (Yin, 1996).

Based on experiment of cultured neonatal rat myocytes, Brunk *et al* proposed that lipofuscinogenesis is the result of interaction between oxidative stress and autophagocytosis. They suggest the reactive oxygen species may diffuse into lysosomes where redox-active, low molecular weight iron is released from autophagocytosed material (Brunk *et al.*, 1992). The results of Chapter 4 clearly showed that extracellular hydrogen peroxide, at as low as 5 femtomol/cell, could

lead immediately to intracellular accumulation. It is very likely that some of the hydrogen peroxide accumulated in the cytoplasm will diffuse into lysosomes to cause oxidation and the production of lipofuscin.

The relationship between lipofuscin accumulation and lack of proliferation has been well established; it only appears in aged or non-proliferating cells. Although it can be used as a parameter of oxidative damage in proliferating cells, it is not a marker of senescence because it also appears in confluent young cells.

Brief exposure of endothelium to low concentrations of hydrogen peroxide has a dual effect: some cells are induced to undergo apoptosis, but others enter a state of chronic growth-inhibition accompanied by characteristic morphological changes of senescence. These surviving but growth inhibited senescent cells may cause long-lasting vascular dysfunction after brief exposure to oxidative stress.

There are several possible mechanisms for accelerated endothelial senescence by Tumour suppressor p53 and other proteins that inhibit the oxidative stress. initiation of DNA synthesis are expressed at higher levers in replicative senescence. (Garkavtsev et al., 1998). The increased expression of the cyclindependent kinase inhibitor p16 may contribute to arresting the growth of senescent cells. Very recently it has been reported that non-lethal dose of hydrogen peroxide induced high level of p53 protein accumulation while hydrogen peroxide at doses of inducing apoptosis actually attenuated the p53 protein accumulation (Jiang et al., 1999). The increase of p53 protein by hydrogen peroxide is very rapid, could be seen at 1.5 hours after 2 hours pulse exposure, which may return to normal level after 48 hours (Chen et al., 1998). Hydrogen peroxide may also translocate p53 from cytosolic to the nuclear compartment (Uberti et al., 1999). More interestingly it has been suggested that genes encoding catalase is very vulnerable to hydrogen peroxide induced damage while p53 gene seems surprisingly resistant (Burdon et al., 1996).

Telomere shortening has now been recognised as one of the most important mechanism of cell senescence. Telomere is a long stretch of simple repeats of (TTAGGG)n present in the ends of all chromosomes and is necessary for chromosome segregation and for protecting chromosome ends against degradation. The number of uninterrupted repeats in human embryonic telomere is about  $2.3 \times 10^3$ , with a high degree of length polymorphism. Between 30 to 200 bp on average, or between 5 to 10 repeats, are lost with each division of human cells because the inability of DNA polymerases to complete replication of the 3' end of the DNA duplex due to the obligate requirement for an upstream RNA primer. In human germline cells the ribonucleoprotein enzyme telomerase is able to synthesise and maintain telomere length, but in somatic tissues there is no telomerase expression and telomere is shortened at every division (Kim, 1997). When the progressive telomere shortening reaches a threshold DNA cannot be duplicated and cells become senescent.

It has been shown that fibroblast telomeres shortened from 9-10 kbp in primary culture to about 6 kbp when cells entered into senescence (Harley *et al.*, 1992). It has also reported that telomere length is predictive of replicative capacity in normal cells (Allsopp *et al.*, 1992). Endothelial telomere is also found to shorten in culture as a function of the number of cell divisions (Chang and Harley, 1995). More evidence comes from immortalisation of human cells, all of which involve either the activation of telomerase or an alternative mechanism that maintains telomeric DNA (Kim, 1997). Very recently Bodnar *et al* have successfully extended the life span of telomerase negative normal human retinal pigment epithelial cells by introduction of telomerase to restore the telomere length (Bodnar *et al.*, 1998), whose work has been hailed as the ending of telomeres and senescence debate (de Lange, 1998).

Hydrogen peroxide has been shown to be able to preferentially accumulate single strand breakage or regions in telomeres of human fibroblasts (Petersen *et al.*,

1998). While the DNA damage in mini satellites or in the bulk of genome has been completely repaired within 24 hours, about 50% of damage in telomeres has been left un-repaired for at least 19 days.

Preliminary experiment in this laboratory also showed that hydrogen peroxide was able to damage endothelial cell telomeres and indicated single stranded breakage accumulated in telomeres. Hydrogen peroxide induced single stranded damage in telomeric DNA may trigger the ultimate loss of cellular proliferative ability via activation of the p53 dependent check point system.

While telomere shortening may have neatly explained the genetic cause for the failure of DNA duplication in senescent cells, there are other questions left unanswered, for example the increased cell size, the accumulation of lipofuscin and altered cell functions in oxidative senescent cells.

One of the possible answers to the above questions is the effect of oxidative stress on the function of proteasome. During oxidative stress oxidised proteins are recognized by proteases and completely degraded to amino acids. New proteins will be synthesized to replace the damaged one (Grune *et al.*, 1995). Oxidised amino acids within oxidatively modified proteins may be eliminated, or used as carbon sources for ATP synthesis. Since an oxidatively modified protein may contain only two or three oxidized amino acids probably most of the amino acids from an oxidized and degraded protein are re-utilised for protein synthesis. When oxidative damage is greater than cellular proteolytic capacity there will be accumulation of oxidised proteins, in which case the functions performed by those proteins may be lost or faulty. If the replacement of damaged proteins is not fast enough the cells will lose functions involving those proteins. Accumulation of damaged proteins is also detrimental to cell functions. In mammalian cells oxidised proteins appear to be recognised and degraded by proteasome (Grune *et al.*, 1995; Grune and Davies, 1997). Proteasome is an unusually large multi subunit complex regarded as a protein degrading organelle or machine (Tanaka and Chiba, 1998). It is an ATP dependent protease with catalytically active threonine residues. Proteasome requires ubiquitin as a partner for the breakdown of a wide variety of target proteins. The 700 kDa (20S) proteasome can combine with other ubiquitin conjugating and ATPase subunits to form a 1500 kDa (26S) proteolytic complex, which is responsible for the ATP and ubiquitin dependent proteolysis in cells. However oxidised proteins are in fact recognised and degraded by the 20S core proteasome in an ATP and ubiquitin independent way (Reinheckel *et al.*, 1998). Sitte *et al* have demonstrated that the degradation of oxidised proteins in hydrogen peroxide treated fibroblasts is proteasome can selectively degrade oxidised histones in nuclei (Ullrich *et al.*, 1999).

When oxidised proteins are selectively degraded by proteasome, proteasome itself is also under various regulations during oxidative stress (Reinheckel *et al.*, 1998). At the same time hydrogen peroxide may also produce cross-linked and/or aggregated proteins, which may be resistant to degradation by proteasome. Overall, it is reasonable to postulate that inhibition of proteasome function may be a potential mechanism of oxidative senescence. Reduced proteolysis explains the accumulation of oxidised proteins, the appearance of lipofuscin inclusion and the increase in cell size.

## 7.7. Conclusion and Future Work

Of all biologically relevant reactive oxygen species hydrogen peroxide is most crucial because of its ability to cross cell membranes. Thus hydrogen peroxide can activate signal transduction systems both extracellularly on the cell membrane but also intracellularly in the cytoplasm, in addition to causing intracellular peroxidation and initialising DNA damage.

Endothelial cell anti-oxidant capacity is weak, which will be overcome by as little as 5 femtomol/cell hydrogen peroxide. Depending on the amount available to each cell hydrogen peroxide can cause a spectrum of endothelial cell damage, range from temporary increase of intracellular peroxidation to altered cell function, inhibition of proliferation, senescence, apoptosis and cell lysis.

To cause immediate necrosis a relatively large amount of high concentration (about 5 picomol/cell or 1 mmol/L) of hydrogen peroxide is needed. Though it is unlikely to have such high concentrations of hydrogen peroxide very often *in vivo* it is possible in situations like ischaemia/reperfusion.

Myoglobin does not produce hydroxyl radical in reaction with hydrogen peroxide. On the contrary it actually protects endothelial cells from membrane damage by hydrogen peroxide. This reaction produces ferrylmyoglobin, which is much less cytotoxic than hydrogen peroxide and can be reduced by comproportionation with oxymyoglobin. Furthermore the redox 'cycling' of myoglobin and ascorbate might act as defence mechanism against oxidative damage.

Extracellular hydrogen peroxide can cause a rapid increase in intracellular peroxidation. At the same time endothelial cells also consume extracellular hydrogen peroxide. Careful examination reveals that endothelial cell damage is directly related to the ratio of hydrogen peroxide available to each cell, but not the concentration.

Exposing endothelial cells to relatively low amount or concentration of hydrogen peroxide (no more than 0.5 picomol/cell or 100  $\mu$ mol/L) leads to cellular function damage. This damage, including reduced mitochondrial activity, delayed re-

endothelialisation, inhibited proliferation and reduced prostacyclin production, can recover gradually after oxidative stress.

At low amount (0.1 to 0.5 picomol/cell) hydrogen peroxide will also induce endothelial apoptosis and senescence on top of functional damage. Apoptosis occurs within 48 hours after oxidative stress while senescence is apparent after 48 hours. Hydrogen peroxide may induce p53 accumulation and translocation to lead to both apoptosis and senescence in endothelial cells. Multiple mechanisms may be involved in hydrogen peroxide-induced apoptosis. Direct DNA damage, activation of caspases and proto-oncogenes may all play important parts.

Endothelial senescence has been linked to atherosclerosis. It has been shown that leucocytes adhering to endothelium is common in many areas of coronary arteries. In addition endothelial cells covering atherosclerotic lesions of all types were irregularly arranged and varied in size and shape, typical morphology of senescence. It is current not known why those endothelial cells covering atherosclerotic lesions showing senescent morphology. It may have entered senescence from exhausted replication duo to continued cell death, which is not very likely. Results in this thesis suggest those cells may have undergone accelerated senescence by oxidative stress, with leucocyte adhering on endothelium as the possible source of hydrogen peroxide *in vivo*.

On the other hand, hydrogen peroxide dismuted from leucocyte released superoxide anion is not the only source of oxidative stress for endothelial cells. Oxidised LDL or oxidised lipoproteins are also capable of damaging endothelial cells.

**Future Work** The most interesting result of this thesis is the accelerated senescence of endothelial cells by low levels of hydrogen peroxide. But more work has to be done to fully understand the mechanisms and eventually to design

ways of intervention to prevent or reduce the premature senescence in endothelial cells.

Telomere damage by hydrogen peroxide has been demonstrated by this and other laboratories, but the mechanism of the damage is not clear. Hydrogen peroxide may directly damage telomere by production of hydroxyl radical, or by the activation of nucleases. Experiments designed to manipulate intracellular metal iron and/or calcium may reveal whether oxidative telomere damage is iron dependent or calcium dependent.

While senescence is one of the manifestations of sublethal oxidative damage, there are many other cells that may have had different degrees of damage without showing senescence. Some of the damage may be repairable in relatively short time, but some damage may be present in endothelial cells for considerably long period of time. At present, most of endothelial cell studies have focused on the short-term (no more than 3 days after oxidative stress) effect of endothelial death or the damage of dying cells, very little attention has been given to the long-term (more then 3 days after oxidative stress) endothelial damage by sublethal amount of reactive oxygen species and the damage in those surviving cells.

According to the response-to-injury hypothesis, endothelial dysfunction is the first step to atherosclerosis (Ross, 1999). The study of the long-term functional change in oxidatively damaged endothelial cells and the mechanism of the dysfunction, for example the synthesis and release of nitric oxide, prostacyclin and t-PA, may contribute to the understanding of atherosclerosis.

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## Abbreviations

APC:	Activated protein C
AT-III:	Antithrombin III
BAEC:	Bovine aorta endothelial cells
BrdU:	5-bromo- 2'-deoxyuridine
DCF:	2'7' dichlorofluorescein
DCF-DA:	2'7' dichlorofluorescein diacetate
DMEM:	Dulbecco's modified eagle medium
DMPO:	5,5'dimethyl-l-pyrroline-N-Oxide
EBM:	Endothelial cell basal medium
EDCF:	Endothelium derived constricting factors
EDRF:	Endothelium-derived relaxing factor
EGM:	Endothelial cell growth medium
EC:	Endothelial cells
ET:	Endothelin
FBS:	Foetal bovine serum
FCS:	Foetal calf serum
FerrylMb:	Ferrylmyoglobin
ESR:	Electron spin resonance spectroscopy
$H_2O_2$ :	Hydrogen peroxide
HUVEC:	Human umbilical vein endothelial Cells
ICAM:	Intracellular adhesion molecule
IL-la:	Interleukin-1 $\alpha$
LDH:	Lactate dehydrogenase
Mb:	Myoglobin
MetMb:	Metmyoglobin
NO:	Nitric oxide
cNOS:	Constitutive NO synthetase
eNOS:	Endothelial NO synthetase
iNOS:	Inducible NO synthetase
O <sub>2</sub> •:	Superoxide anion
OxyMb:	Oxymyoglobin
PA:	Plasminogen activator
PAF:	Platelet-activating factor
PAI:	Plasminogen activator inhibitor
t-PA:	Tissue type plasminogen activator
u-PA:	Urokinase-type plasminogen activator
<b>PAR 1</b> :	Protease-activated receptor-1
PI:	Propidium iodide
PBN:	1-alpha-phenyl-tert-butyl nitrone
PBS:	Dulbecco's phosphate buffered saline, pH 7.3
PD:	Population doubling time

.

PGI <sub>2</sub> :	Prostacyclin
ROS:	Reactive oxygen species
SA-β-Gal:	Senescence associated $\beta$ -galactosidase activity
SOD:	Superoxide dismutase
TdT:	Terminal deoxynucleotidyl transferase
TEMPO:	2,2,6,6 tetramethylpiperidine-N-oxyl
TF:	Tissue factor
TFPI:	Tissue factor pathway inhibitor
TM:	thrombomodulin
TMPO:	3,3,5,5 tetramethyl-l-pyrroline-N-oxide
vWF:	von Willebrand factor

## **List of Publications**

- 1. DP de Bono and W Yang. Vascular Endothelial Growth Factor and Fibroblast Growth Factors increase Endothelial Resistance to Intracellular Oxidative Stress but also Increase Susceptibility to Cell Death after Oxidative Injury. In press
- 2. RL Boultby, W. Yang and DP de Bono. Oxidative Stress-Mediated Telomere Loss: A Potential Mechanism for the Accelerated Senescence of Endothelial Cells in Atherosclerosis. Circulation 100 (18, sup) I-260, 1999
- W. Yang and DP de Bono. A New Role for Vascular Endothelial Growth Factor and Fibroblast Growth Factors: Increasing Endothelial Resistance to Oxidative Stress. FEBS Letters 403:139-142, 1997
- 4. W. Yang and DP de Bono. Vascular Endothelial Growth Factor Modulates Endothelial Resistance to Hydrogen Peroxide. European Heart Journal 17(sup) 196, 1996
- W. Yang and DP de Bono. Intracellular Oxidative Status of Endothelial Cells Exposed to Low Hydrogen Peroxide Concentrations. Circulation 92(8, sup 1) I-560, 1995
- 6. DP de Bono and W Yang. Exposure to Low Concentrations of Hydrogen Peroxide Causes Delayed Endothelial Cell Death and Inhibits Proliferation of Surviving Cells. Atherosclerosis. 114:235-245, 1995
- 7. W. Yang and DP. de Bono. Effects of Reactive Oxygen Species on Endothelial Cell Proliferation and Senescence. Euro Heart J. 15 (Sup) 488, 1994.
- DP. de Bono, W. Yang, et al. Effects of NMHH and Myoglobin on Endothelial Cells Damage by Hydrogen Peroxide. Cardiovasc. Res. 28(9):1641-1646, 1994
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- 10. W. Yang, DP. de Bono and MCR. Symons. Myoglobin Protects Against Endothelial Cell Membrane Damage Associated with Free Radical Generating Systems. FEBS Letters 319 (1,2): 145-150, 1993.
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- W. Yang et al. Direct detection of Oxygen Free Radicals Produced by Myocardial Reperfusion by Electron Spin Resonance Spectroscopy. Chi J Cardiol 17(3):178, 1989.