THE EFFECT OF 3-HYDROXY-3-METHYLGLUTARYL COENZYME A REDUCTASE INHIBITORS ON OXIDATIVE AND HYPOXIC STRESS IN THE VASCULAR ENDOTHELIUM

by

Mr Kurian Joseph Mylankal MBBS FRCS Edin

Submitted in accordance with the requirement for the Degree of Medicine

University of Hull, February 2008

The candidate confirms that the work submitted is his and the appropriate credit has been given where reference has been made to the work of others.

Acknowledgements

At the very outset I would like to thank Professor Peter Thomas McCollum for giving me the opportunity to do research under his guidance. He has been a very supportive and understanding supervisor and provided assistance in funding this project. He was an easily approachable individual who clarified and refined my thought process. He has been the one figure who has helped me realize my dream of a career in Vascular Surgery.

Secondly, I am indebted to Dr Camille Ettelaie, Lecturer at Department of Biological Sciences, University of Hull for taking me on under his wings in an entirely alien environment of basic science. He has been extremely patient throughout the duration of this work and has been a constant source of encouragement.

Mr Ian Chetter, Senior Lecturer Department of Vascular Surgery, Hull Royal Infirmary will be remembered as being instrumental in having this work completed. He has been constant and relentless in his efforts to help me focus my thoughts on this work.

Miss Amandine Pradier, PhD student at the Biological Sciences, University of Hull is acknowledged for her part in training me to perform cell cultures and the various assays described in the methodology.

My sister Dr Ida Thomas, a Periodontic Surgeon is thanked for the painstaking task of proof reading this document.

Lastly, I would like to thank my wife Zoe for being a very understanding person and for all the sacrifices she made during my time in research. Her continuous support and encouragement have been an inspiration for me. As for our children Kian and Imogen, they have been a source of cheer and happiness through the times of despair.

CONTENTS **PAGE**

List of figures Pages

List of tables Pages

Abbreviations

ADP-ribosylation factor	ARF
Angiotensin Converting Enzyme	ACE
Apoptosis inhibiting factor	AIF
Baciloviral IAP repeat	BIR
Basic fibroblast growth factor	bFGF
c-jun N-terminal kinase	JNK
Cyclin Dependent Kinase	CDK
Cyclic Guanine Mono Phosphate	cGMP
Death-inducing signaling complex	DISC
Diacylglycerol	DG
Direct IAP binding protein with low pI	DIABLO
Endothelin 1/2/3	ET1/2/3
Endothelial cells	EC
endothelial Nitric Oxide synthase	eNOs
Extra- cellular signal regulated kinases1/2	ERK1/2
Fas associated death domain	FADD
Human umbilical vein endothelial cell	HUVEC
Human saphenous vein endothelial cell	HSVEC
Hypoxia inducible factor-1	HIF-1
Hypertension	HT
inducible Nitric Oxide synthase	iNOS
Inhibitors of Apoptosis Proteins	IAP

Inositol triphosphate	IP3
Intercellular adhesion Molecule-1	ICAM- 1
Interleukin-1/6	IL-1, IL-6,
6-Methyltetrahydorbiopterin	BH4
Mitochondrial outer membrane permeabilization	MOMP
Mitogen Activated Protein Kinase	MAPK
Murine leukaemia cellular oncogene	cAbl
Nicotinamide adenine dinucleotide	NAD
Nicotinamide adenine dinucleotide phosphate	NADP
Nitric Oxide	NO
Permeability transition pore	PTP
Phophatidyl inositol 4, 5 –biphosphate	PIP2
tissue Plasminogen activator	tPA
Plasminogen activator inhibitor	PAI
Platelet Activating Factors	PAF
Platelet–Endothelial Cell Adhesion Molecule	PECAM
Platelet transforming growth factor B	TGF-β
Prostacyclin	PGI_2
p38 kinase	p38
Retinoblastoma family of proteins	pRb
Second mitochondria derived activator of caspase	Smac
Sequence specific DNA binding	SSDB
Vascular Cell Adhesion Molecule-1	VCAM-1

Vascular Endothelial Growth Factor	VEGF
Von Willebrand Factor	vWF

Author; Mr KJ Mylankal MBBS, FRCS

Thesis title; The effect of 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitors on oxidative and hypoxic stress in the vascular endothelium

Degree Concerned; Submitted for MD, February 2008

Abstract: 3-Hydroxy-3-methylglutaryl coenzyme A reductase inhibitors (statins) improve endothelial cell (EC) function by enhancing the synthesis of Nitric Oxide (NO) and attenuating the endothelial leucocyte interaction and platelet aggregation. However the effects of statins on endothelial cell proliferation, apoptosis and the mediators of these responses are not clearly defined.

The aims of this research were:

1) To determine the effect of statin on EC proliferation and apoptosis.

2) To assess these cellular processes in the presence of oxidative stress and hypoxia.

3) To study the cellular response to these stresses in the presence of a statin.

4) To assess the effect of sudden withdrawal of statin on the endothelial cell proliferation and apoptosis

Statins exert a proliferative effect on EC at low concentrations and induce apoptosis at higher doses. Oxidative stress and hypoxia induce apoptosis in the EC, mediated via enhanced expression of an apoptotic protein, Bax. Statins abrogate the anti-proliferative and pro-apoptotic effects of oxidative and hypoxic stress by modulating the expression of Bax and cell cycle regulator protein Cyclin D. Acute withdrawal of statins reverses the protective effects on EC survival by promoting apoptosis and inhibiting the proliferative activity.

Publications

1) Oxidative and Hypoxic Stress on the Vascular Endothelium – A Protective Role of Statins. Mylankal K J; Ray B; Venkatasubramaniam A K; Mehta T; Pradier A; Ettelaie C; Chetter I C;McCollum P T; British Journal of Surgery 2004, 91: 1077-1088

2) Statins offer a protective role against Oxidative and Hypoxic stress induced vascular endothelial apoptosis. K J Mylankal, A Pradier, B Ray, A K Venkatatsubramaniam, T Mehta, I Chetter, C Ettalaie, P T McCollum; Atherosclerosis 2003, 170: S13

3) Effect of Statins on the Expression of Bax, cyclin D and tissue factor in response to stress. K J Mylankal, A Pradier, B Ray, C Ettelaie, IC Chetter, PT McCollum; Irish Journal of Medical Science, 2003 Volume 173. No 1. Supplement 1. p19

Oral Presentations

1) Acute Statin Withdrawal and its effect on the Vascular Endothelium. K J Mylankal, A Pradier, B Ray, C Ettelaie, IC Chetter, PT McCollum. XXIXth Sir Peter Freyer Memorial Lecture and Surgical Symposium Galway, September 2004

2) Acute Statin Withdrawal and its effect on the Vascular Endothelium. K J Mylankal, A Pradier, B Ray, C Ettelaie, IC Chetter, PT McCollum. Yorkshire Vascular Surgical Day, Hull, May 2004

3) Effect of Statins on the Expression of Bax, cyclin D and tissue factor in response to stress. K J Mylankal, A Pradier, B Ray, C Ettelaie, IC Chetter, PT McCollum. Sylvester O'Halloran Surgical Scientific Meeting, Limerick, Ireland, March 2004

4) Oxidative and Hypoxic Stress on the Vascular Endothelium – A Protective Role of Statins. Mylankal K J; Ray B; Venkatasubramaniam A K; Mehta T; Pradier A; Ettelaie C; Chetter I C; McCollum P T. Vascular Surgical Society of Great Britain & Ireland (VSSGBI) – Glasgow, November 2003

5) Oxidative and Hypoxic Stress on the Vascular Endothelium – A Protective Role of Statins. Mylankal K J; Ray B; Venkatasubramaniam A K; Mehta T; Pradier A; EttelaieC; Chetter I C; McCollum P T. Royal College of Surgeons of Edinburgh Clinical and Scientific Meeting, November 2003

Poster Presentations

1) Expression of Cellular Mediators in response to Oxidative and Hypoxic Stress- Role for Statins? Mylankal KJ, Pradier A, Ray B, Ettelaie C, Chetter IC, PT McCollum. Association of Surgeons of Great Britain and Ireland, Harrogate April 2004

2) Statins Offer A Protective Role Against Oxidative and Hypoxic Stress Induced Vascular Endothelial Apoptosis. Mylankal KJ, Ray B, Pradier A, Chetter IC, Ettelaie C, McCollum PT. Joint BAS/BSHT Meeting, UK, Sept 2003

3) Role of Statins in the Proliferation and Apoptosis of Vascular Endothelial Cells. Mylankal KJ, Mehta T, Venkatasubramaniam AK, Ray B, Chetter IC, McCollum PT. XXVIIIth Sir Peter Freyer Memorial Lecture and Surgical Symposium meeting, Ireland, Sept 2003

Chapter 1. INTRODUCTION

1.1 Vascular Endothelium

Wilhelm His, Sr., in 1865 first coined the term endothelium [Greek for endos (within) and (thelois) nipple] to describe cells lining surfaces within the body (1).Vascular EC form the inner lining of all blood vessels and act as a dynamic barrier separating the blood and blood cells from the smooth muscle cells. There are over 10^{12} endothelial cells in an adult human being which occupies 1000 m^2 in surface area weighting approximately 1 to 1.5 kg (2).

Although the endothelial cell barrier was initially thought to be inert, it is now known that this layer has a complex function. It generates factors that control vasorelaxation and vasoconstriction, thrombosis and thrombolysis, platelet aggregation and inhibition. A balance of these contrasting functions is vital for the maintenance of blood pressure, tissue blood flow and patency of the blood vessel. When EC fail to maintain this equilibrium chaos results with deranged blood pressure control, tissue hypoperfusion and end-organ failure. It is now clear that endothelial function is deranged in conditions such as hypertension, diabetes, hypercholesterolaemia, aging and smoking with resultant manifestations of end organ damage.

1.1.2 Endothelial Structure

Endothelial cells are polygonal squamous cells measuring 25-50μm long and 10-15μm wide (3). The cells and their elongated nuclei are arranged in the long axis of the vessels due to the longitudinal shear force of the blood flow. They have two faces, the luminal one exposed to the blood flow and the abluminal front bathed in the interstitial fluid (4). The endothelial cells are attached to each other by intercellular junctions of two different kinds.

Occluding junctions (tight junctions) give anchorage to adjoining cells and the communicating junctions (gap junctions) promote intercellular communication. There are cell membrane infoldings called plasmalemmal vesicles which help in transendothelial transport of molecules. Besides the nuclei, the cells also comprise of other cellular organelles such as mitochondria, endoplasmic reticulum, ribosomes and golgi apparatus. There are also certain rod shaped granules in the endothelial cells called Weibel Palade bodies which are the sites of synthesis of Von Willebrand Factor. At the abluminal surface, the cell rests on a matrix 30-50nm wide. There are fine microfibrils within the matrix which anchors the endothelial cells to the basal lamina. The basal lamina is predominantly composed of collagen type IV, V and VIII along with proteoglycans and glycoproteins. In vessels with an internal elastic lamina the endothelial cells may have processes extending through the elastic lamina to form myoendothelial junctions. Vascular smooth muscle forms the contractile outer layer of arterioles and these cells are arranged in a helical pattern in the media and longitudinally in the intima and adventitia.

1.1.3. Physiological Functions of the Vascular Endothelium

1.1.3.1. Vascular Tone

This is dependent on the constrictive and relaxing effects on the vascular smooth muscle cells. The endothelium produces factors which have effect on both these functions.

Endothelium Derived Relaxing Factor

Stimulation of endothelial cells by neurotransmitters, hormones, platelet and coagulation derived substances, and shear forces from blood flow cause the release of a factor which results in relaxation of the vascular smooth muscle cells (5). This factor has now been

identified as a free radical Nitric Oxide (NO). It is freely diffusible and has a half life of a few seconds (5). It is easily destroyed by reactive oxygen species but potent anti-oxidant systems such as catalase and superoxide dismutases improve tissue availability. NO synthesis is catalysed by the enzyme endothelial NO synthase (eNOs) and this enzyme occurs in various tissues including platelets, macrophages, vascular smooth muscle cells and the brain (6). An inducible form of this enzyme (iNOS) is also produced by the endothelial cells in response to endotoxins and cytokines (7). This can result in the synthesis of large amounts of NO over a prolonged period of time which can result in systemic cytotoxicity as in septic shock (8, 9).

In response to vasodilatory stimuli, the agonist binds to the cell membrane receptor and activates regulatory G proteins. They activate phospholipase C to generate diacylglycerol (DG) and inositol triphosphate (IP3) from phophatidyl inositol 4, 5-biphosphate (PIP2). IP3 mobilizes intracellular Ca2+ and DG activates protein kinase C and transport of extracellular Ca2+ to maintain high intracellular Ca2+ levels. Elevated Ca2+ levels along with calmodulin are essential for activation of the enzyme NO synthase. L-arginine is the precursor to NO synthesis and this step wise transformation is dependent on reducing agents 6-methyltetrahydorbiopterin (BH4) and NADPH (10). Nitric oxide is freely diffusible and it activates soluble guanylate cyclase to produce cyclic Guanine Mono Phosphate (cGMP). cGMP mediates relaxation of the smooth muscle cells by inhibiting the Ca^{2+} influx in the sarcolemma (11) or by activating the sarcolemmal Ca^{2+} extrusion pump (12).

Other Endothelium Derived Relaxing Factor

Prostacyclin is another factor largely produced by the endothelial cells and in smaller quantities by platelets and smooth muscle cells (13, 14). It is metabolised from arachidonic acid by prostacyclin synthase and has a short half life of 2-3 minutes (15). It has an inhibitory effect on platelet aggregation, promotes platelet disaggregation and also causes vasodilatation by relaxation of the vascular smooth muscle (16-18). The latter effect is mediated by increasing the levels of cyclic adenosine monophosphate in smooth muscle cells.

Endothelial Derived Contracting Factor

Endothelins are the most potent vasoconstrictors known. They exist in 3 isomeric forms endothelin1(ET1), endothelin 2(ET2) and endothelin 3(ET3); of which ET1 is produced by the vascular endothelium (19). It is a 21 amino-acid chain peptide which is synthesized from its precursor preproendothelin. This is initially converted to big endothelin (bET1) and then further by endothelin converting enzyme to ET1. Some of the stimuli for inducing ET1 synthesis are thrombin (20), platelet transforming growth factor B-1(TGF-β) (21), hypoxia (22), epinephrine (23), angiotensin II (23), vasopressin (24) and shear stress (25). These stimuli mediate their effect through two endothelin specific receptors namely Endothelin A (ETA) and Endothelin B (ETB). These receptors have G proteins coupled to them which activate the phophatidyl inositol system in vascular smooth muscle cells to release intracellular Ca^{2+} and thus effect smooth muscle contraction. Although ET1 has a half life of only 2 minutes, it has a protracted duration of action for over an hour and this is due to its slow release from the receptors in the smooth muscle cells (26, 27). In intact endothelial cells the endothelin related responses are diminished. This is effected by basal

NO synthesis which inhibits stimulated endothelin synthesis and constrictor responses (28). In contrast the ET1 can stimulate synthesis of NO and prostacyclin as a negative feedback mechanism to attenuate its own vasoconstrictive effects (29).

Other Endothelial Derived Contracting Factors

Thromboxane A2 and Prostaglandin H2 are products of the cyclooxygenase pathway in endotheial cells that can mediate vasoconstrictive effects. In response to stimuli from factors such as arachidonic acid, histamine, acetylcholine and serotonin the cycloxygenase pathway within endothelial cells generate Thromboxane A2 and Prostaglandin H2 (30). These activate the thromboxane receptors in vascular smooth muscle cells and counteract the vasodilatory effects of NO and prostacyclin. Besides this, the cycloxygenase pathway is a source of reactive oxygen species which can also inactivate NO.

The renin angiotensin system is also regulated by the endothelium. Angiotensin Converting Enzyme (ACE) which converts Angiotensin I to Angiotensin II is localised in the endothelial cell membrane. Activated receptors for Angiotensin II in the endothelial cells can upregulate the synthesis of ET1 with resultant vasoconstriction (31).

1.1.3.2. Homeostasis and Coagulation Antiplatelet Effects

The EC through different mechanisms exerts effect on the platelets. They prevent platelet activation and adhesion to the EC wall and also aggregation to form platelet plugs.

Surface Charges

The vascular endothelial surface has a negative surface charge which repels the negative charge of the platelet cell surface membrane. This acts as deterrent to platelet adhesion to endothelial surface (32). However when the integrity of the vascular endothelium wall is

disrupted, the exposed subendothelial matrix with a positive charge promotes platelet adhesion due to its surface.

Nitric Oxide

Nitric Oxide modulates the interaction between platelets and endothelium. It inhibits human platelet activation and adhesion to endothelial cells and promotes disaggregation. Thus it plays a vital role in preventing thrombosis. The platelet inhibitory effects are mediated by activation of the enzyme guanylate cyclase which results in accumulation of cyclic GMP (33). Platelet activation and adhesion are dependent on low cGMP levels. Increased cGMP levels diminish the cytosolic Ca2+ levels and thus inhibit platelet activation.

Prostacyclin

Prostacyclin (PGI_2) which is a derivative of the cyclooxygenase pathway is a potent vasodilator and has an inhibitory effect on platelet function (13, 16, 34). It is synthesised by the endothelial cells from arachidonic acid in response to chemical stimuli such as bradykinin (35), lipoproteins (36), thrombin (37) and histamine (38). Although PGI_2 has no influence on adhesive properties of unstimulated platelets, it inhibits adhesion of stimulated platelets to the endothelial wall. This effect is mediated through increased cAMP levels within platelets.

Other mediators

Adenosine is a potent vasodilator and inhibitor of platelet aggregation. Aggregating platelets release ADP which promotes further aggregation and platelet plug formation. EC have membrane bound ectoenzyme which rapidly inactivates this ADP to AMP and adenosine (39). Thus adenosine contributes to antiplatelet activity.

Anticoagulant effects

The anticoagulant pathway helps prevent formation of microthrombi that interrupt blood flow. This pathway comprises of thrombomodulin, antithrombin, protein C and protein S. Following tissue injury mediated via endotoxins, there is enhanced tissue factor expression with factor VIIIa-IXa and Va-Xa complex formation. This leads to thrombin generation which activates the coagulation pathway.

Thrombomodulin is a protein bound to EC membrane that binds to thrombin. The thrombin-thrombomodulin complex inactivates thrombin which is then easily removed from circulation (40). Thrombomodulin has anti inflammatory effect by inhibiting cytokine synthesis and decreasing leucocyte-endothelial interaction. This complex also activates Protein C to promote receptor binding (41). Activated protein C inhibits factor V and VIII of the coagulation cascade (42, 43). Subsequently the activated protein C is released from its receptor and binds to Protein S. This complex inactivates factor Va-VIIa preventing further thrombin release (44).

Antithrombin is another mediator of the anticoagulant pathway which is synthesized by the hepatocytes. It is activated by heparin sulphate within the EC (45). Antithrombin attaches to the EC membrane and acts as a scavenger for thrombin with which it forms a covalent bond (46). Thus the circulating thrombin is neutralised. Antithrombin also inactivates Factors Xa and has similar effects on IXa, XIa and XIIa of the coagulation cascade.

Procoagulant effects

von Willebrand Factor is a large glycoprotein synthesized by the EC and megakaryocytes. It is stored in the Weibel Palade bodies within the EC and secreted into plasma and the extracellular matrix. It serves as an adhesion protein that helps to aggregate platelets at

sites of endothelial damage with exposed extracellular matrix (47, 48). vWF from serum attaches to exposed collagen with in the exposed subendothelial extracellular matrix and uncoils the collagen structure. This enables trapping of platelets to collagen and the activation of platelet membrane glycoproteins which in turn facilitate platelet to vessel wall and platelet to platelet adhesion to form platelet plugs.

Platelet Activating Factors (PAF) are a group of structurally related phospholipids synthesized predominantly by EC and leucocytes with a potent proinflammatory potential (49, 50). PAF is a common mediator to many upstream signalling pathways and is synthesized predominantly in response to oxidants which release peroxidised lipids from cell membrane (51). These in turn activate p38 kinase enzyme with release of PAF by acetyltransferase and phopholipase A2 (52). PAF mediates its effect through PAF receptor which mobilises Ca2+ and depletes cAMP levels. This activates platelets and promotes chemotaxis and adhesion of leucocytes.

Fibrinolytic Pathway

This is vital to maintain the equilibrium between bleeding and thrombosis. During fibrinolysis, plasminogen is converted to active plasmin by endothelial derived plasminogen activator (tPA). Plasmin can effect lysis of fibrinogen and restrict clot formation. tPA synthesis can also be activated by PAF which further ensures that clot formation remains under check (53). Plasminogen activator inhibitor (PAI) synthesized by EC regulates synthesis of plasmin and hence the lysis of fibrinogen (54). Thus the activity of these two components of the pathway, determine the net fibrinolytic effect.

1.1.3.3 Host Responses

The EC being the first line of defence against blood borne stimuli, plays a vital role in balancing the propagation and inhibition of inflammatory responses. This is brought about through the release of cytokines and by promoting leucocyte adhesion.

Cytokines

EC release small peptides called cytokines in response to noxious stimuli and cytokines target specific cells and modify their function (55). They communicate with the target cells through receptors. The primary role of cytokines is to promote responses in order to minimise damage and maintain a steady state. Based on their functions they may be pro-inflammatory cytokines which are interleukin 1 (IL-1) , IL-6, tumor necrosis factor alpha [TNF-α], and transforming growth factor beta [TGF-β]) (56, 57). These pro-inflammatory cytokines propagate the inflamatory response. Immunoregulatory cytokines (IL-4, IL-10) help regulate the immune response by inhibiting synthesis of cytokines and enhancing the synthesis of neutralizing antibodies to foreign stimuli (58, 59). The role of chemokines (IL-8) is to attract immune cells such as neutrophils to sites of infection (60).

Leucocyte interactions

The EC generates various membrane bound molecules which promote circulating leucocytes to target sites of inflammation. Selectins are a group of glycoproteins that initiate the first step of EC-leucocyte interaction. Selectins are of three forms, E Selectin (endothelium derived), L selectin (leucocyte) and P selectin (platelet and endothelium). P selectin expression which is enhanced by TNFα and interleukin-1 accelerates leucocytes rolling phenomenon before their adhesion to EC surface. The EC also express adhesion molecules which promote adhesion of circulating leucocytes. ICAM- 1 (Intercellular

adhesion Molecule-1) expression on endothelial cells is upregulated by interleukin-1, interferon-gamma or TNF-alpha (61, 62) and it binds to leucocyte beta 2 integrins (CD11a/CD 18) and CD11b/CD 18 which mediate leukocyte-endothelial cell adhesion and transendothelial migration of leukocytes into tissues (63). VCAM-1 (Vascular Cell Adhesion Molecule-1) expression in EC is enhanced by TNF-alpha, interleukin-1 or interleukin-4 (64). VCAM-1 promotes EC binding to lymphocytes, monocytes, eosinophils and Langerhans cells.

1.1.3.4 Synthesis of Growth Factors

The EC synthesises a melieu of growth regulators. Under physiological states, the net effect of the influences of these growth regulators favours an overall inhibitory effect so as to maintain luminal patency of the vessel and blood flow. Heparin Sulphate and NO are potent inhibitors of smooth muscle cell proliferation (65). Some of the growth promoters are vascular endothelial growth factor, platelet derived growth factors, endothelin, epidermal growth factor and angiotensin II (66-69).

1.1.3.5 Metabolic Functions

The EC has metabolic functions such as presenting receptors for LDL, HDL and chylomicrons (70, 71). EC membrane incorporates lipoprotein lipase synthesized in macrophages and smooth muscle cells to hydrolyze glycerol groups from very low density lipoproteins and chylomicrons. Thus LDL is transformed to promote uptake and further metabolism by the macrophages.

1.1.3.6 Connective Tissue Synthesis

The basement membrane which anchors the EC comprises of collagen, elastin, laminin, mucopolysaccharides, fibronectin and thrombospondin. EC secrete predominantly collagen type IV and V to form the extracellular matrix (72). Elastin is synthesized as a single chain tropoelastin by EC which subsequently transforms to the highly cross linked elastin molecule (73). Synthesis of Laminin by EC, another component of the extracellular matrix is enhanced when the EC are not confluent(74, 75). Mucopolysaccharides such as heparin sulphate, dermatan sulphate and chondrotin sulphate form the extracellular matrix whilst the cell membrane is predominantly composed of heparin sulphate (76, 77). Fibronectin and thrombospondin are proteins secreted by the EC which form fibrillar structures within the extracellular matrix that anchor EC to the basement membrane (78, 79).

1.2. Endothelial Dysfunction

Endothelial dysfunction is characterised by an imbalance in the EC mediated vasodilator and constrictor effects. The various risk factors for atherosclerosis such as smoking, hypercholesterolaemia, hypertension and diabetes predispose to EC dysfunction which is now recognised as the first step in the atherosclerotic process (80). EC dysfunction precedes any morphological alteration to the vessel wall.

1.2.1 Vascular Aging

Aging is a physiological process where cardiovascular mortality is increased even in the absence of any significant risks. This may be due to the cumulative effects of prolonged exposure of EC to reactive oxygen species with resultant impairment in endothelium

dependent vasodilatation (81). Studies have demonstrated ageing to decrease the basal (82) and stimulated (83) NO synthesis by reduced expression of endothelial NO synthase gene. This effect may be compounded by a decrease in NO bioavailability due to scavenging by increased levels of free radical molecules (84).

1.2.2. Smoking

Smoking has a deleterious effect on the endothelial function. This is due to an impaired vasodilatory response through reduced NO synthesis, although the synthesis of ET remains unaffected by smoking (85). It also has an inhibitory effect on Prostacyclin synthesis which attenuates vasodilatation and promotes platelet aggregation (86). Cigarette smoke has been shown to activate Platelet–Endothelial Cell Adhesion Molecule (PECAM) which promotes monocyte migration across the vascular endothelium and platelet adhesion (87). Apart from these effects on the EC, it alters the prothrombotic and fibrinolytic pathways mediated by tissue-plasminogen activator (t-PA) and tissue factor plasminogen inhibitor1 (TFPI-1) (85). Nicotine causes intimal hyperplasia by smooth muscle cell proliferation which is mediated through mitogens such as basic fibroblast growth factor (bFGF) and transforming growth factor-b1 (TGF-b1) (88).

1.2.3. Hypercholesterolaemia

It is now well recognised that hypercholesterolaemia results in alteration of the vasodilatation and vasoconstrictor effects mediated by the vascular endothelium (89). There is increased generation of oxidised LDL in hypercholesterolaemia and this results in endothelial dysfunction (90). Oxidised LDL inhibits the gene expression for eNOS in EC and hence attenuates the vasodilatory effect (91). This effect is further compounded by an increased production of superoxide anions in hypercholesterolaemia which scavenge the

NO to form peroxynitrite (92). Peroxyntrite is a potent cytotoxic and proinflammatory mediator (93, 94). Upregulation of enzyme superoxide dismutase to raise the antioxidant capacity by exogenous supplementation in rabbits helps to reverse these effects of imbalance between the superoxide anion generation and NO synthesis (95). There is a simultaneous increase in the expression of inducible nitric oxide synthase (iNOS) and this together with peroxynitrite could initiate lipid peroxidation. Oxidised -LDL also enhances the gene expression of adhesion molecules such as ICAM-1, VCAM-1, P and E selectin to promote leucocyte and monocyte adhesion to EC. It has procoagulant effects and inhibits the fibrinolytic pathway, thus promoting thrombosis. There are specific receptors for uptake of oxidised-LDL by EC known as LOX-1. This receptor enhances expression of cellular adhesion molecules, cellular apoptosis and downregulates the gene expression of eNOS.

1.2.4. Hypertension

EC dysfunction in hypertension results in the various manifestations of the disease in the blood vessels and end organs. EC dysfunction related to hypertension (HT) is characterised by an impaired vasodilatory effect. Animal studies blocking NOS synthesis have demonstrated acute onset HT (96). An imbalance in NO bioactivity can be due to decrease in the availability of substrate L arginine; competitive inhibition by L -arginine antagonist; decreased activity of eNOS; and accelerated NO scavenging. Although experimental studies on humans and animals have shown that arginine supplementation does increase the vasodilatory effects dependent on EC in hypercholesterolaemia, a similar effect is not observed in HT. This suggests that in HT, substrate deficiency does not affect the attenuated vasodilation. To the contrary it has been documented that the NOS activity is

enhanced in HT. However the NO bioavailabilty is affected due to scavenging by the superoxide anions. In hypertensive patients there is an exaggerated synthesis of superoxide anions and this is mediated through Angiotensin II. Angiotensin II is a major stimulus for activating the xanthine oxidase system within the EC (97). Once activated the oxidases utilise Nicotinamide adenine dinucleotide (NAD) and Nicotinamide adenine dinucleotide phosphate (NADP) as substrates for electron transfer to molecular oxygen to form superoxides. The superoxides combine with NO to form peroxynitrites thus depleting the NO bioactivity. Peroxynitrite may have a further action by oxidising arachidonic acid and releasing prostanoids which have a potent vasoconstrictor effects. .

1.2.5. Diabetes Mellitus

Elevated serum glucose levels predispose to EC dysfunction. This is due to an imbalance in the NO pathway. There is a glucose induced increase in generation of superoxide anions due to enhanced NAD(P)H oxidase activity (98). Recent studies have also shown that this EC dysfunction may be further amplified by myeloperoxidase (MPO) which is leucocyte derived protein (99). H2O2, the substrate of MPO oxidises chlorides to generate HOCL which reacts with molecules to produce chlorinated L-arginine, chlorotyrosine and modified LDL. The concentration of these molecules in atherosclerotic plaques is elevated in hyperglycaemia and this furthers accentuates the NO deficit in hyperglycaemia (100). The expression of LOX-1 receptor on EC membrane is increased in hyperglycaemia and this mediates the enhanced monocyte and neutrophil adhesion to EC (33).

Another feature of EC dysfunction noted in hyperglycaemia is an increased cellular permeability to macromolecules (101). This effect is mediated through Vascular Endothelial Growth Factor (VEGF), a cytokine synthesised by smooth muscle cells (102).

mRNA transcription for VEGF is mediated by Protein Kinase C (PKC). PKC levels are elevated with high serum glucose levels and this mediates mRNA transcription to synthesise VEGF (103). There are specific EC receptors for VEGF on the EC membrane. VEGF has a potent mitogenic effect on EC and angiogenesis is another characteristic feauture of EC dysfunction in diabetes (104, 105). Activated PKC has an inhibitory effect on eNOS and promotes iNOS gene activity (106).

1.3. Cellular Proliferation and Apoptosis

The risk factors of EC dysfunction can also result in derangement of the proliferative and apoptotic mechanisms of the cellular components of the vessel wall. Cellular proliferation is vital to replace dead cells and for neovascularisation to maintain a confluent monolayer of ECs. Besides this, cellular proliferation is characterised by the interaction of various extracellular and intracellular mediators which determine the fate of the cell. Interference to this highly complex signalling pathway would reflect in the ability of the cells to multiply. In contrast, the apoptotic pathway is critical to maintain a constant cell number among the various cellular components of the vessel wall. A derangement to this pathway can result in denudation of the vascular endothelial monolayer and expose the subendothelial matrix to the cellular blood components.

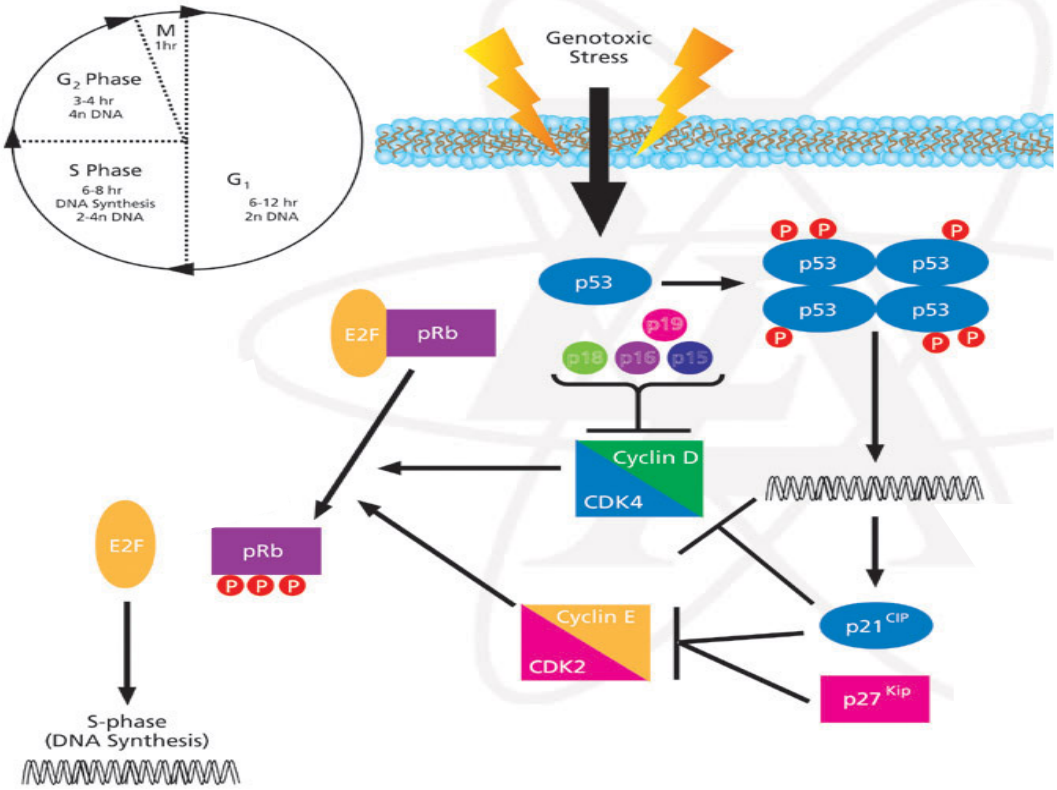
1.3.1. Cellular proliferation

Cell division is a highly regulated event and depends on the presence of multiple regulator molecules through out the cell cycle.

1.3.1.1. Cell Cycle

The eukaryotic cell cycle comprises of four phases, gap (G1), synthesis (S), second gap phase (G2) and mitosis (M). G1 phase is vital in that it determines the fate of the cell. The cell decides to progress through the cell cycle or exits depending on the positive and inhibitory signals in this phase. In mammalian cells, there is a restriction point in G1 phase beyond which the cell is committed to completion of the cell cycle. S phase is the interval for DNA synthesis. The cell prepares itself to divide in G2 and during the M phase, two daughter cells are formed with individual nuclei and intracellular organelles. There is a further phase called G0 where the cells have divided and remain quiescent. These cell cycle phases are interrupted by check points to ensure that the complex process of cell division is completed correctly.

Figure 1. Cell Cycle Regulators



Cyclins regulate the passage of cells through the phases of the cell cycle by interacting with various types of Cyclin Dependent Kinases (CDK) to form complexes. Stress induced DNA damage results in p53 accumulation in the cell which inhibit the cyclin/CDK complex via p21 and p27. Retinoblastoma protein (pRB) when bound to transcription factor E2F is in an inactive form. Phophorylation of pRB releases E2F which is free to initiate DNA synthsis.

(Reproduced with permission from Sigma Aldrich, http://www.sigmaaldrich.com/life-science/cell-biology/learning-center/pathway-slides-and/g1-and-s-phases-of-the-cell-cycle.html)

1.3.1.2. Regulators of the Cell Cycle

The cell cycle is regulated by the close interaction of promoters and inhibitors. The concentrations of these regulators peak at different stages of the cell cycle to promote specific molecular pathways. Thus they act as check points to prevent aberrations in the end product.

Cyclins

Cyclins are proteins which play a pivotal role in the cell cycle and their levels fluctuate through the various stages of the cell cycle (107). Cyclins were first identified in marine invertebrates. In contrast to the number of cyclins seen in lower eukaryotic cells, there are over 16 types of cyclins identified in mammalian cells (A, B1, B2, C, D1, D2, D3, E, F, G1, G2, H, I, K, T1 and T2) . All these cyclins do not have a promoting function on the cell cycle, some serve as catalyst for gene transcription, DNA repair and apoptosis. Cyclin D is the rate limiting factor in the progression of the mammalian cell from the G1 phase. Unlike others, the Cyclin D levels remain relatively static through the cell cycle. Cyclin D synthesis is enhanced by mitogens such as growth factor and withdrawal of this stimulus early in the G1 phase will prompt the cell to exit the cell cycle (108, 109). However once the cell has passed the restriction point, then Cyclin D withdrawal does not affect the progress of the committed cell. Cyclin E activity is maximal in the transition phase between G1 and S and subsequently their levels taper (110). On the other hand Cyclin A is active through the G1-S transition phase and the S phase (111).

CDK/Cyclin complex

Cyclin Dependent Kinase (CDK) are enzymes that can phosphorylate various substrates such as retinoblastoma family of proteins (pRb), p53, $p27^{Kip1}$ and cyclin kinase inhibitors

(CKI). CDK is activated by complexing with specific cyclins and phosphorylation of their threonine residues. There are various combinations of Cyclin /CDK complexes that phophorylate the retinoblastoma family of proteins during the cell cycle. During the early G1 phase cdk4/cyclinD and cdk6/cyclinD complexes are active, cdk2/cyclinA and cdk2/cyclinE through the G1/S phase, cdk2/cyclinA through S phase and cdc2/cyclin A, cdc2/cyclin B through G2 phase (110, 112).

Retinoblastoma family of proteins

The retinoblastoma family of proteins (pRb) are phosphoproteins that are vital for the regulation of the cell cycle. CDK-cyclin enzyme complex has substrate specificity to pRb which it inactivates by phosphorylation. The hypophosporylated pRb is the active form which has a cell cycle inhibitory role. The active pRb binds to a transcription factor which activates various genes in preparation for the S phase. Thus phosphorylation state of pRb determines the course of the cell cycle. pRb is in the active hypophosphorylated form in G0 and early G1 phase. Towards the late G1 phase it is hyperphophorylated and thus inactivated (113, 114).

Cyclin-Dependent Kinase Inhibitors (CKI)

There are two different groups of CKI, the Cip/Kip family and the INK4 family. They act on specific CDK to inhibit their complexing with cyclins. The Cip/Kip family comprises of small protein units that have an inhibitory function by their ability to bind to cyclin complexes and cdk. Absence of these growth regulators have been implicated in tumour cell generation. The INK4 group of proteins prevent the interaction of cyclin D with cdk4 and cdk6 to form complexes and also has an inhibitory effect on preformed complexes of

cdk4/cyclinD and cdk6/cyclinD. The role of these proteins is vital in the cell cycle by its suppressive role in the emergence of tumour cells (115).

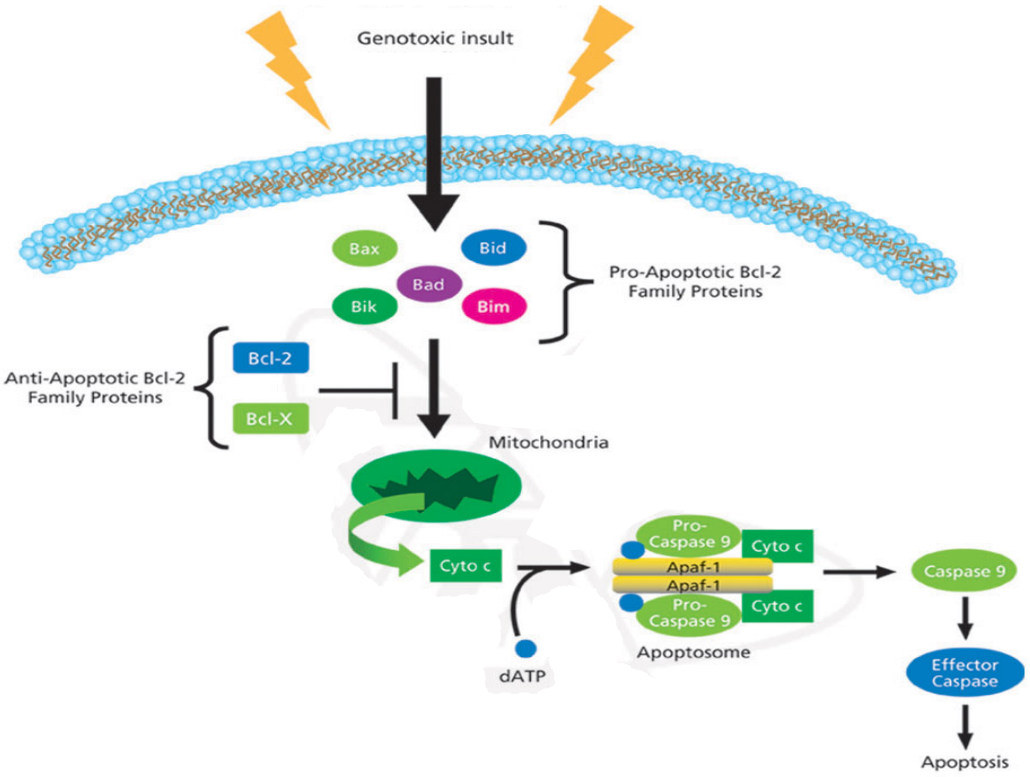
p53 Pathway

p53 is a tumour suppressing protein which can regulate cellular proliferation and apoptosis. It is a phosphoprotein encoded by a gene located on chromosome 17q13 and it is composed of multiple domains which determine the function and interaction of this protein (116). In physiological state it is present in low levels and is bound to its inhibitor, human double minute 2 (HDM2). However in response to DNA damage and other apoptotic stimuli such as hypoxia, metabolic changes and nucleotide depletion, p53 expression is amplified in the nucleus where it activates the transcription factor for genes which mediate cell cycle arrest, DNA repair or apoptosis (117). p53 can arrest the cell cycle at the G1/S phase by inhibiting transcription factors vital for activation of genes required for the transition from G1 to S. p53 can also arrest the cell cycle at the G2/M phase. This transition requires activation of the Cyclin B/CDK1 complex by a kinase. p53 inhibits this activation and can cause arrest of the cell cycle at the G2 phase (118). The other vital function of p53 is DNA repair. This is mediated through various nucleotide excision repair factors and DNA polymerase alpha primase (119, 120). p53 also has an exonuclease activity which is vital in identifying nucleotide mismatches, mediating excision of nucleotides and repair (121).

1.3.2. Cellular apoptosis

The term apoptosis was coined by Wyllie, Kerr and Currie to describe programmed cell death which removes unwanted and damaged cells without recruitment of the immune system (122).

Figure 2. Apoptotic Pathway



The pro-apoptotic proteins Bax, Bad, Bid and Bim can bind with the anti-apoptotic proteins Bcl-2 and Bcl-X. This complex inactivates the cell survival promoting function of Bcl-2 and Bcl-X. The pro-apoptotic proteins cause leakage of mitochondrial cytochrome c (cyto c) which combines with Apaf-1 in the presence of ATP to form an oligomere called Apoptosome. Caspase 9 is activated by this oligmerisation process which activates effector caspases (3, 6 and 7) resulting in cell apoptosis.

(Reproduced with permission from Sigma Aldrich, http://www.sigmaaldrich.com/life-science/cell-biology/learning-center/pathway-slides-and/programmed-cell-death.html)

1.3.2.1 Apoptotic Pathway

There are two separate pathways involved in apoptosis, the death receptor (extrinsic) pathway and the mitochondrial (intrinsic) pathway, which converge downstream to execute the final phase.

The extrinsic pathway is initiated by direct activation of the death receptors by ligands such as TNF. This activation leads to the formation of a protein called death-inducing signaling complex (DISC), comprising of a death receptor with attracts an intracellular adaptor protein called Fas associated death domain (FADD). Activation of the adapter protein by oligomerisation follows and this activates the initiator caspases 8 and 10 (123). These in turn catalyze activation of downstream executioner caspases 3 and 7.

The intrinsic pathway which is dependent on mitochondria is the predominant pathway in eukaryotic cells and is initiated by stimuli such as oxidative stress, hypoxia, irradiation, cytotoxic drugs and DNA damage (124). These stimuli mediate their effect on the mitochondrial membrane permeability which is vital for the continuation of the intrinsic pathway. There are two different mechanisms by which the mitochondrial membrane permeability is relaxed, also known as mitochondrial outer membrane permeabilization (MOMP). In the former pathway, proapoptotic Bcl-$_2$ proteins can stimulate the formation of pores in the mitochondrial membrane (125). This causes the release of many proteins including cytochrome c which is a vital component of the mitochondrial respiratory chain into the cytoplasm. In the second pathway the inner mitochondrial membrane is permeabilized by permeability transition pore (PTP) causing the release of cytochrome c and other proteins. Cytochrome c is vital for further progress of the pathway as it activates Apaf 1 which is an inactivated monomer in the cytosol. This activation requires ATP and

consequently Apaf 1 undergoes conformational change to form an oligomer "apoptosome". This oligomer activates caspase 9 which in turn regulates caspase 3. The death receptor and the mitochondrial pathways converge downstream at the level of caspase 3 which activate effector caspases 6 and 7 (33). The pathway further branches into multiple enzyme mediated cleaving reactions which involve targeting specific substrates from the cell to effect apoptosis. This is characterised by cell shrinkage, chromatin condensation, DNA fragmentation and membrane blebbing (126).

1.3.2.2. Mediators of cellular apoptosis

Cellular apoptosis is regulated by the participation of numerous molecules and there are a few rate limiting steps that determine the fate of the cell.

Bcl2 family of proteins

This family of proteins include both proapoptotic and antiapoptotic members. The proapoptotic subgroup include proteins such as Bax, Bak, Bok, Bad, Bid and the antiapoptotic members are Bcl-$_2$ and Bcl-x_L. They share homologous regions BH1, BH2, BH3 and BH4 (BH means Bcl_2 Homology) which are vital towards their function and interaction with other proteins. Based on the BH domains, the Bcl-2 family is divided into Bcl-$_2$ subfamily which comprises of BH1, BH2 and BH4 domains, Bax subfamily (BH1, BH2 and BH3) and BH3 only subfamily (127). These domains play a vital role in protein interaction and communication. All proapoptotic members have the BH3 domain and any mutational defect to this domain can result in inhibition of the apoptotic pathway (127). The Bcl-$_2$ family has a vital role in regulating mitochondrial permeability. Bcl-$_2$ proteins are mostly attached to the intracellular mitochondrial membrane. Other proapoptotic proteins migrate from the mitochondrial cytosol to incorporate into the mitochondrial

membrane after priming by apoptotic stimuli. This results in the formation or inhibition of pores depending on proapoptotic or antiapoptotic stimuli. As a mediator of MOMP, Bcl-$_2$ promote release of endonucleases and cytochrome c (128, 129).

MOMP

This is known as the point of no return in the mitochondrial apoptotic pathway as it mediates the release of vital proteins from the mitochondrial matrix which determine the fate of the cell. Importantly, release of cytochrome c activates Apaf 1 to form apoptosome which regulates the downstream effector caspases. As described earlier, MOMP is dependent on two separate pathways. In the Bcl-2 dependent pathway, proapoptotic proteins help in pore formation in the mitochondrial membrane. MOMP is dependent on the BH3 domain of the Bcl2 family of proteins and is mediated by Bax and Bak. Other proapoptotic proteins may trigger MOMP by indirectly activating these two proteins. On the other hand the antiapoptotic proteins inhibit MOMP by inactivating the BH3 domain of the proapoptotic members. In the second pathway, the inner mitochondrial membrane is permeabilized by PTP to proteins smaller than 1.5Da. The PTP is a complex spanning the entire width from the inner to outer mitochondrial membrane. An apoptotic stimuli causes Ca2+ influx into the mitochondria which activates the PTP (130). Diffusion of water and ions through the PTP causes swelling of the mitochondrial matrix and rupture of the mitochondrial outer membrane and release of cytochrome c and other proteins such as inhibitors of apoptosis proteins, caspase-2 and caspase 9. Apoptotic stimuli such as oxidative stress, hypoxia, ischaemia reperfusion and calcium overload mediate cytochrome c release through PTP (131).

Inhibitors of Apoptosis Proteins (IAP)

These proteins are important in regulating apoptosis by their effect on activated caspases. There are 8 identified IAP in mammals and they have domains called Baciloviral IAP repeat (BIR) which help in their recognition. The BIR domains are vital to the IAP for recognition and binding to activated caspases and this in turn renders the caspases inactive. There are certain IAP binding proteins within the mitochondria which can inactivate IAP and thereby relieve the caspases of the inhibitory effect from IAP (132).

Heat Shock Proteins (HSP)

HSP are a family of protective proteins expressed by cells in response to stimuli such as heat, hypoxia, ischaemia, free radicals and hypothermia. HSP are named according to their molecular weight. They protect the cell by folding of denatured proteins and clearance of irreversibly denatured proteins. HS vary in their localisation within the cell and the distribution changes in response to stimuli. HSP have a predominantly antiapoptotic activity by inhibiting proteolytic reactions and caspase activity (133, 134). HSP60 complexes with Bax and Bak in the cytosol and apoptosis is inhibited when HSP60 expression is enhanced.

p53 Pathway

p53 plays a vital role in apoptosis. There are certain protein kinases that sense cellular DNA damage and they phosphorylate p53. This activates the DNA binding capacity of p53. In response to DNA damage, p53 stimulates repair of the damage and when the extent of damage reaches a certain threshold, it stimulates apoptosis (135, 136). p53 activates transcription of proapoptotic genes of Bax to effect apoptosis (110, 137, 138).

1.4. Effect of Oxidative Stress and Hypoxia on the Endothelial Cell

1.4.1. Reactive Oxygen Species and Oxidative Stress

Reactive oxygen species (ROS) are intermediates in the oxidation reduction (redox) reaction between oxygen molecules (O2) and water molecule (H2O) and there are a variety of ROS synthesised as a result of various enzymatic reactions. Although ROS is a toxic derivative of O_2 metabolism, contrary to popular belief, it has a vital role in cellular signalling and regulation of gene transcription and protein synthesis (139-141).

The major ROS include superoxide ($O2^{\cdot}$), hydroxyl radical ($HO^{\cdot}$), hydrogen peroxide (H2O2), peroxynitrite ($ONOO^{\cdot}$) and lipid radicals. $O2^{\cdot}$ has an unpaired electron which makes it unstable and highly reactive. It is water soluble and can act as an oxidising agent forming H2O2 or as a reducing agent donating the uncoupled electron to NO to form $ONOO^{\cdot}$ (142, 143). Under physiological conditions the former reaction is favoured although in the presence of excess superoxide, there is a large amount of $ONOO^{\cdot}$ generated (142). $ONOO^{-\cdot}$ is a potent cytotoxic agent which oxidises arachidonic acid to form isoprostanes which have potent vasoconstrictor effects. H2O2 is not a free radical and is relatively stable with a longer half life. It is soluble in lipids and can hence cross cell membranes. In the presence of metal containing molecules such as Fe^{2+} it can generate $HO^{\cdot}$ which is a highly reactive radical (143). $HO^{\cdot}$ can produce localised damage at the site of synthesis.

The primary sources of ROS are the oxidase system and NOS. The various oxidase systems within the mammalian cells are named according to their substrate specificity such as NAD(P)H oxidase and xanthine oxidase. NAD(P)H oxidase system is the main source of

ROS synthesis in neutrophils, vascular EC and smooth muscle cells (144-146). It is an enzyme that comprises multiple subunits which bind to flavin adenine nucleotide (FAD). This complex with FAD forms cytochrome which is vital for the mitochondrial electron transfer chain. They transfer electron from substrate NADPH to FAD, then to heme and finally to molecular oxygen to form superoxide. On activation by stimuli such as growth factors, hormones, metabolic and mechanical factors, the enzyme subunits are phosphorylated and transported to the cell membrane to form active oxidase (147, 148). The NAD(P)H oxidase system is a key target for Angiotensin II which up regulates expression of the oxidase subunit. Excessive Angiotensin II stimulus enhances the NAD(P)H oxidase activity and increases the generation of superoxide (147). It has been implicated in the endothelial dysfunction as a consequence of hypertension. In hyperglycaemia, accelerated metabolism of glucose through glycolysis and the tricarboxylic acid cycle results in mitochondrial synthesis of excessive ROS (149). This mobilizes protein kinase c which activates NAD(P)H oxidase and furthers the generation of ROS (150).

Xanthine oxidase is a flavoprotein that oxidizes hypoxanthine to xanthine and then to uric acid. In this reaction, molecular O_2 which acts as an oxidant is reduced to H_2O_2 which subsequently disassociates to H_2O and O_2. In aortic smooth muscle cells xanthine oxidase produces a significant increase in ROS generation (151).

NOS which is the source of NO synthesis can also generate ROS under circumstances of substrate (arginine) or cofactor (tetrahydrobiopterin) deficiency (152). High levels of ROS can cause oxidative inactivation of tetrahydrobiopterin and the electron flow from NOS reductase domain to oxygenase domain is diverted to molecular oxygen rather than L-

arginine. This phenomenon known as uncoupling of NOS results in exaggerated synthesis of superoxide and is observed in atherosclerosis, diabetes and hypertension (153-155). Overproduction of ROS combined with NO can result in peroxynitrite generation which is cytotoxic. It oxidizes proteins and lipids and thereby interferes with vital cellular metabolic and signalling pathways (156).

There are various anti-oxidant mechanisms in place which help regulate the activity of ROS within the cellular environment. In pathological conditions, an excessive generation of reactive oxygen species (ROS) within the tissues overwhelms the neutralising effects of the antioxidant mechanisms, resulting in oxidative stress (157, 158). ROS reacts with NO at a rate of 6.7×10^9 M^{-1} sec^{-1} which is quicker than the antioxidant mechanisms and this preferential scavenging for NO, nullifies NO bioactivity within the tissues (146, 159).

Oxidative stress is implicated in the development of and progression of endothelial dysfunction and atherosclerosis. High ROS concentration can cause DNA single strand breakage which can activate poly(ADP-ribose) polymerase (160). This reduces the intracellular concentration of substrate NAD^+ which slows electron transport chain and ATP formation and predispose to EC dysfunction (161). ROS can induce vascular remodelling, cellular proliferation and plaque formation. It induces synthesis of VCAM-1 and ICAM-1 which promote leukocyte endothelial interactions, IL-6 which is a pro-inflammatory cytokine, LOX-1 an EC receptor for oxidized LDL and Id3 a protein involved in proliferation of vascular smooth muscle cells (162-166). Oxidative stress is a potent inducer of cellular apoptosis. The key target for ROS is the mitochondria, where it effects lipid peroxidation, protein oxidation and DNA mutations. This can impede the respiratory chain enzymes and ATP synthesis. Excessive presence of ROS can activate

PTP by modulating the redox sensitive sites in the mitochondrial channel to promote the release of mitochondrial cytochrome c to activate the caspase pathway.

1.4.2. Hypoxic Stress

A reduction in the tissue oxygen levels can derange the cellular metabolic and signalling pathways. This state of increased cellular stress is associated with depletion of high energy metabolites, accumulation of lactate and intracellular Ca^{2+} (167). There is also a coincident increase in the generation of free radicals in hypoxia through the cycloxygenase, lipoxygenase, lipid peroxidation and NOS pathways (168-170). This can result in cellular dysfunction due to reduced bioavailability of the NO which is scavenged by the ROS. In human umbilical vein endothelial cells hypoxia can enhance the synthesis of the potent vasoconstrictor endothelin which has a prothrombotic and mitogenic influence (171). Hypoxic stress can also induce apoptosis and, Bcl2 and Bax proteins play a crucial role in determining the fate of the cell. Hypoxia induces phosphorylation of Bcl_2 and Bax proteins in animal models. This can alter the function of Bcl2 and render it less anti-apoptotic (172). Bcl_2 is unable to regulate the intranuclear $Ca2^+$ concentration once phosphorylated and this may permit accelerated $Ca2^+$ influx and opening up of the PTP to promote apoptosis (173). Other mechanisms that contribute to the effects seen in hypoxia include HSP mediated responses. HSP60 and the proapoptotic protein Bax are complexed in the cytosol under normal physiological states. However with a hypoxic stimulus this complex is split and the HSP60 translocates to the membrane. The freed up Bax in the cytosol is oligomerized which subsequently mediates activation of the PTP (174, 175). This induces mitochondrial release of cytochrome c which activates the effector caspases. The p53 pathway also contributes to hypoxia induced cellular apoptosis. Hypoxia triggers the accumulation of

p53 protein within the nucleus which acts as a transcription factor for the gene expression vital for cell cycle arrest or apoptosis (117).

1.4.3. Potential Modulators of Oxidative Stress and Hypoxia

There are various agents that can regulate or inhibit the enzymatic reactions that generate oxidative and hypoxic stress and thus these agents may also have a regulatory effect on the downstream effects of these metabolic pathways. By their regulatory role in abrogating these stress effects, they may also qualify as drugs for specific diseases related to the impaired metabolic processes. The effects of both oxidative and hypoxic stress are mediated through the excessive generation of ROS and its effect on the mitochondrial metabolism. Although there are numerous agents with identified roles in interrupting the pathways to ROS generation, there are only a handful of agents in clinical use with desired effect on oxidative and hypoxic stress.

NO synthesis

An enhanced generation of NO can improve its tissue bioavailability and abrogate the inhibitory effects of ROS. NO is synthesized by three isomeric forms of NOS; eNOS, iNOS and nNOS which are activated by intracellular Ca2+ mediated by stimuli such as shear stress, bradykinin and acetylcholine. Increased availability of L-Arginine, the substrate for NO synthesis, enhances the NO generation. L-Arginine supplementation has shown to improve the flow mediated vasodilatation in peripheral arterial disease (176) and also improve cardiac ischaemic symptoms (177, 178). Nitro-glycerine and sodium nitroprusside which are used in clinical settings for treatment of hypertension and congestive cardiac failure have a potent vasodilator effect by donating NO.

Antioxidant systems

There are numerous cellular mechanisms involved in the breakdown of highly toxic ROS to water and O_2. Superoxide dismutase (SOD), glutathione peroxide (GPXs) and catalase (CAT) are the vital enzymes in this antioxidant system. SOD are of three isomeric forms, mitochondrial manganese SOD (MnSOD), cytosolic copper -zinc SOD (CuZnSOD) and extracellular SOD (ECSOD). SOD are the only enzymes that catalyze the degradation of $\cdot O_2^-$ to H_2O_2 (179, 180). H_2O_2 is further degraded by GPXs and CAT to H_2O (181). Under normal physiological state, the rate of production of ROS is balanced by the rate of degradation by the various antioxidant systems. However any disruption here, from excessive generation of ROS or ineffective antioxidant mechanisms can result in a state of oxidative stress.

Hypoxic Preconditioning

Exposure to hypoxia may initiate the synthesis of various proteins which may have a protective effect and impart a survival benefit to the cell from future exposure to the stimuli. Hypoxia inducible factor 1alpha (HIF-1α) protein which is a transcription product of the HIF-1α gene plays a vital role in this hypoxic preconditioning. HIF-1α is found in very low basal levels during normoxia and is bound to HSP90 (182). In the presence of oxygen, an enzyme called prolyl hydroxylase hyroxylates HIF-1α followed by acetylation of the molecule. It is then bound to von Hippel Lindau protein. This complex molecule is acted upon by enzyme ubiquitin ligase and subsequently degraded by proteasome (183). This mechanism maintains the low basal levels of HIF-1α during normoxia. Normoxia also prevents HIF-1α from activating the target genes by hydroxylation changes mediated via enzyme asparginyl hydroxylase. This induces conformational changes to the HIF-1α

protein, preventing complex formation which is critical to stimulate target genes (184, 185). In a hypoxic environment, HIF-1α is activated by kinase and it then undergoes dimerisation (186-188). This activates various genes regulating vasomotor control, angiogenesis, erythropoiesis, iron metabolism, cell cycle and apoptosis (183). HIF-1α thus prepares the cell to survive hypoxic stress by inducing cellular adaptations.

Bcl2 family

This family of proteins comprise of both antiapoptotic and proapoptotic proteins. Over expression of these subgroups of proteins can result in inhibition or activation of the apoptotic pathway. This creates opportunities to control the mechanisms of apoptosis through molecules that mimic BH domains which are vital for the function and interaction of the Bcl2 family of proteins. There are compounds such as antimycin which activate Bcl2 protein via the BH3 domain and activate the apoptosis pathway (189). Another vital role for these molecules with regulatory effects on the apoptosis pathway is in the development of drugs against tumour cells.

MOMP

It is a vital step in the apoptotic pathway which determines the fate of the cell. High levels of ROS can result in raised intracellular Ca2+ and this can increase the PTP activity which triggers caspase activation through the release of cytochrome c. MOMP is a rate limiting step in this pathway and hence a target for manipulation by synthetic molecules to regulate apoptosis. Current research into MOMP regulation is focused primarily on compounds with antineoplastic activity.

p53

This protein is vital for its regulatory role in cell cycle progression, cell cycle arrest and apoptosis. Human double minute 2 (HDM2) molecule is vital in regulating p53 activity. Binding to HDM2 renders p53 inactive and it presents p53 for degradation by the proteasome enzymes. Activation of p53 is dependent on various proteins including Abelson Murine leukaemia cellular oncogene (cAbl), ADP-ribosylation factor (ARF) and hypoxia inducible factor 1 alpha (HIF-1) (190, 191). ARF specifically binds to HDM2 and promotes its degradation. Regulating the p53-HDM2 interaction is an area of great promise in clinically manipulating the vital cell cycle and apoptosis pathway.

1.5. 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitors

3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitors or **s**tatins inhibit the mevalonate pathway for cholesterol synthesis and for this reason they have been utilized in the clinical setting of high serum cholesterol. Although statins have been in use since the early 1990's for their lipid lowering effect, some of the noticed benefits in cardiovascular morbidity and mortality were not completely explained solely by their lipid lowering effect. Numerous randomised trials have since concluded that statins have beneficial effects independent of their lipid lowering effect and these are now recognised as "pleotropic effects"(192-197).

1.5.1. Structure and types of statins

Statins share the 3-hydroxy 3-methylglutaryl (HMG) like moiety in their chemical structure. All statins are administered in their active form apart from simvastatin and

lovastatin which remain in an inactive lactone form and are hydrolyzed by enzymatic reaction in vivo to the active drug (198). Covalently attached to the HMG moiety are hydrophobic molecules which give rise to different types of statins. Based on this hydrophobic molecule, statins are categorized into two types. Type 1, which comprise of Lovastatin, Pravastatin and Simvastatin, have a hydrophobic molecule covalently bound to the HMG moiety. Type 2 includes Atorvastatin, Cerivastatin, Fluvastatin and Rosuvastatin which are wholly synthetic with a larger hydrophobic molecule covalently bound to the HMG moiety. Another distinguishing feature of the Type 2 compounds is the presence of a flourophenyl group within the hydrophobic molecule.

1.5.2. Mechanism of action

Statins block the mevalonate pathway of cholesterol synthesis by competitively inhibiting the enzyme 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMGR). This rate limiting step is the deacylation of HMG CoenzymeA to reduced coenzyme A and mevalonate which is catalyzed by HMGCoA reductase.

The reaction is described as follows.

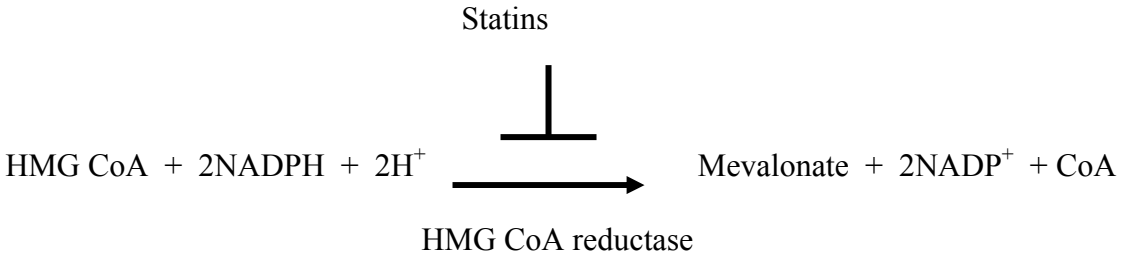
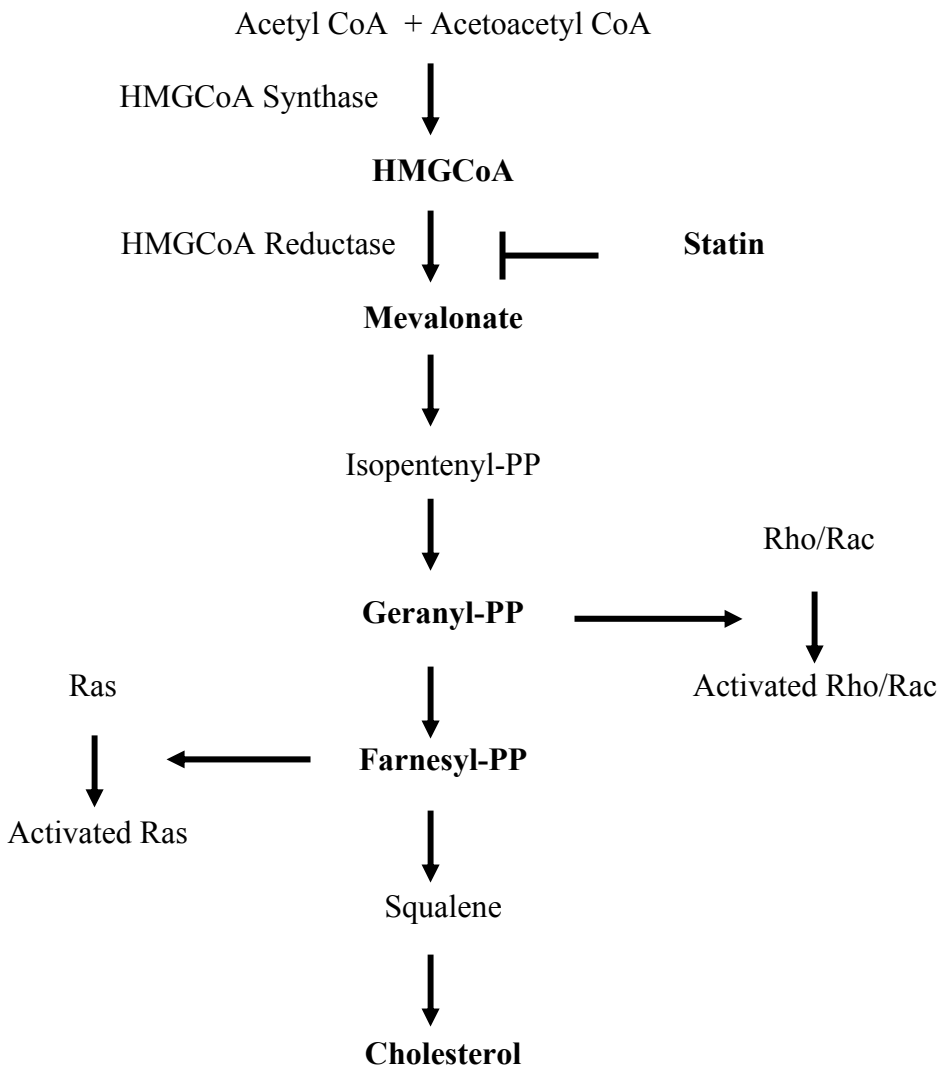


Figure.3. The rate limiting step in the mevalonate/cholesterol synthesis pathway.

The competitive inhibition by statin occurs by binding of its HMG moiety to the corresponding active site in HMGR designed for recognition of HMGCoA. Although the

statin molecule is bulky and rigid, the HMGR binding site is relatively flexible to accommodate the statin molecule. The tight binding between the HMGR and the inhibitor is facilitated by van der Waals forces and this blocks the contact between HMGR and HMGCoA (199). The liver is the principal organ of action for all statins. Inhibition of cholesterol synthesis enhances LDL receptor presentation in liver. This promotes clearance of LDL and other derivatives from circulation. Statins also inhibit hepatic LDL and apolipoprotein B-100 synthesis and enhance the synthesis of HDL which has antiatherogenic properties. The other intermediates of the mevalonate pathway are geranyl pyrophosphate and farnesyl pyrophosphate which are also inhibited by statins. They transform a variety of proteins such as Rho, Rac and Ras to a lipophilic state which is vital for interaction of these proteins with the cell membrane. The Rho protein is activated by attachment to geranylgeranyl pyrophosphate and Ras by farnesylpyrophosphate and this reaction is generically termed as geranylation and farnesylation respectively. This promotes translocation of the proteins from the cytosol to the cell membrane. Statins, by blocking the synthesis of isoprenoids, inhibit this translocation and also the metabolic effects mediated by the activated proteins. The pleiotropic manifestations of statins are due to the downstream effects of inhibition of the critical pathways regulated by these proteins.

Figure.4. The mevalonate cholesterol synthesis pathway



Statins competitively inhibit the conversion of HMGCoA to mevalonate by HMGCoA reductase. The end product of the pathway is cholesterol synthesis. Intermediate products in the pathway are vital to the synthesis of intra cellular regulatory proteins such as Ras, Rho and Rac.

1.5.3. Metabolism of Statins

Statins are extensively metabolized in the liver by a super family of enzymes to the active form via multiple intermediary forms. These enzymes, known as cytochrome P450's (CYP) are vital to the overall metabolic function of the liver. CYP super family is further divided into families (eg. CYP1, CYP2) based on 40% homology of their amino acid chains and subdivided into subfamilies with 55% homology of their amino acid chain. CYP2C, CYP2D and CYP3A are the most clinically relevant enzymes of the 26 known mammalian subfamilies (200). Lovastatin, Simvastain, Cerivastatin and Atorvastatin are substrates of CYP3A4 (201, 202) and fluvastatin is metabolized by CYP2C9 (203).

1.5.4. Side effects

Although statins are relatively safe, there is one uncommon but important side effect related to intake of this drug. Skeletal muscle abnormalities which have been defined as statin myopathy (any muscle complaints related to statins), myalgia (any muscle complaint without elevated serum creatinine kinase), myositis (muscle symptoms with CK elevations) and rhabdomyolysis (CK elevated 10 times more than the upper limit of normal with elevated creatinine levels from myoglobin related nephropathy) (204). Although the incidence of the less serious adverse effects from large trials have shown no significant difference in comparison with placebo group, there is a small but significant incidence of fatal rhabdomyolysis which is estimated at 0.15 deaths per 1 million prescriptions. This death rate from cerivastatin was 16 to 80 times greater than that of other statins (205). Certain drug interactions contribute to the risk of rhabdomyolysis. Drugs that inhibit specific hepatic CYP enzymes may increase the serum concentrations of statins and risk of

rhabdomyolysis. Cerivastatin was withdrawn from the market in 2001 for 31 cases of fatal rhabdomyolysis as a result of drug interaction with lipid lowering drug gemfibrozil (206). The mechanism by which high serum concentration of statin causes skeletal muscle manifestations is not fully understood. It may be manifest by the inhibitory effect of statins on the regulatory proteins Ras, Rho and Rac which promote cell growth and inhibit apoptosis (207-209). This effect on the skeletal muscle cell is reversed by supplementing mevalonate, farnesyl pyrophosphate and geranylgeranyl pyrophosphate (210).

1.5.5. Pleiotropic effects of Statins

Endothelial Function

Statins have a vital role in reversing endothelial dysfunction and this is mediated through multiple pathways. Statins enhance the bioavailability of NO by up regulating NO synthesis. Statins prolong the half life of eNOS mRNA by blocking the activity of Rho proteins (211). Noxious stimuli such as hypoxia, cytokines and oxidized LDL reduce the half life of eNOS mRNA and here statins have a vital role in counteracting the effects of these stimuli. Statins have an alternate mechanism to activate eNOS through protein kinase Akt. This kinase is activated by various growth factors (VEGF, angiopoetin-1, fibroblast growth factor) and shear stress (212). Statins activate the kinase via phosphoinositol-3-kinase with resultant enhanced NO generation (213). One further mechanism by which statins enhance NO synthesis is by inhibiting caveolins. These are small plasma membrane pockets which play a vital role in cellular signaling and they bind to eNOS and inhibit them. Statins inhibit caveolin and free eNOS for NO generation (214).

Statins attenuate ROS generation and thus reduces the cellular oxidative stress. Reduction of serum lipid levels in itself reduces the oxidized LDL concentration which is a potent

source of ROS. However a more important mechanism by which statins reduce the cellular oxidative state is by influencing the NAD(P)H oxidase system. The NAD(P)H oxidase is the most potent source of ROS generation in the vascular endothelium. Angiotensin II which is elevated in hypertension activates Angiotensin1 (AT1) receptor and induces over expression of cytosolic protein subunits of NAD(P)H oxidase. Statin attenuate the expression of AT1 receptor and thus NO generation (215). Isoprenylation of the Rac protein is essential for translocation of these cytosolic proteins to the membrane and their assembly to the NAD(P)H oxidase system. Statins, by blocking the isoprenylation of these Rac proteins and other NAD(P)H subunit proteins, block $\cdot O2^{+}$ generation (216, 217). In addition statins also enhance the activity of the ROS scavenger, catalase. A net increase in the NO synthesis with a concurrent reduction in the ROS generation improves the NO bioactivity and promotes reversal of the endothelial dysfunction. Statins also inhibit the expression of endothelin which is a potent vasoconstrictor and mitogen.

Anti-thrombotic activity

Tissue factor is a glycoprotein which binds to factor VII/VIIa and activates the coagulation pathway. Tissue factor expression is normally restricted to the adventitial layer and to a lesser extent in the media. However this expression is more widespread in atherosclerotic lesions with an increased predisposition to thrombogenesis. Thrombin from the coagulation cascade is a potent inducer of tissue factor synthesis. In animal studies, statin therapy reduces the expression of tissue factors in response to thrombin. This effect is mediated by statin via inhibition of Rho kinase and activation of Akt (218).

Platelet activation and aggregation is vital to thrombus formation. Platelets are activated by an increase in thromboxane A2 activity and a rise in cytosolic Ca^{2+} levels. Statins inhibit

this activation by a negative effect on the thromboxane pathway (219). This in turn inhibits platelet aggregation and thrombus formation.

Atherosclerotic plaque rupture is the cause for acute ischemic effects in vital organs such as the heart. The plaques have a fibrin cap which is prone to rupture due to the activity of matrix metalloproteinases (MMP) expressed from macrophages. Statins contribute to plaque stability by inhibiting macrophage activity and the synthesis of MMP 9 (220).

Statins can also influence the fibrinolytic system. It enhances the activity of plasminogen activator and inhibits plasminogen activator inhibitor and thus interupts the progression of atherosclerotic plaques (221).

Anti-inflammatory effects

Statins reduce the leucocyte/EC interaction which is one of the earliest steps in the cascade of events leading to atherosclerosis. It attenuates the expression of ICAM-1, VCAM-1 and cytokines IL 6/ 8 which are critical to the recruitment of inflammatory cells to the EC (222, 223). Statins directly bind to a regulatory site on β2 integrin called leucocyte function antigen-1 (LFA-1) which is a counter receptor for ICAM-1 on leucocytes (224). This further reduces the EC/leucocyte interaction.

C-Reactive protein (CRP) is an acute phase reactant synthesized by the liver in response to proinflammatory cytokines. It aggravates the atheroscelorotic process by binding to LDL within atherosclerotic lesions and attracting complements to the site. It promotes endothelial dysfunction by inhibiting eNOS activity. Clinical trials have demonstrated a significant reduction in CRP levels in response to statin therapy at the end of one year and over a 5 year period (225, 226). This role of statins may be of significance in reducing the systemic and vascular inflammatory response.

Cell Cycle effects

Vascular smooth muscle cell (VSMC) proliferation results in narrowing of the vessel lumen and decrease in blood flow which is a key feature of atherosclerosis. Statin mediated inhibition of isoprenylation results in reduced DNA synthesis in response to stimulation by Platelet Derived Growth Factor (PDGF) (227). Statin hypophosphorylates pRB (inactive form) and inactivates CDK 4 and 6 which are vital for progression of the cell cycle from G1 to S phase. In addition, it enhances the levels of CDK inhibitor p27.

The activated Rho and Ras proteins are also implicated in promoting cell cycle progression. Rho promotes cellular proliferation by inactivating p27 and Ras acts via Mitogen Activated Protein Kinase (MAPK) (228, 229). Statins block this influence by inhibiting the isoprenylation of these proteins.

In contrast to the effects of statin on VSMC, recent in-vitro studies in EC have demonstrated that statins promote proliferation, migration and cell survival (230). The signaling pathway for this effect is the protein kinase Akt (213). This effect of statins is vital to promote re-endothelialization of denuded vascular wall secondary to injury.

Cellular Signaling Pathway

Statins have influence on multiple cellular signaling pathways and many of the pleiotropic effects of statins are dependent on these pathways. The two main pathways influenced by statins are GTPases and MAPK.

The former comprises of three GTP binding proteins Ras, Rho and Rac. Ras remains in a GDP bound inactive phase and GTP bound active phase. Activation requires farnesylation, and then Ras is translocated from cytosol to the cell membrane. Activated Ras is vital for transduction of growth promoting signals from cell membrane to nucleus. Blocking

farnesylation by statins inhibits SMC proliferaton and migration mediated by Ras (231). Rho activation is by prenylation and this mediates organization of cell cytoskeleton, cell shape, motility and proliferation (232). Geranylation of Rac protein is essential for the activation of NAD(P)H oxidase system (233). Statins block these activities by inhibiting the synthesis of isoprenoids.

MAPK is a serine/threonine family of protein which is essential for translocation of signals from activated proteins such as Ras from the cell membrane to the nucleus. This leads to phosphorylation of various proteins that lead to gene transcription. MAPK promotes cellular proliferation, differentiation and migration by these pathways and causes intimal hyperplasia (234). Statins, by blocking MAPK activation, inhibits this signaling pathway.

Chapter 2. MATERIALS AND METHODS

2.1. Materials

Suppliers of various reagents, kits and equipments are cited in the text. Concentration of the various reagents and the solvents used are described under the appropriate headings. The reagents and kits were stored as per the manufacturer's instructions. Solid chemicals were weighed using an electronic scale with a resolution of 0.001gm. Liquid reagents were measured using Gilson's pipettes for volumes less than 1ml. Disposable plastic pipettes and graduated glass cylinders were used for larger volumes.

2.2. Methods

2.2.1. Cell culture media

The reagents and chemicals used for preparation of the cell culture media were medium 199, endothelial cell growth supplement (50µg/ml), heparin (0.1mg/ml), antibiotic (penicillin 5units/ml, streptomycin 5µg/ml and amphotericin 25ng/ml) and 20% (v/v) fetal calf serum. All the above were supplied by Sigma-Aldrich Company Ltd.UK. The prepared media was stored in sterile glass bottles after filtering through a disposable bacterial filter. . Cell culture media was stored in a refrigerator and used within three days of preparation.

2.2.2. Preparation of Statins

Cerivastatin was kindly donated by Bayer AG, Germany and was dissolved in distilled water to make up a 1millimole/litre solution. It was aliquoted in plastic microtubes and

stored at -70°C. Simvastain was granted by Merck Research Laboratories, USA in the inactive prodrug form. It was activated by dissolving 4mg of the inactive simvastatin prodrug in 100µl of ethanol. Then, 150µl of 0.1 N NaOH was added to the solution. This was incubated at 50°C for 2 h. The pH of the solution was neutralised by 0.1N HCl, and the final concentration of the stock solution was adjusted to give 4 mg/ml (235, 236). Aliquotes of the stock solution were prepared in micro tubes and stored at -70°C.

2.2.3. Cell Culture Technique

Human Umbilical Vein Endothelial Cells (HUVEC) were supplied by Promocell GmbH, Germany. They were pelleted in sterile micro tubes with 1million cells per milliliter (ml) and stored at -70°c in liquid nitrogen bath. For propagation of the cells, the aliquots were thawed in a water bath maintained at 37°c. The cell culture preparations were transferred to an aseptic environment in a laminar air flow chamber. The media was removed from the flask and the cells were washed with 8ml of phosphate buffered solution (PBS), (Sigma-Aldrich Company Ltd. UK) to remove all traces of the media. Subsequently Tryp sin/EDTA (5ml) was added to the flask and cells were incubated for 4 minutes to separate them from the flask surface. To release the cells, the flask was firmly tapped and then 5ml of prepared complete media was added to neutralize the trypsin. After transferring the cell suspension to a sterilin tube, it was centrifuged at 2500rpm for 5 minutes to pellet the cells. The media was then carefully drained without disturbing the pellet and subsequently the cells were resuspended in freshly prepared cell culture media. Total number of cells in the suspension was calculated by mounting 20µl of the suspension on a haemocytometer and counting the cells in the squares. The calculation is described in Fig. 3 and Fig. 4. A 75cc cell culture flask with 15mls of freshly prepared culture media was seeded with 25,000

cells. The cells were incubated in a standard CO2 incubator and were supplemented with fresh media on alternate days. HUVEC's between the second and fourth generation of cells from the initial parent cells were used for the experiments. Cells were harvested when there was a confluent colony formation. The adherent cells were released from the surface, pelleted by centrifuging and resuspended in fresh culture media as described earlier. Cell count was performed with a haemocytometer before seeding the required number of cells on to plates. The experiments were initiated after letting the newly transferred cells stabilize in the incubator for 48 hours.

2.2.4. Characterisation of Human Umbilical Vein Endothelial Cells

Morphologic criteria using microscopy at 40x100 magnifications was used to confirm the growth of HUVEC. This was performed at 24hr intervals during cell growth in culture flasks and in microtitre plates. They have a characteristic cobblestone appearance with individual cells appearing elongated and flattened with a prominent nucleus (237, 238).

Cell Counting using a Haemocytometer

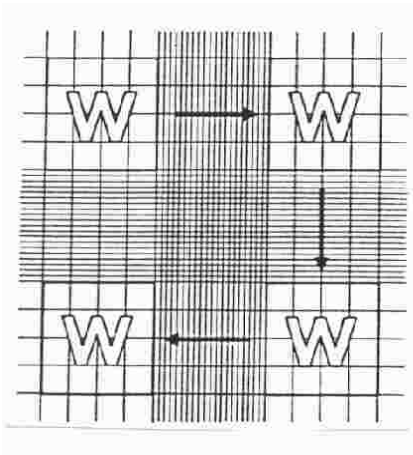


Figure 5

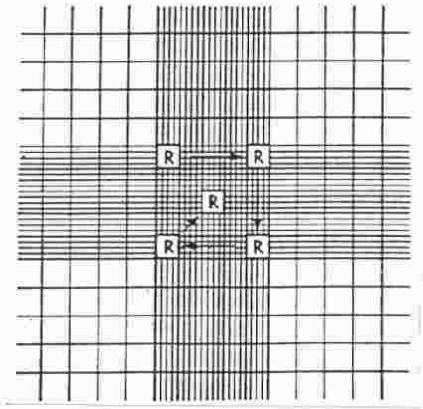


Figure 6

Method 1. Count total number of cells in the 4 outer squares (Fig.5). Cell concentration per ml = total cell count x 2500 x dilution factor. **Method 2**. Count the total number of cells in the 5 squares (Fig.6). Cell concentration per ml = total cell count x 50000 x dilution factor. (Dilution factor is the number of times one aliquot of cell suspension is diluted.)

2.2.5. Exposure to Oxidative Stress

Oxidative stress was induced in HUVEC's by administering Hydrogen Peroxide (H_2O_2) supplied by Sigma Aldrich Co Ltd, UK. H_2O_2 is a ROS produced as an intermediate of the NAD(P)H oxidase system. Oxidative stress has been successfully induced by administering H_2O_2 in cell culture studies (239-241). H_2O_2 provided as a 1 molar solution was freshly diluted with sterile water to achieve a range of final concentrations in the cell culture media.

2.2.6. Exposure to Hypoxia

Hypoxia in a cell culture environment can be induced by hypoxic hypoxia and chemical hypoxia. Cobalt Chloride has been shown to mimic hypoxia at a cellular level (242-244). It is associated with induction of HIF-1 protein synthesis and accumulation of the protein within the cell (245). In this study hypoxia was simulated chemically with Cobalt chloride ($CoCl_2.6H_2O$) supplied by Sigma Aldrich Co Ltd, UK. Crystalline cobalt chloride was diluted with sterile water to achieve a range of final concentrations in the cell culture media.

2.2.7. Live cell counting

A quantitative assessment of cell survival and death was done at the end of the experiments by counting for live cells using a microscope at 40x10 resolutions. A live cell was identified by characteristic cell membrane and nuclear morphology. This includes an intact cell membrane with a definite nuclear margin with no evidence of cell membrane blebbing

or nuclear condensation (246). Total number of live cells from five random microscopic fields was used as an index of the total number of live HUVEC's in a plate.

2.2.8. Cellular Proliferation Assay

CellTiter 96 Aqueous One Solution Cell Proliferation Assay kit (Promega UK) was used for assay of proliferating cells in the plate subsequent to the experiment. It is a quantitative technique that determines the number of viable cells. It contains an active tetrazolium compound [3-(4,5-dimethlthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, innersalt; MTS] and an electron coupling stabilizing reagent (phenazine etholsulphate; PES). The tetrazolium compound is reduced by live cells to a coloured formazan product which is soluble in cell culture media. The co–factors NADPH and NADH in metabolically active cells are responsible for this biochemical reduction to the formazan compound. The quantity of the active compound formazan is directly proportional to the number of live cells in culture (Technical Bulletin No 245 1999, Promega Corporation)

The microtitre plates were seeded with 3 X 10^4 HUVEC per well and allowed to stabilize for 48 hours before the preliminary test. After treatment with the test drugs for the desired duration, the assay was performed. The old culture media was removed and 200 microlitres of fresh media and 40 microlitres of CellTiter 96 Aqueous One Solution reagent were added to each well. The plate was incubated for an hour at 37^0C in a humidified 5% CO_2 and 95% air atmosphere and then 760 microlitres of PBS was added to the wells to make up the volume to 1ml. The cellular proliferation which is a measure of the soluble

formazan produced by reduction of MTS was assayed by a spectrophotometer measuring absorbance at 490nm. A blank with 200 microlitres of plain media, 40 microlitres of CellTiter 96 Aqueous One Solution reagent and 760 µl of PBS was used as the reference.

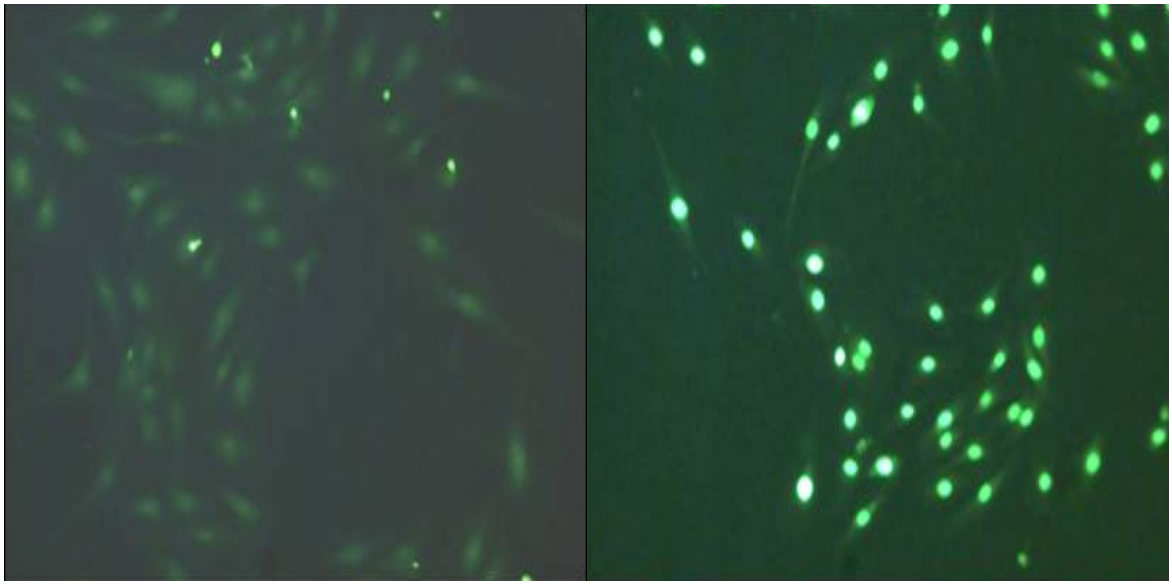
2.2.9. Cellular Apoptosis Assay

DeadEnd™ Fluorometric TUNEL System (Promega UK) kit was used for assay of cellular apoptosis following treatment with the various agents in the experiment. It is a test designed for the detection and quantification of apoptotic cells within a cell colony and it measures nuclear DNA fragmentation which is an important character of apoptotic cells. The DeadEnd™ Fluorometric TUNEL System measures the fragmented DNA of apoptotic cells by catalytically incorporating fluorescein-12-dUridine Tri Phosphate at 3'-OH DNA ends using the enzyme Terminal Deoxynucletidyl Transferase, Recombinant enzyme (rTdT). This enzyme forms a polymeric tail using the principal of the TdT-mediated dUTP Nick-End Labelling (TUNEL) assay (247). The fluorescein -12-dUTP-labeled DNA can be visualized directly by fluorescence microscopy (Technical Bulletin No 235, 2003; Promega.Corporation).

The microtitre plates were seeded with 3 X 10^4 HUVEC per well and allowed to stabilize for 48 hours before the preliminary test. After treatment with the test drugs for the desired duration the assay was performed. Controls were chosen and are discussed in the respective chapters. The plates were washed twice with PBS and following this the HUVEC were fixed by immersing in a 4% methanol free formaldehyde solution in PBS for 25 minutes at 4^{o}C. The plates were washed by immersing in fresh PBS for 5 minutes and this was repeated once again. The HUVEC were permeabilized by immersing the plates in 0.2%

Triton X solution in PBS for 5 minutes followed by rinsing in PBS twice for 5 minutes. Equilibration buffer was added to cover the cells and the HUVEC were equilibrated at room temperature for 10 minutes. Next, 50µl of freshly prepared rTdT incubation buffer is added to the plates. The rTdT incubation buffer is prepared by mixing 45µl of equilibration buffer, 5µl of nucleotide mix and 1µl of rTdT enzyme, in multiples dependent on the number of plates. The plates were incubated at 37^{o}C for 60 minutes for the tail reaction to take place. Care was taken to prevent the plates from drying out and the plates were covered with aluminium foil to protect the light sensitive rTdT incubation buffer. Subsequently the reaction was terminated by immersing the plates in 2X SSC solution (prepared by diluting 20X SSC 1:10 in deionized water) for 15 minutes. The plates were rinsed in PBS for 5 minutes to remove unincorporated fluorescein-12-dUTP. The plates were analyzed under fluorescent microscope using a flourescein filter set to view green fluorescence at 520 ± 20 nm. The total numbers of apoptotic cells were counted from five random microscopic fields at 40 x 10 resolutions.

Figure 7. Apoptotic cells under fluorescent microscope



Flourescein uptake by cells on the left demonstrates fewer apoptotic cells and on the right the uptake is enhanced by a higher number of apoptotic cells

2.2.10. Assay of modulators of cell cycle and apoptotic pathway.

Cyclin D are a family of proteins vital to the progression of the cell cycle and it is the rate limiting factor in the progression of the mammalian cell from the G1 phase. Cyclin D synthesis is enhanced by mitogens such as growth factor and withdrawal of this stimulus early in the G1 phase will prompt the cell to exit the cell cycle. In contrast Bax proteins are a subfamily of the Bcl2 family of apoptotic proteins. They are pro-apototic proteins that play a vital role in mediating apoptosis by enhancing MOMP to promote cytochrome c release and activate the caspase pathway. The expression of Cyclin D and Bax were assayed by Reverse Transcriptase Polymerase Chain Reaction (RT PCR) after HUVEC exposure to the stress stimuli and statins.

2.2.10.1. RNA isolation

GenElute^{TM} Mammalian Total RNA kit (SigmaAldrich, UK) was used to isolate total RNA from HUVEC. HUVEC were lysed and homogenized for denaturation of macromolecules and inactivation of RNAases by using a guanidine thiocyanate containing buffer. RNA binds to a silica membrane in a micro centrifuge tube when the lysate is spun in ethanol. RNA is eluted in the elution solution after washing to remove any contaminants. The purified RNA is then ready for reverse transcription (Technical Bulletin Product Codes RTN10, RTN70 and RTN350, Sigma Aldrich, UK).

All work surfaces were carefully cleaned with RNAase Away to eliminate ribonuclease degradation of RNA. HUVEC subjected to stress stimulus and/or statin in 25cc cell culture flasks were released from the support surface with trypsin. They were pelletted and the culture medium discarded. The HUVEC were loosened in a vortex. A mixture of freshly

prepared lysis solution 250µl and 2-mercaptoethanol (2ME) 2µl was added to the pellet and agitated in a vortex until all clumps disappeared. Next, the lysed cells were pipetted into a GenElute Filtration column (blue insert with a 2ml receiving tube) and centrifuged at 16000 x g for 2 minutes. After discarding the filtration column, 250 µl of 70% ethanol was added to the lysate followed by agitation in the vortex. The solution was pipetted out into a GenElute Binding Column (red o-ring seated in a 2ml receiving tube), centrifuged at maximum speed for 15 seconds and the flow-through liquid discarded. Subsequently 500 µl of wash solution 1 was pippetted into the column, centrifuged for 15 seconds at maximum speed. After discarding the flow through liquid, the column was transferred into a fresh 2ml collection tube. 500µl of diluted wash solution 2 (wash solution 2 diluted with 200 proof ethanol 1:4) was added to the column and centrifuged at maximum speed for 15 seconds. After discarding the flow through liquid, a further 500µl of wash solution 2 was added to the column and centrifuged at maximum speed for 2 minutes. The column was next transferred on to a fresh 2ml tube and 60µl of elution fluid was added to it and centrifuged at maximum speed for 1 minute. The purified RNA collects in the tube and was used immediately or stored at -70^{o}C.

The concentration of the RNA yield was determined by measuring the absorption at 260nm on a spectrophotometer. After initial calibration of the spectrophotometer using 54µl of distilled water as a blank, 6µl of RNA sample was added to the cuvette and mixed thoroughly by pipetting. RNA concentration was measured from the absorption values at 260nm.

RNA concentration (µg/ml) = Absorption (260nm) × 40 × dilution factor

Purity of the RNA sample is determined by the 260:280 ratios and a value >1.3 indicates purity sufficient for subsequent RT-PCR analysis. Lower value samples were unsuitable for the experiment.

2.2.10.2. Reverse transcriptase-polymerase chain reaction (RT-PCR)

The Cyclin D and Bax cDNA sequence were obtained from published sequences on Pubmed and basepair program was used for designing primers. Based on the basepair program, expected DNA fragment sizes produced by these primers were 484 bp for Cyclin D and 195 bp for Bax.

Cyclin D primer sequence

Forward: 5'- GAG ACC ATC CCC CTG ACG GC-3'

Reverse: 5'- TCT TCC TCC TCC TCG GCG GC-3'

Bax primer sequence

Forward: 5'-TGG CAG CTG ACA TGT TTT CTG AC-3'

Reverse: 5'-TCA CCC AAC CAC CCT GGT CTT-3'

β Actin primer sequence

Forward: 5'-CCAGAGCAAGAGAGGCATCC-3'

Reverse: 5'-CTGTGGTGGTGAAGCTGTAG-3'

RNAase free water was used to reconstitute the primers to a final concentration of 100pmol/µl. Ready-to-go RT-PCR beads (SigmaAldrich, UK) containing Taq DNA

polymerase, Maloney Murine Leukemia Virus (M-MuLV) reverse transcriptase, nucleotides and ribonuclease inhibitors were used for RT-PCR. The RNA samples were amplified with Cyclin D, Bax and also β Actin primers which being a house keeping gene, is constitutively expressed by cells and hence used as the reference. A specific volume of each RNA solution (based on calculated RNA concentrations) was added to each RT-PCR tube in triplicates. Subsequently forward and reverse primers for Cyclin D, Bax and β Actin were added to sets of tubes and finally the volume in each tube was made up to 50µl using RNAase free water. The three sets of mRNA were amplified separately using different programs based on the variation in annealing temperatures of the primers.

Table 1. Annealing temperatures and cycle lengths used for RT-PCR analysis of Cyclin D, Bax and β Actin

Primer sets	Annealing temperature (^{o}C)	Number of cycles
Cyclin D	65^{o}C	22
Bax	58^{o}C	28
β Actin	55^{o}C	30

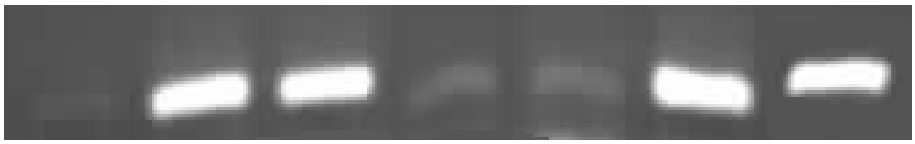
2.2.10.3. Agarose gel electrophoresis for amplified DNA analysis

Gel electrophoresis was used to analyse the amplified DNA fragments produced by RT-PCR to determine the expression of Cyclin D and Bax in the samples. A 2% (w/v) agarose gel was prepared by dissolving 1g of agarose in 50ml of Tris-borate EDTA (TBE) buffer pH 8.3 and heating in a microwave until clear. The gel was allowed to set in an electrophoresis tray at 4^{o}C for one hour and once the gel had set, the combs were removed and the gel was placed in an electrophoresis tank and covered with the TBE buffer. The samples for electrophoresis were prepared with 10µl RT-PCR amplified DNA, 2µl loading dye and 1µl SYBR Green I. A DNA marker was prepared with 5µl PCR ranger DNA marker, 2µl loading dye and 1µl SYBR Green I. The marker and samples were loaded onto separate wells in the gel and electrophoresis carried out at 100V until the bands were 1cm away from the end of the gel. The bands were visualised using the UV transilluminator using the GeneSnap imaging system. The GeneTool imaging programme was used to quantify the intensity of the bands in the sample and this was normalised against the intensities of the β Actin bands.

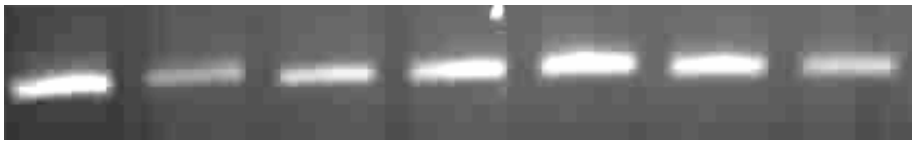
Figure 8. Agarose gel electrophoresis

Bax

Cyclin D



β **Actin**



The bands are visualised under UV illuminator and they represent amplified DNA fragments following RT-PCR. The cellular expression of Bax, Cyclin D and β Actin (reference protein) in response to various experimental stimuli was quantified using GeneTool imaging programme

2.2.11. Statistical Analysis

Statistical analysis was performed using the SPSS v13 software for parametric data. Experimental data was expressed in means ± SEM. A Student's t test was used to compare two groups of data. A value of $p < 0.05$ was considered to indicate statistical significance.

Chapter 3. Effect of Statins on HUVEC

Introduction

Re-endothelialisation is of great significance in the event of denudation of the vascular endothelial cell lining. A stimulus for this mechanism would be of immense therapeutic value in the context of critical ischemia, ischemia reperfusion injury and post interventional damage to the vascular wall.

The antiatherosclerotic effects of statins are partly attributable to its effects on the VSMC. It initiates apoptosis of the VSMC and causes regression of atherosclerotic plaques and also inhibits myointimal hyperplasia. This effect of statins is mediated through its inhibitory effect on the isoprenylation of the GTPase proteins (Rho and Ras) and the MAPK pathway (228, 229). Rho and Ras are cytosolic proteins that require post translational modification for incorporation to the membrane. Ras acts as a transmitter of cell growth signals across the cell membrane for cellular proliferation (231) and Rho activation helps reorganisation of the cellular cytoskeleton, motility and proliferation (232). MAPK helps propagate and amplify the signals from GTPase proteins. Activated MAPK is transported to the cell nucleus where it phosphorylates various gene regulatory proteins to promote gene transcription (248). This is mediated through specific pathways which involve kinases1/2 (ERK1/2), p38 kinase (p38) and c-jun N-terminal kinase (JNK) (234). Statins inhihit intimal hyperplasia by specifically blocking ERK1/2 mediated cellular proliferation, differentiation and migration (231).

Statins enhance EC function by increasing the synthesis of NO, attenuate the ROS generation and reduce the EC-leucocyte interactions. In contrast to its effect on VSMC, a

similar apoptotic effect on EC is not desired. In fact in vitro studies on endothelial progenitor cells (EPC) have shown that statins evoke proliferation, migration and cell survival (230). There is an enhanced synthesis of cell cycle promoter proteins such as cyclins after statin therapy and simultaneous reduction in the levels of cell cycle inhibitors such as p27 (249). In contrast to these effects in EPC, the effect of statins on fully differentiated endothelial cells is not well elucidated.

Aim

The aim of this experiment was to determine the effects of exposure to Simvastatin and Cerivastatin on HUVEC proliferation and apoptosis. Expression of the cell cycle pathway promoter Cyclin D and pro-apoptotic protein Bax in response to HUVEC exposure to various doses of statins was studied.

Materials and Methods

HUVEC between 2^{nd} and 4^{th} generation were seeded onto 48 well plates with 5 $x10^4$ cells per well. Each well accommodates approximately $5x10^4$ cells to form a single layer of cells. They were allowed to stabilize for 48 hours in the incubator followed by supplementation with fresh culture media. Simvastatin and Cerivastatin were added to the plates to achieve a final concentration of 1nmoles/l, 5nmoles/l, 10nmoles/l, 1000nmoles/l and 5000nmoles/litre with three repeats for each concentration and a control well. Live cell counting, cellular proliferation assay using the CellTiter 96 Aqueous One Solution Cell Proliferation Assay kit (Promega UK) and cellular apoptosis assay using the DeadEnd™ Fluorometric TUNEL System kit (Promega UK) were performed at 24 hours after

treatment with the drug. RT-PCR for Bax and Cyclin D assay was performed on HUVEC propagated in 25cc culture flasks following treatment with 5nmol/l and 5000nmol/l of statins at 24 hours, with a control receiving no treatment.

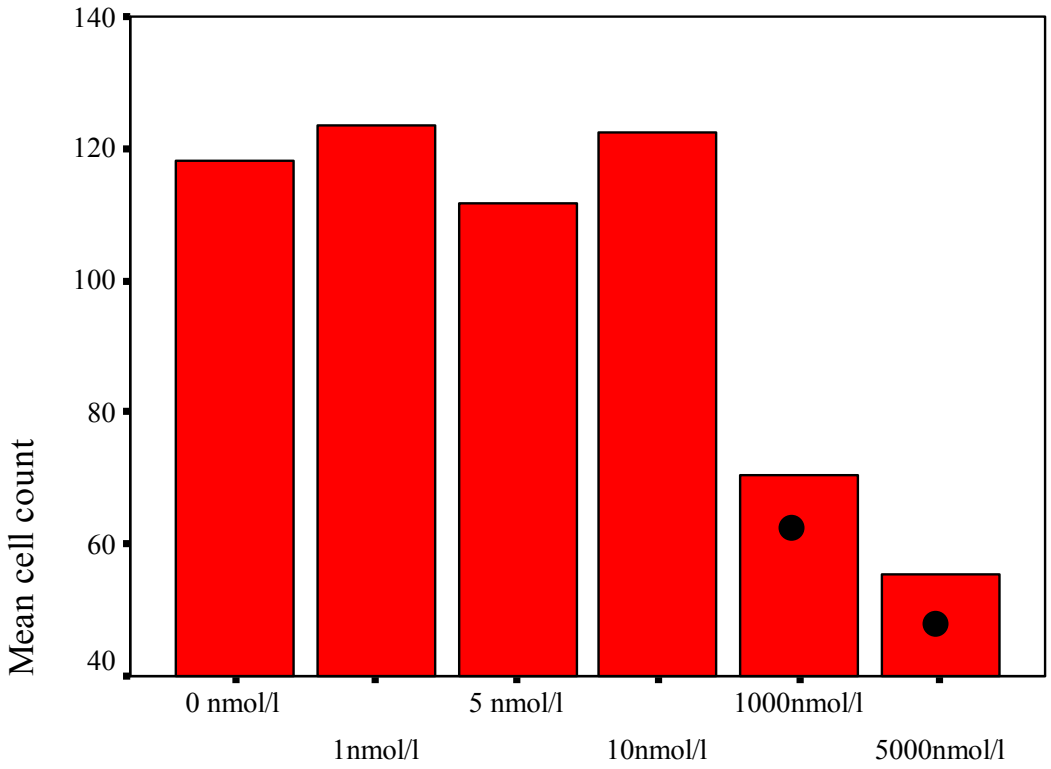
Data was expressed in means ± SEM. The paired samples t test was used to compare the groups. $P < 0.05$ was considered to be statistically significant.

Results

Live cell count

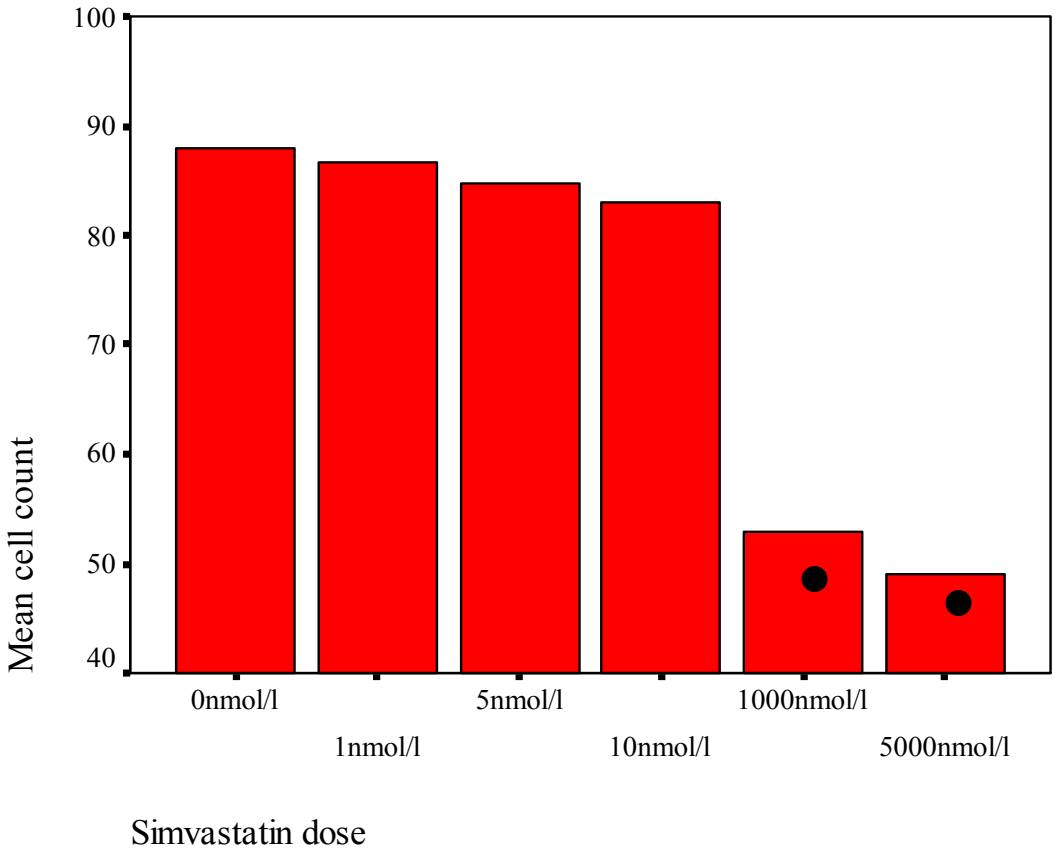
Although in comparison with the control group there was no significant difference in the cell numbers at lower doses of simvastatin and cerivastatin, there was a significant reduction ($p<0.05$) in the total cell count at 24 hours in both statin groups treated with 1000nm/l and 5000nm/l doses (Figure. 9 and Figure. 10).

Figure 9. Cell count 24 hours after treatment with Cerivastatin



[● = p < 0.05, paired t test]

Figure 10. Cell count 24 hours after treatment with Simvastatin



[● = p < 0.05, paired t test]

Cellular proliferation assay

Assay of cellular proliferation at the end of the 24 hour period of treatment showed an increase in the spectrophotometric absorption in both statin groups at lower doses in comparison to the control group. In contrast, the cellular absorption at higher doses was lower than the control group in both statin groups (Table.2).

Apoptosis cell count

There was a significant increase in the number of HUVEC undergoing apoptosis at higher treatment doses in both statin groups although no similar response was noted at the lower dose concentration (Table. 3)

RT PCR

Cyclin D expression

HUVEC expression of cyclin D after treatment with both statins for 24 hours showed an enhanced expression at lower statin doses. This reached significance in the Cerivastatin group at 1nmol/l and in the Simvastatin group, at 5nmol/l (Table. 4)

Bax expression

HUVEC treated with high dose (1000nmol/l) Cerivastatin and Simvastatin had a significant increase ($p<0.05$) in the expression of Bax protein. Low dose treatment did not show any HUVEC response to Bax expression (Table 5).

Table 2. Spectrophotometric absorption 24 hours after statin treatment

	Control	1nmol/l	5nmol/l	10nmol/l	1000nmol/l	5000nmol/l
Cerivastatin	29.9 (1.86)	35.8(1.28)	46.7(2.07)*	46 (1.51)*	23 (4.24)	18 (1.65)*
Simvastatin	78 (1.36)	82 (1.52)	86 (1.17)*	82.9 (1.46)	69 (1.9) *	65 (3) *

[* = $p<0.05$ Student's t- test, values expressed as mean (Standard Error of Mean)]

Table 3. Apoptosis cell count 24 hours after statin treatment

	Control	1nmol/l	5nmol/l	10nmol/l	1000nmol/l	5000nmol/l
Cerivastatin	14.3(1.2)	14(1.15)	13.6(2.18)	14.6(1.6)	38.66(2.02)*	42.6(4.9)*
Simvastatin	15.3(1.45)	18.3(0.8)	18(1.52)	16(1.52)	32.6(2.02)*	37.6(1.45)*

[* = $p<0.05$ Student's t- test, values expressed as mean (Standard Error of Mean)]

Table 4. Cyclin D expression 24 hours after statin treatment

	Control	1nmol/l	5nmol//l	1000nmol/l
Cerivastatin	0.7(0.03)	0.85(0.02)*	0.89(0.03)	0.68(0.02)
Simvastatin	0.7(0.03)	0.75(0.03)	0.91(0.03)*	0.64(0.03)

[* = $p<0.05$ Student's t- test, values expressed as mean (Standard Error of Mean)]

Table 5. Bax expression 24 hours after statin treatment

	Control	1nmol/l	5nmol//l	1000nmol/l
Cerivastatin	1.53(0.26)	1.56(0.25)	1.55(0.23)	3.29(0.29)*
Simvastatin	1.53(0.26)	1.55(0.28)	1.6(0.29)	2.84(0.10)*

[* = $p<0.05$ Student's t- test, values expressed as mean (Standard Error of Mean)]

Conclusion

Higher doses of statins (1000 and 5000 nmol/l) cause cellular apoptosis as demonstrated by a significant reduction in the number of live HUVEC at the end of the 24 hour treatment period. Although there was no increase in cell number noted during the same treatment duration for the lower statin doses (1, 5 and 10 nmol/l), an increased absorption rate from the proliferative assay suggests metabolically active cells in the low dose category. TUNEL assay for apoptosis demonstrated an increased fluorescent uptake by a significantly higher number of HUVEC undergoing apoptosis at high statin doses. Bax expression was enhanced in the high dose group suggesting that it may have a role in inducing apoptosis in HUVEC. In addition, cyclin D expression was elevated in the low dose group suggesting an enhanced cellular proliferative response to statins.

Chapter 4. - Effect of Oxidative Stress on HUVEC

Introduction

The reactive oxygen species (ROS) are highly unstable molecules derived from the metabolism of oxygen molecules. They play a vital role in the intracellular communication and signalling mechanisms in various metabolic pathways including gene transcription and protein synthesis (139, 140). Although it has a vital role in the normal physiology of the EC, it is also involved in the pathophysiological processes of atherogenesis in cardiovascular diseases such as hypercholesterolemia, hypertension, diabetes and ischemia reperfusion injury (250, 251). These risk factors induce accelerated generation of ROS with inability of the antioxidant mechanisms to balance their oxidant effect. This results in "oxidative stress" within the cellular environment.

ROS is generated as a consequence of oxidative phosphorylation in mitochondrial aerobic respiration (252). The oxygen consumed in this reaction is converted to water through a series of reactions requiring 4 electrons. This sequential reaction involving donation of electrons can result in ROS generation at each step (253, 254). Oxygen molecule accepts the first electron to transform to a superoxide molecule ($\cdot O2^{+}$). This subsequently transforms into hydrogen peroxide (H2O2) after second electron transfer. The third electron transfer known as the Fenton reaction requires an iron molecule which converts the superoxide to the highly reactive hydroxyl ion (·OH). Finally the ·OH accepts the fourth electron to transform to water. The antioxidant systems such as NAD(P)H oxidase, uncoupling of eNOS and iNOS are all potent sources of ROS generation (255-257). Surplus ROS reduces the bioactivity of NO and this results in endothelial dysfunction (146,

258). This is characterised by attenuation of the NO mediated vasodilatory effects, enhanced leucocyte endothelial interaction, smooth muscle cell proliferation and platelet aggregation (33, 259-261).

Oxidative stress in itself can also activate apoptosis with resultant effects on the MOMP which triggers the release of cytochrome c. This can activate the effector caspases and result in apoptosis of the cell. The cellular manifestations of oxidative stress can thus range from endothelial dysfunction to apoptosis. These effects can result in an attenuated EC capacity to re-endothelialize in response to cellular injury. There is inadequate information on the mechanism by which ROS affects EC proliferation and apoptosis. This understanding is critical when considering treatment options for the widespread effects of ROS on the EC.

Aim

The aim of this experiment was to determine the effect of varying doses of H_2O_2 on HUVEC survival. The response of the cell cycle pathway regulators, Cyclin D and Bax to oxidative stress was studied.

Materials and Methods

HUVEC between passage 2 and 4 were utilized for this experiment. Cells were seeded onto 48 well plates with 5 $x10^4$ cells per well. They were allowed to stabilize for 48 hours in the incubator followed by supplementation with fresh culture media. For induction of oxidative stress, H_2O_2 was added to the plates to achieve a final concentration of 50µmol/l, 100µmol/l and 200µmol/l with three repeats for each concentration and a control well. Live

cell counting, cellular proliferation assay using the CellTiter 96 Aqueous One Solution Cell Proliferation Assay kit (Promega UK) and cellular apoptosis assay using the DeadEnd^{TM} Fluorometric TUNEL System kit (Promega UK) were performed at the termination of the experiment 24 hours after initiation of drug treatment. RT-PCR for Bax and Cyclin D assay was performed on HUVEC propagated in 25cc culture flasks following treatment with 50 and 100μmol/l of H_2O_2 with a control receiving no drug.

Data was expressed in means ± SEM. The paired samples t test was used to compare the groups. $P < 0.05$ was considered to be statistically significant.

Results

Live cell count

There was a steady decline in the number of live cells with increasing concentration of H_2O_2 in the test group over a 24 hour treatment period in comparison with the control group. This reached statistical significance at 100μmol/l of H_2O_2 (Table 6).

Table 6. Live cell count 24 hours after H_2O_2 treatment

	Control	50µmol/l	100µmol/l	200µmol/l
H_2O_2	109.6(5.7)	103(5.7)	87(2.5)*	59(3.5)*

[* = $p<0.05$ Student's t- test, values expressed as mean (Standard Error of Mean)]

Cellular proliferation assay

Assay for cellular proliferation at the end of the 24 hour treatment period showed a significant reduction in the spectrophotometric absorption with higher dose concentrations of H_2O_2 in comparison to the control (Table 7).

Apoptosis cell count

There was a significant increase in the number of HUVEC undergoing apoptosis at higher treatment doses of H_2O_2 in comparison with the control (Table 8).

RT PCR

For assay of Cyclin D and Bax expression, 50μmol/l and 100 μmol/l of H_2O_2 were used as they exhibited optimal stress response, avoiding low strength treatment doses with inadequate stress response and also overwhelming toxic response from high strength dose.

Cyclin D expression

HUVEC expression of Cyclin D after treatment with H_2O_2 for 24 hours showed a downward trend with increasing dose strength although this did not reach statistical significance (Table 9).

Table 7. Spectrophotometric absorption 24 hours after H_2O_2 treatment

	Control	50µmol/l	100µmol/l	200µmol/l
H_2O_2	64.66(3.28)	61.66(4.05)	53.33(1.85)*	38(2.08)*

[* = $p<0.05$ Student's t- test, values expressed as mean (Standard Error of Mean)]

Table 8. Apoptosis cell count 24 hours after H_2O_2 treatment

	Control	50µmol/l	100µmol/l	200µmol/l
H_2O_2	9.66(0.88)	10.66(0.66)	15.66(0.88)*	29(1.73)*

[* = $p<0.05$ Student's t- test, values expressed as mean (Standard Error of Mean)]

Table 9. Cyclin D expression 24 hours after H_2O_2 treatment

	Control	50µmol/l	100µmol/l	200µmol/l
H_2O_2	0.7(0.17)	0.66(0.01)	0.63(0.02)	_

[* = $p<0.05$ Student's t test, values expressed as mean (Standard Error of Mean)]

Bax expression

HUVEC treated with higher dose of H_2O_2 had an enhanced generation of Bax protein which was statistically significant. Although there was an increase in the Bax expression with lower treatment doses in comparison with the control group, this was not statistically significant (Table 10).

Table 10. Bax expression 24 hours after H_2O_2 treatment

	Control	50µmol/l	100µmol/l
H_2O_2	1.51(0.03)	1.59(0.05)	2.03(0.03)*

[* = $p<0.05$ Student's t test, values expressed as mean (Standard Error of Mean)]

Conclusion

The survival of HUVEC declined with higher dose strengths of oxidative stress in the form of H_2O_2. There was a reduction in the total number of live cells at 100/200μmol/l of H_2O_2. In addition, HUVEC subjected to these dose strengths had a significant reduction in the spectrophotometric absorption to the cellular proliferation assay, suggesting a reduced proliferative activity of these cells. TUNEL assay for cellular apoptosis confirms the above observations. Higher doses of H_2O_2 induced HUVEC to undergo apoptosis with an increase in fluorescent uptake. The number of HUVEC undergoing apoptosis was significantly higher than the control group. The expression of the proapoptotic Bax proteins by HUVEC was significantly enhanced by H_2O_2, suggesting that the apoptosis pathway is mediated by Bax proteins in response to H_2O_2. However the expression of Cyclin D, the cell cycle pathway promoter, was not significantly affected by oxidative stress in HUVEC.

Chapter 5. Effect of Statins on Cellular Proliferation and Apoptosis modulated by Oxidative Stress

Introduction

Oxidative stress, as described earlier, is pivotal to the pathogenesis of atherosclerosis. An excessive generation of ROS in the absence of an efficient antioxidant mechanism results in endothelial dysfunction which is characterised by attenuated vasodilatory effects, enhanced leucocyte endothelial interaction, VSMC proliferation and platelet aggregation. In addition to this effect, oxidative stress is also a potent inducer of apoptosis, which is mediated via the mitochondria which activates the caspase pathway. In fact, atherosclerosis is characterised by an increased incidence of apoptosis of EC along areas prone to plaque formation. (262).

Statins have a wide ranging effect on the EC which help enhance endothelial function. It has a potent antioxidant activity derived from increasing the synthesis of NO and the antioxidant mechanisms. It promotes VSMC apoptosis by inhibiting the isoprenylation of GTPase proteins Ras and Rho, and the MAPK pathway which are vital to transmission of growth signals to the cellular nucleus in order to promote gene activation and protein synthesis for cellular proliferation. However the effects of statins on the EC may be different to this apoptotic effect on the VSMC. In vitro studies on endothelial progenitor cells have demonstrated proliferative response and an increased cell survival as a result of statin treatment. This may be mediated through the cell cycle promoter cyclin D. The experiment in Chapter 3 did reveal an enhanced synthesis of cyclin D in response to statin treatment in HUVEC. The hypothesis to this experiment was that statins offer protection

against oxidative stress induced apoptosis in HUVEC. Secondly it may reverse the anti-proliferative effects of oxidative stress and promote cellular proliferation.

Aim

The aim of this experiment was to determine whether statins offer any protection against oxidative stress induced antiproliferative effects and apoptosis in HUVECs. Secondly, the effect of statins on the cell cycle promoter cyclin D and the proapoptotic protein Bax were studied in the context of oxidative stress.

Materials and Methods

HUVEC were prepared as described earlier, in chapter 3. After allowing the cells to stabilise for 48 hours they were treated with Cerivastatin and Simvastatin to obtain a final concentration of 5nmol/l. Oxidative stress was induced in these HUVEC with H_2O_2 100μmole/l, 24 hours after treatment with statin. The experiments were performed in triplicates with a control group receiving treatment with H_2O_2 alone. Live cell counting, cellular proliferation assay using the CellTiter 96 Aqueous One Solution Cell Proliferation Assay kit (Promega UK) and cellular apoptosis assay using the DeadEnd^{TM} Fluorometric TUNEL System kit (Promega UK) were performed at termination of the experiment, 24 hours after treatment with H_2O_2. RT-PCR was performed for Bax and Cyclin D assay on HUVEC propagated in 25cc culture flasks and treated as described above. The negative control was treated with H_2O_2 and the positive control received activated mevalonate 10μmol/l (downstream metabolite of the mevalonate pathway) in addition to statin and H_2O_2.

Data was expressed in means ± SEM. The paired samples t test was used to compare the groups. $P < 0.05$ was considered to be statistically significant.

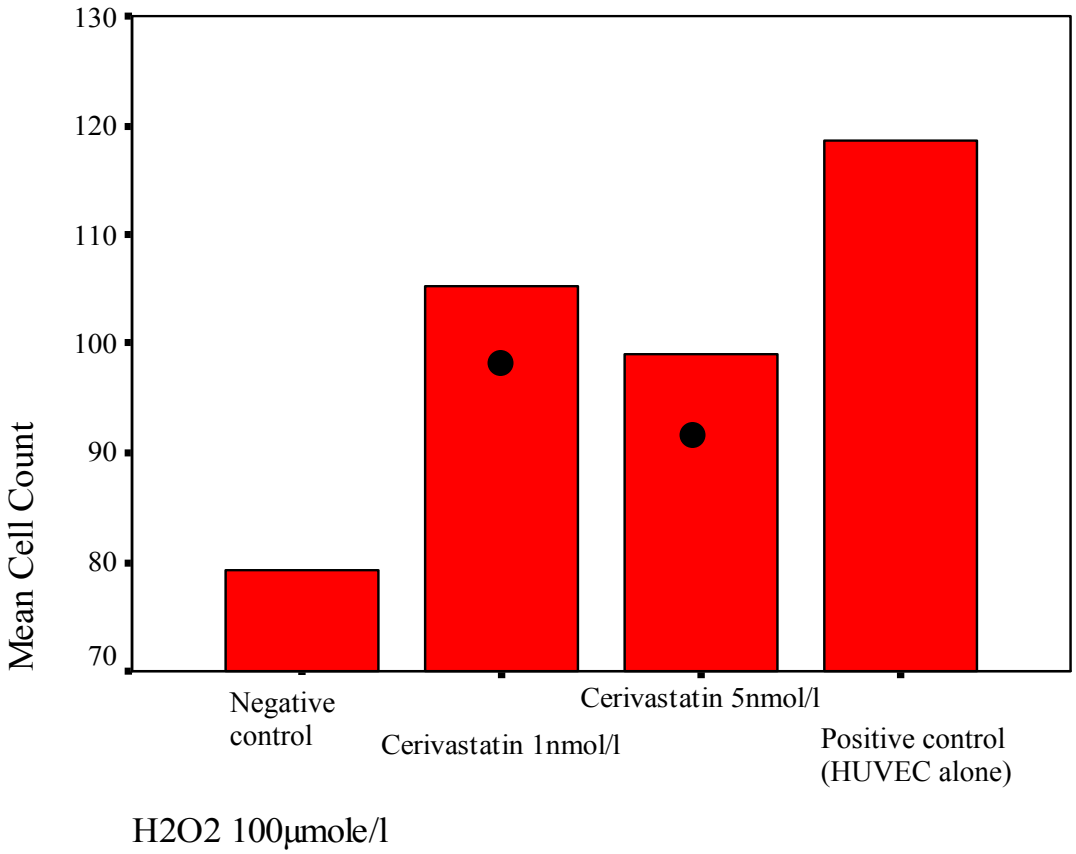
Results

Live cell count

There was a significantly higher number of live cells when HUVEC were pre-treated with Cerivastatin before induction of oxidative stress. This cell survival benefit was enhanced at the lower Cerivastatin dose of 1nmol/l (Figure 11).

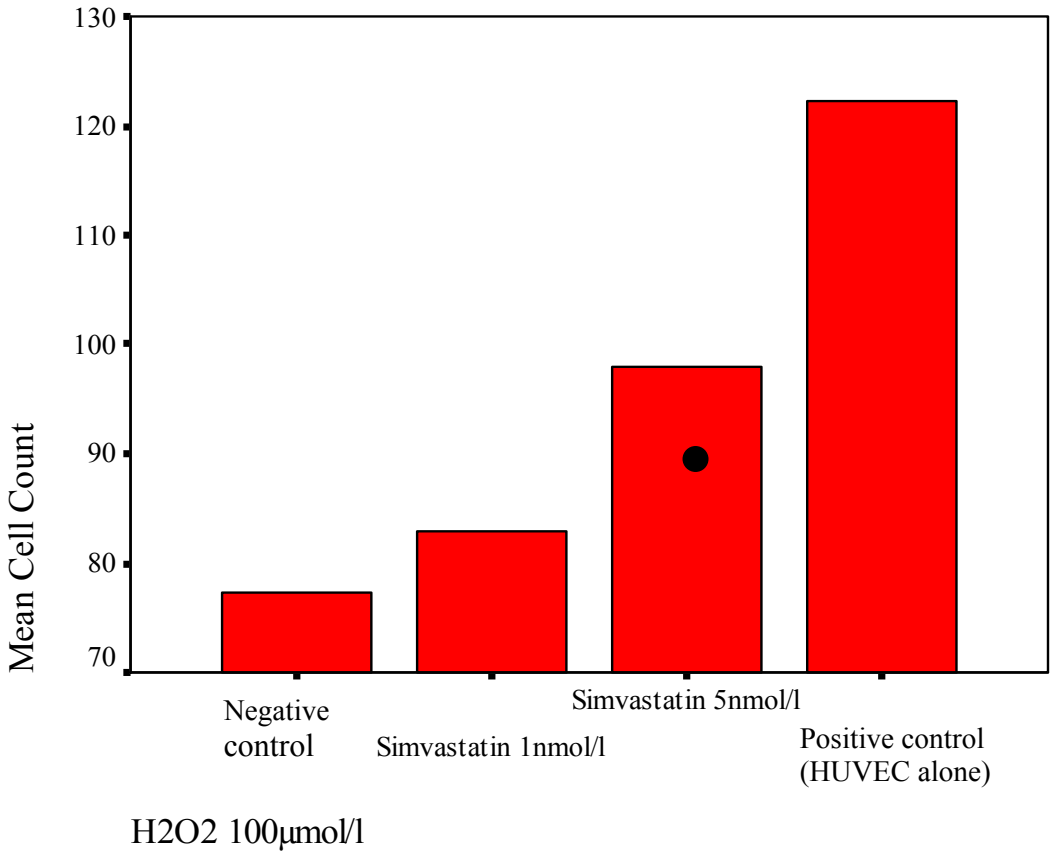
HUVEC treatment with Simvastain showed a similar survival benefit with a higher number of live cells in comparison with the control group. Statistical significance was reached in cells treated with 5nmol/l of Simvastatin (Figure 12).

Figure 11. Live Cell Count after treatment with Cerivastatin and H_2O_2



[Statin vs Negative control, ● = p < 0.05 (paired t test)]

Figure 12. Live Cell Count after treatment with Simvastatin and H_2O_2



[Statin vs Negative control, ● = p < 0.05 (paired t test)]

Cellular proliferation assay

Spectrophotometric absorption showed an enhanced proliferative response when HUVEC were treated with statins before induction of oxidative stress. There was a significant increase in the proliferative response to both 1nmol/l and 5nmol/l doses of cerivastatin as opposed to only 5nmol/l with Simvastatin (Table 11).

Apoptosis cell count

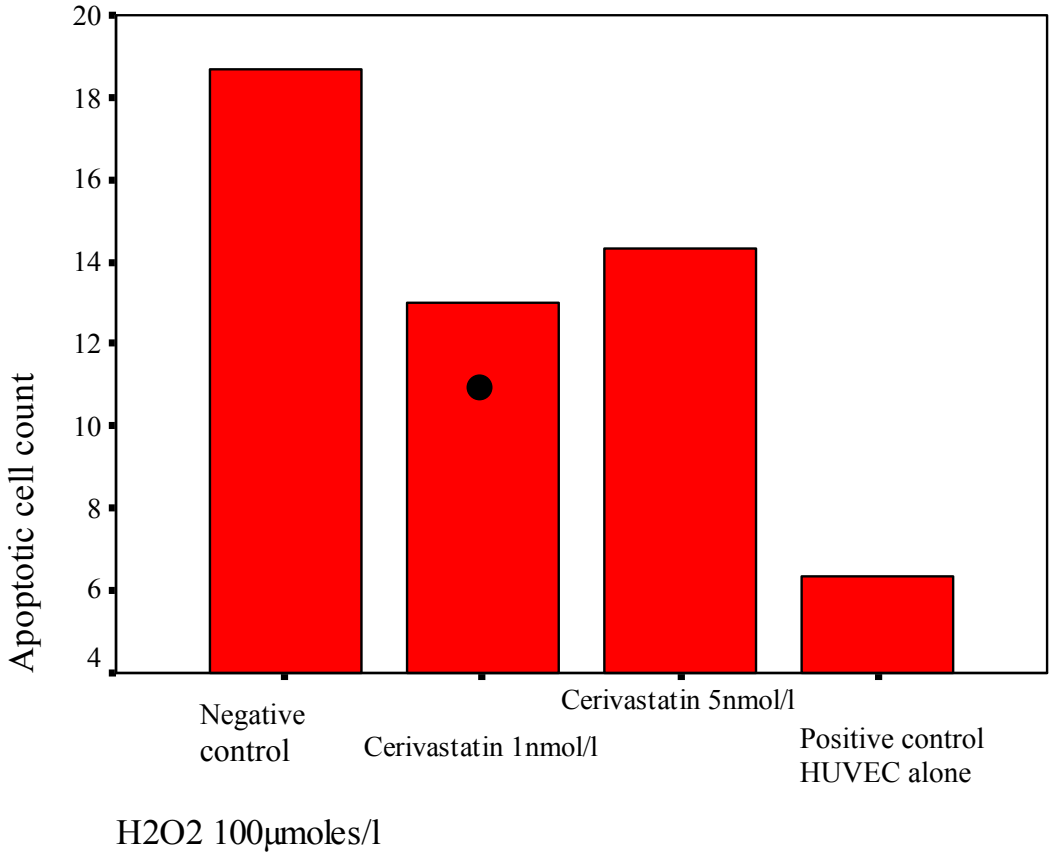
In comparison with the negative control group, there was a reduction in the number of HUVEC undergoing apoptosis as a consequence of exposure to H_2O_2 100µmol/l when they were pre-treated with a statin. This was statistically significant for 1nmol/l dose concentration of Cerivastatin (Figure 13). There was similar effect noted with Simvastatin treatment in HUVEC exposed to oxidative stress with H_2O_2 100µmol/l although this did not reach statistical significance (Figure 14).

Table 11. Spectrophotometric absorption after statin and H_2O_2 (100µmol/l) treatment

	Negative control (HUVEC + H_2O_2)	Statin 1nmol/l + H_2O_2	Statin 5nmol/l + H_2O_2	Positive control (HUVEC alone)
Cerivastatin Group	55(1.73)	68(1.2)*	63(1.15)*	71.6(1.45)
Simvastatin Group	55(1.73)	62(1.73)	66(0.57)*	71.6(1.45)

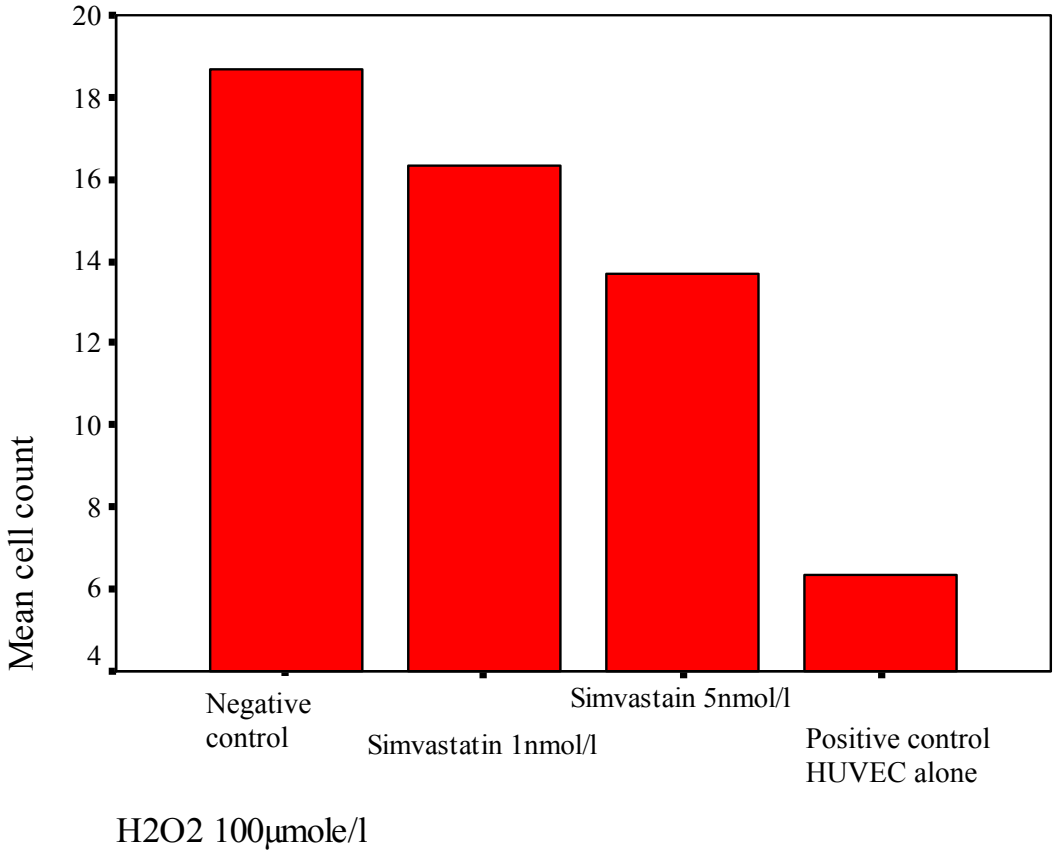
[Statin vs Negative control, (* = $p<0.05$ Student's t-test) values expressed as mean (Standard Error of Mean)]

Figure 13. Apoptosis cell count after Cerivastatin and H_2O_2 (100µmol/l) treatment



[Statin vs Negative control, ● = p < 0.05 (paired t test)]

Figure 14. Apoptosis cell count after Simvastatin and H_2O_2 (100µmol/l) treatment



[Statin vs Negative control, ● = p < 0.05 (paired t test)]

RT PCR

Cyclin D expression

There was enhanced expression of Cyclin D in cells that were preincubated with statin before exposure to H_2O_2 100µmol/l (Table 12). The difference of the means in comparison with the negative control which received only H_2O_2 100µmole/l was significant for Cerivastatin 1nmol/l and Simvastatin 5nmol/l. Addition of mevalonate 10µmol/l to the cells to reverse the effects of statin resulted in abrogation of the Cyclin D expression (Figure 15 and 16).

Bax expression

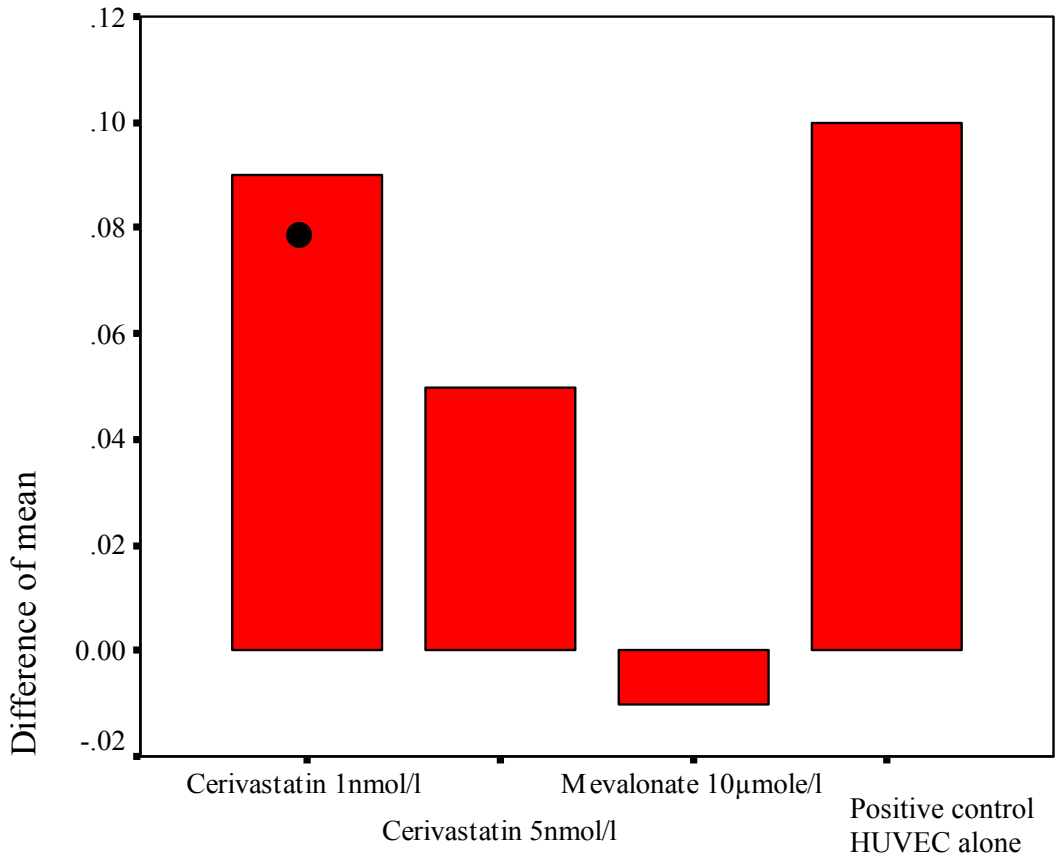
Expression of Bax protein in response to H_2O_2 100µmol/l administration was attenuated by preincubation of HUVEC in statin (Table 13). This reached statistical significance with all the dose concentrations used with Cerivastatin and Simvastatin, in comparison with positive control with HUVEC alone. Mevalonate treatment reversed the effect of statin on Bax expression (Figure 17 and 18).

Table 12. Cyclin D expression after statin and H_2O_2 (100µmol/l) treatment

	Negative Control (HUVECs + H_2O_2)	Statin 1nmol/l + H_2O_2	Statin 5nmol/l + H_2O_2	Statin 1nmol/l + H_2O_2 + Mevalonate 10µmole/l	Positive control (HUVECs alone)
Cerivastatin Group	0.62(0.01)	0.71(0.01) *	0.67((0)	0.61(0.01)	0.72(0)
Simvastatin Group	0.62(0.01)	0.64(0)	0.71(0) *	0.61(0)	0.72(0)

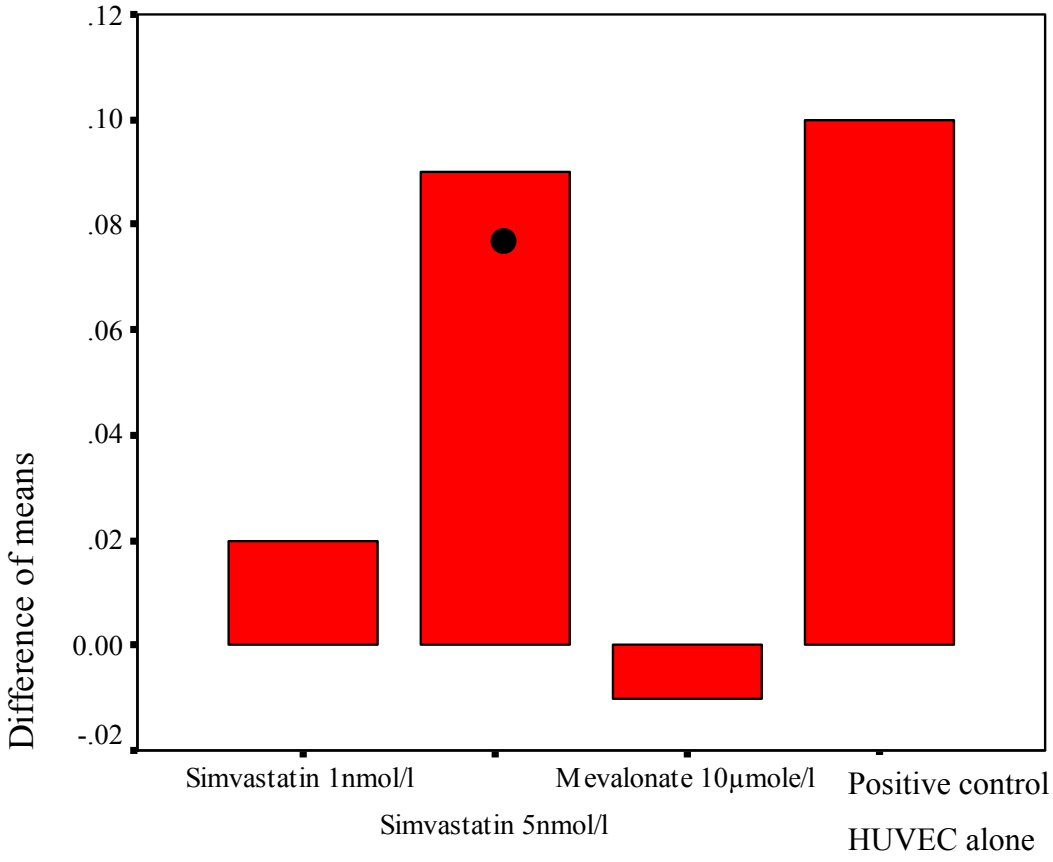
[Statin vs Negative control, * = $p<0.05$ Student's t- test, values expressed as mean (Standard Error of Mean)]

Figure 15. Difference in Cyclin D expression in comparison with negative control (H_2O_2 100µmole/l) after treatment with Cerivastatin and H_2O_2 100µmol/l



[Statin vs Negative control, ● = p < 0.05 , paired t test]

Figure 16. Difference in Cyclin D expression in comparison with negative control (H_2O_2 100µmol/l) after treatment with Simvastatin and H_2O_2 100µmol/l



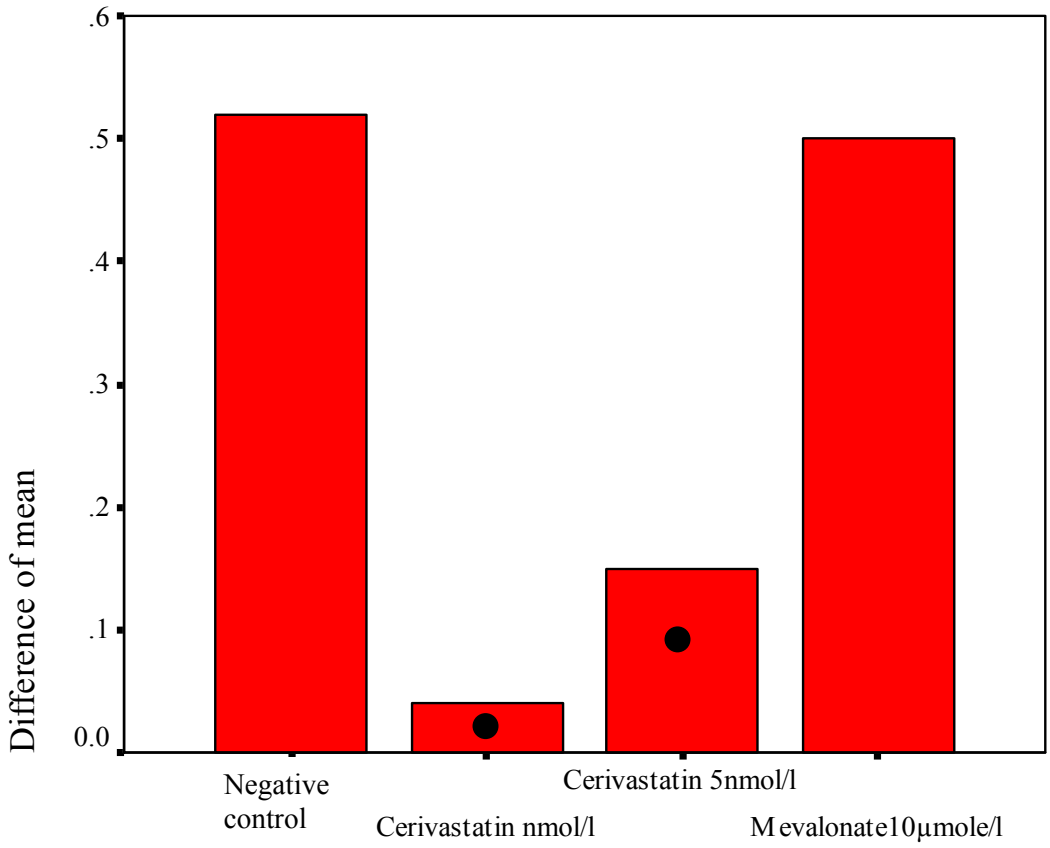
[Statin vs Negative control, ● = p < 0.05, paired t test]

Table 13. Bax expression after statin and H_2O_2 (100µmol/l) treatment

	Negative control (HUVEC + H_2O_2)	Statin 1nmol/l + H_2O_2	Statin 5nmol/l + H_2O_2	Statin 1nmol/l + H_2O_2 + Mevalonate 10µmole/l	Positive control (HUVEC alone)
Cerivastatin Group	2.03(0.03)	1.55(0.04) *	1.66(0.05)*	2.01(0.05)	1.51(0.03)
Simvastatin Group	2.03(0.03)	1.75(0.02) *	1.6(0.01) *	1.97(0.10)	1.51(0.3)

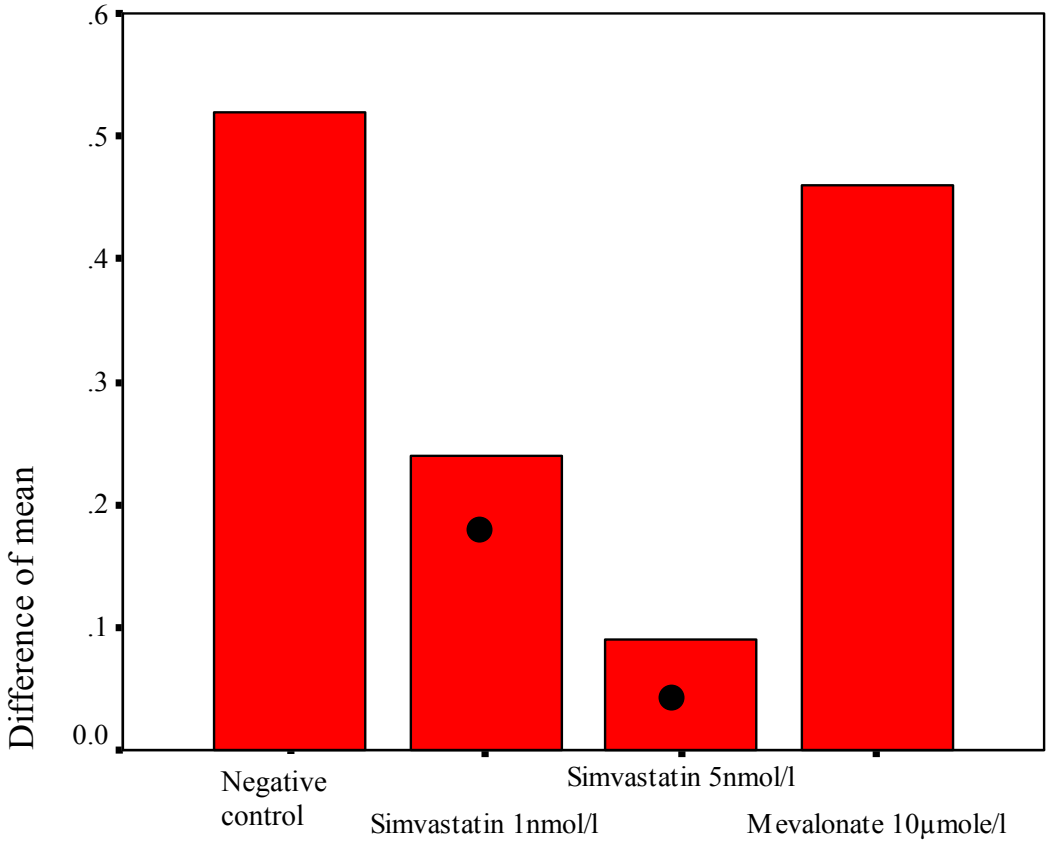
[Statin vs Positive control, * = $p<0.05$ Student's t- test, values expressed as mean (Standard Error of Mean)]

Figure 17. Difference in Bax expression after treatment with Cerivastatin and H_2O_2 100µmol/l in comparison with positive control (healthy HUVEC)



[Statin vs Positive control, ● = p < 0.05, paired t test]

Figure 18. Difference in Bax expression after treatment with Simvastatin and H_2O_2 100µmol/l in comparison with positive control (healthy HUVEC)



[Statin vs Positive control, ● = p < 0.05, paired t test]

Conclusion

There was a significant increase in the number of live cells when HUVEC were preincubated with a statin prior to H_2O_2 exposure. This reached statistical significance in both statin groups. The spectrophotometric absorption was also enhanced in HUVEC treated with statins indicating that the proliferative activity in the cells exposed to oxidative stress was increased by statins. The number of HUVEC undergoing apoptosis consequent to oxidative stress was reduced by statin pre-treatment although this reached statistical significance in the cerivastatin group alone. Cyclin D expression was increased in HUVEC preincubated with statin which may in turn enhance the proliferative stimulus on the HUVEC. There was a simultaneous reduction in the Bax expression in HUVEC suggesting that statin may inhibit activation of this pro-apoptotic protein and therefore enhance the cell survival capabilities.

Chapter 6 – Effect of Hypoxic Stress on HUVEC

Introduction

An optimal oxygen level is vital for the normal cellular physiology and metabolism. A reduction in tissue oxygen availability can adversely affect the various cellular metabolic pathways. The vascular endothelium is subject to these effects of hypoxia in pathophysiological states such as atherosclerotic diseases where there is a compromised blood supply with consequent reduction in the oxygen delivery. Myocardial infarction, stroke, peripheral vascular disease and acute/chronic limb ischemic episodes are all examples of tissue hypoxia.

Hypoxic effects have been extensively studied in neuronal tissues. Here hypoxia mediates its effects through multiple pathways. There is depletion of the energy reserves within the cell, accumulation of lactic acid with consequent acidosis, reduction in the Na K ATPase activity, intracellular accumulation of Ca^{2+} and lipid peroxidation (167, 263, 264). It affects the expression of endothelial vasoconstrictor endothelin and also alters the vasodilator response of the VSMC to NO (265, 266). Hypoxia also results in enhanced generation of ROS by the cycloxygenase, lipoxygenase and NOS pathways (168, 169, 267). These effects can result in endothelial dysfunction.

In addition, hypoxia can also trigger mechanisms to induce apoptosis. It opens the mitochondrial PTP to large molecules with consequent loss of electrochemical gradient, uncoupling of oxidative phophorylation, ATP hydrolysis and mitochondrial swelling (268). PTP can also promote cytochrome c release which activates the caspase pathway with resultant apoptosis. In pig neuronal cells, hypoxia induced apoptosis is accelerated by

inactivation of the antiapoptotic Bcl-2 protein with consequent enhancement of apoptosis from the unopposed effects of Bax (172). These effects are mostly from studies on neuronal tissues. Although hypoxic stress is a potent and ubiquitous modulator of the vascular endothelial function, its effect on the vascular endothelial cell cycle pathway and apoptosis is not clear. Understanding the effects of hypoxia in EC and the mechanisms by which these effects are mediated is vital.

Aim

This experiment was set to determine the effect of varying doses of $CoCl_2$ on the cell cycle and apoptosis. The response of cell cycle promoter Cyclin D and pro-apoptotic protein Bax to $CoCl_2$ induced hypoxia was also determined.

Materials and Methods

HUVEC were prepared as described in the earlier chapters. For induction of hypoxia, HUVEC seeded onto 48 well plates were treated with 100µmol/l, 200µmol/l and 500µmol/l of $CoCl_2$ with three repeats for each drug concentration and a control sample. Live cell counting, cellular proliferation assay using the CellTiter 96 Aqueous One Solution Cell Proliferation Assay kit (Promega UK) and cellular apoptosis assay using the

DeadEnd™ Fluorometric TUNEL System kit (Promega UK) was performed at termination of the experiment 24 hours after initiation of treatment with $CoCl_2$. Bax and Cyclin D assay were performed by RT-PCR on HUVEC propagated in 25cc culture flasks following treatment with 100 and 200µmol/l of $CoCl_2$ with a control receiving no drug.

Data was expressed in means ± SEM. The paired samples t test was used to compare the groups. $P < 0.05$ was considered to be statistically significant.

Results

Live cell count

Following $CoCl_2$ treatment there was a significant reduction in live HUVEC numbers in comparison with the control group for strengths of 200μmol/l and 500μmol/l (Table 14).

Cellular proliferation assay

Spectrophotometric absorption showed a significant reduction in cellular proliferation of HUVEC subjected to higher dose concentrations of $CoCl_2$ in comparison to the control (Table 15).

Apoptosis cell count

There was a significant increase in the number of HUVEC undergoing apoptosis when treated with 200μmol/l and 500μmol/l of $CoCl_2$ in comparison with the control (Table 16).

Table 14. Live cell count 24 hours after $CoCl_2$ treatment

	Control	100µmol/l	200µmol/l	500µmol/l
$CoCl_2$	109.6(5.7)	103.3(6.48)	79(3.6)*	61(4.16)*

[$CoCl_2$ vs Control, * = $p<0.05$ Student's t- test, values expressed as mean (Standard Error of Mean)]

Table 15. Spectrophotometric absorption 24 hours after $CoCl_2$ treatment

	Control	100µmol/l	200µmol/l	500µmol/l
$CoCl_2$	64.66(3.28)	63.33(2.84)	54.33(2.6)*	43.66(2.6)*

[$CoCl_2$ vs Control, * = $p<0.05$ Student's t- test, values expressed as mean (Standard Error of Mean)]

Table 16. Apoptosis cell count 24 hours after $CoCl_2$ treatment

	Control	100µmol/l	200µmol/l	500µmol/l
$CoCl_2$	9.66(0.88)	12.33(0.88)	19.33(0.88)*	26(2.08)*

[$CoCl_2$ vs Control,* = $p<0.05$ Student's t- test, values expressed as mean (Standard Error of Mean)]

RT PCR

$CoCl_2$ dose concentrations of 100μmol/l and 200μmol/l were used to study the expression of Bax and Cyclin D as response to hypoxic stress.

Cyclin D expression

There was no significant change to HUVEC expression of Cyclin D after treatment with $CoCl_2$ in comparison with the control group (Table 17).

Bax expression

Expression of Bax protein in response to $CoCl_2$ administration reached statistical significance at 200μmol/l (Table 18).

Table 17. Cyclin D expression 24 hours after $CoCl_2$ treatment

	Control	100µmol/l	200µmol/l
$CoCl_2$	0.7(0.17)	0.69(0.02)	0.69(0.01)

[$CoCl_2$ vs Control, * = $p<0.05$ Student's t- test, values expressed as mean (Standard Error of Mean)]

Table 18. Bax expression 24 hours after $CoCl_2$ treatment

	Control	100µmol/l	200µmol/l
$CoCl_2$	1.51(0.03)	1.59(0.03)	2.02(0.04)*

[$CoCl_2$ vs Control, * = $p<0.05$ Student's t- test, values expressed as mean (Standard Error of Mean)]

Conclusion

Hypoxia induced by $CoCl_2$ resulted in reduction of the total number of live cells after 24 hour period of treatment. This reduction was significant at doses 200µmol/l and 500µmol/l. These higher doses of $CoCl_2$ also resulted in reduction of the cellular proliferative activity as shown by a reduced spectrophotometric absorption values. In addition, these higher doses induced apoptosis in a significant number of HUVEC in comparison to the control group. The expression of the cell cycle promoter Cyclin D remained unaffected by $CoCl_2$ treatment doses although the proapoptotic protein Bax levels were enhanced with higher doses. This study shows that hypoxia inhibits the proliferative activity of HUVEC and induces apoptosis. Bax expression plays a vital role in induction of hypoxia mediated apoptosis. However Cyclin D expression was unaffected by hypoxia which implies that hypoxic stimulus does not have a negative impact on Cyclin D expression.

Chapter 7. Effect of Statins on Cellular Proliferation and Apoptosis modulated by Hypoxic Stress

Introduction

Hypoxia is a common occurrence in the vascular endothelium as a manifestation of the atherosclerotic processes. It deranges various cellular metabolic pathways and results in depletion of the energy reserves of the cell (269). More importantly it is also a stimulus for accelerated ROS generation with consequent oxidative stress and endothelial dysfunction (170, 270). Acute and chronic hypoxia are potent inducers of cellular apoptosis in cell culture models (271). Hypoxia can induce apoptosis by directly promoting the PTP to open and allow passage of molecules across the mitochondrial membrane, including cytochrome c which activates the caspase pathway (268). Hypoxia may also induce apoptosis by modifying the expression of the apoptotic proteins Bax and Bcl-2. In neuronal tissues, there is increased inactivation of the anti-apoptotic BcL-2 protein with worsening degrees of hypoxia (172). Thus hypoxia has effects on the EC ranging from metabolic derangement and endothelial dysfunction to apoptosis.

There are various mechanisms which abrogate the metabolic effects of hypoxia on the cell. Preconditioning to hypoxia induces activation of various metabolic pathways by transcription of proteins. This enhances the cellular adaptive mechanism to hypoxic stimuli (272). HIF-1 is the key mediator of hypoxic preconditioning through its effects on various metabolic pathways which promotes cell survival (187, 188). Statins are pleiotropic drugs which enhance endothelial function and have a potent antioxidant activity. Thus statins may influence the hypoxia induced accelerated synthesis of ROS and consequent oxidative

stress. As mentioned in chapter 3, statins induce a proliferative response in endothelial progenitor cells by promoting the cell cycle regulator proteins such as cyclin D and inhibit apoptosis by inactivating the proapoptotic proteins. The hypothesis of this study was that statins, by up regulating their effects on endothelial survival and proliferation, may offer protection against hypoxia induced antiproliferative and apoptotic mechanisms.

Aim

This experiment was designed to determine if statins offered any protection against antiproliferative and apoptotic effects induced by hypoxia in HUVEC. Expression of the cell cycle promoter cyclin D and the proapoptotic protein Bax were studied in a hypoxic environment.

Materials and Methods

HUVEC were prepared as described earlier in chapter 3. After allowing the cells to stabilise for 48 hours they were treated with Cerivastatin and Simvastatin to obtain a final concentration of 5nmol/l. Hypoxia was induced in these HUVEC with $CoCl_2$, 200µmol/l, 24 hours after treatment with statin. The experiments were performed in triplicates with a control group receiving treatment with $CoCl_2$ alone. Live cell counting, cellular proliferation assay using the CellTiter 96 Aqueous One Solution Cell Proliferation Assay kit (Promega UK) and cellular apoptosis assay using the DeadEnd^{TM} Fluorometric TUNEL System kit (Promega UK) were performed at termination of the experiment 24 hours after treatment with $CoCl_2$. RT-PCR was performed for Bax and Cyclin D assay on HUVEC propagated in 25cc culture flasks and treated as described above. A negative control was

treated with $CoCl_2$ alone and a positive control was treated with activated mevalonate 10µmol/l (downstream metabolite of the mevalonate pathway) in addition to statin and $CoCl_2$.

Data was expressed in means ± SEM. The paired samples t test was used to compare the groups. $P < 0.05$ was considered to be statistically significant.

Results

Live cell count

HUVEC pre-incubated with statin before treatment with $CoCl_2$ to induce hypoxia had a higher number of live cells compared to the control group which received only $CoCl_2$. This increased cell survival capability reached statistical significance with a Simvastatin dose of 5nmol/l (Figure 19 and 20)).

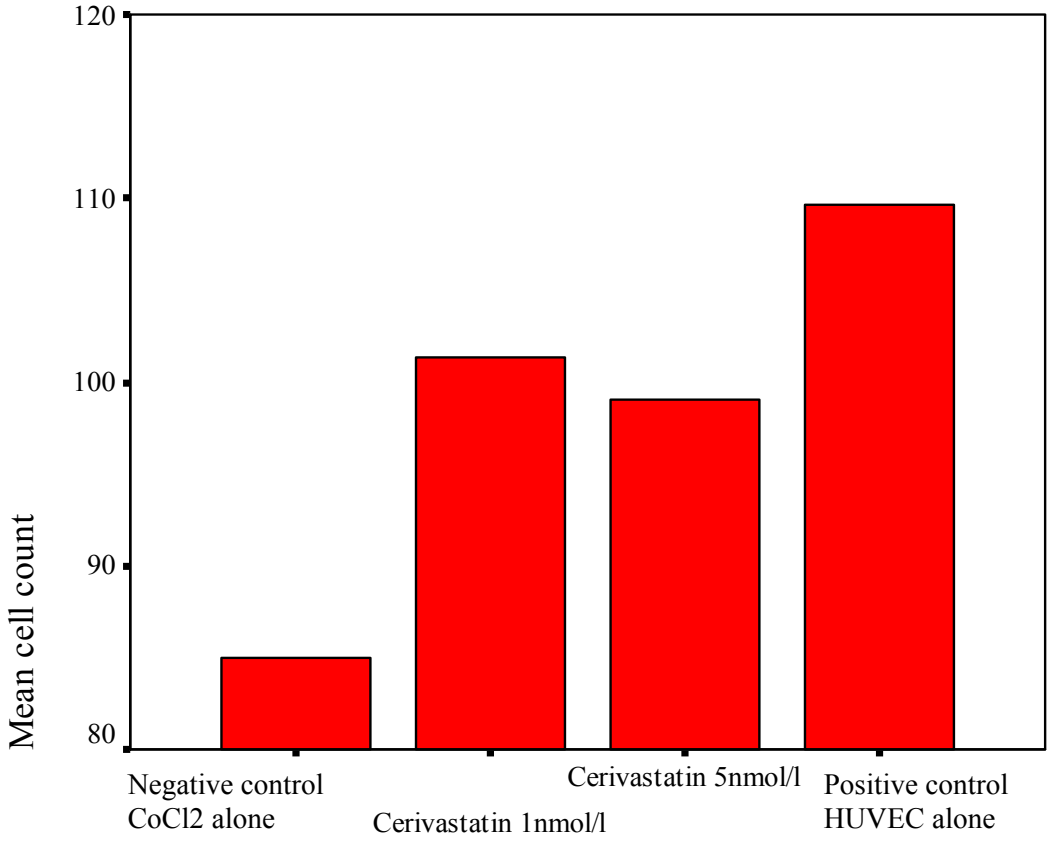
Cellular proliferation assay

Spectrophotometric absorption showed an enhanced proliferative response when HUVEC were treated with either of the statins before induction of hypoxia (Table 19).

Apoptosis cell count

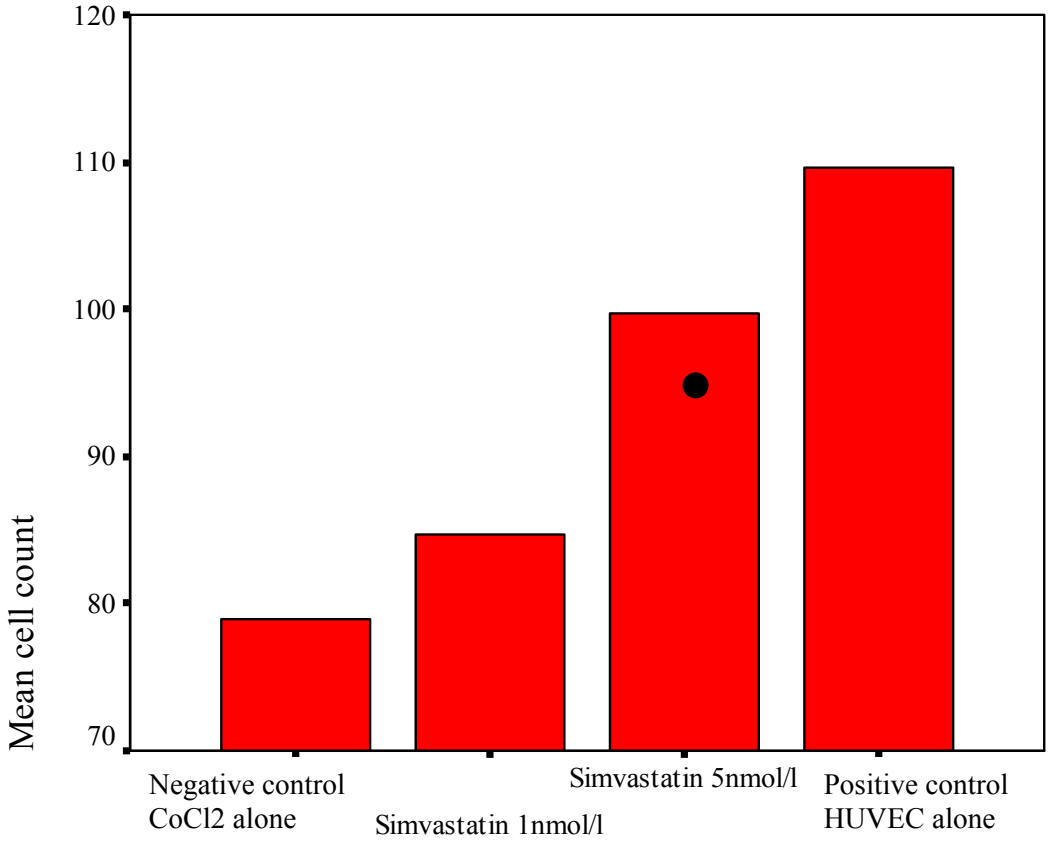
In comparison with the control group there was a reduction in the number of HUVEC undergoing apoptosis as a consequence of exposure to $CoCl_2$ 200µmole/l when they were pre-treated with a statin. This was statistically significant for Cerivastatin and Simvastatin in both dose strengths (Figure 21 and 22).

Figure 19. Live Cell Count after treatment with Cerivastatin and $CoCl_2$ 200µmol/l



[Statin vs Negative control, ● = p < 0.05, paired t test]

Figure 20. Live Cell Count after treatment with Simvastatin and $CoCl_2$ 200µmol/l



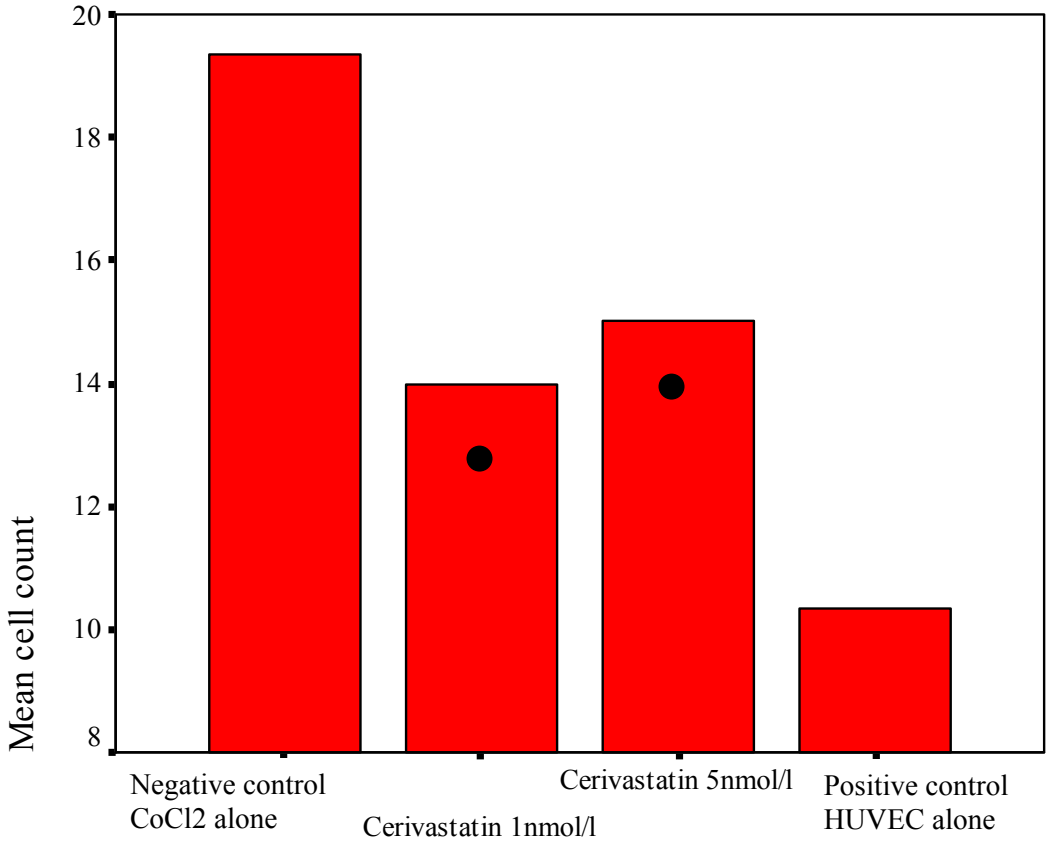
[Statin vs Negative control, ● = p < 0.05, paired t test]

Table 19. Spectrophotometric absorption after statin and $CoCl_2$ 200µmol/l

	Negative control (HUVEC + $CoCl_2$)	Statin 1nmol/l + $CoCl_2$	Statin 5nmol/l + $CoCl_2$	Positive control HUVEC alone
Cerivastatin Group	53(2.08)	57.3(2.02)*	55.6(1.76)	64.6(3.28)
Simvastatin Group	54.3(2.6)	57(2.08)	60(3.21)*	64.66(3.28)

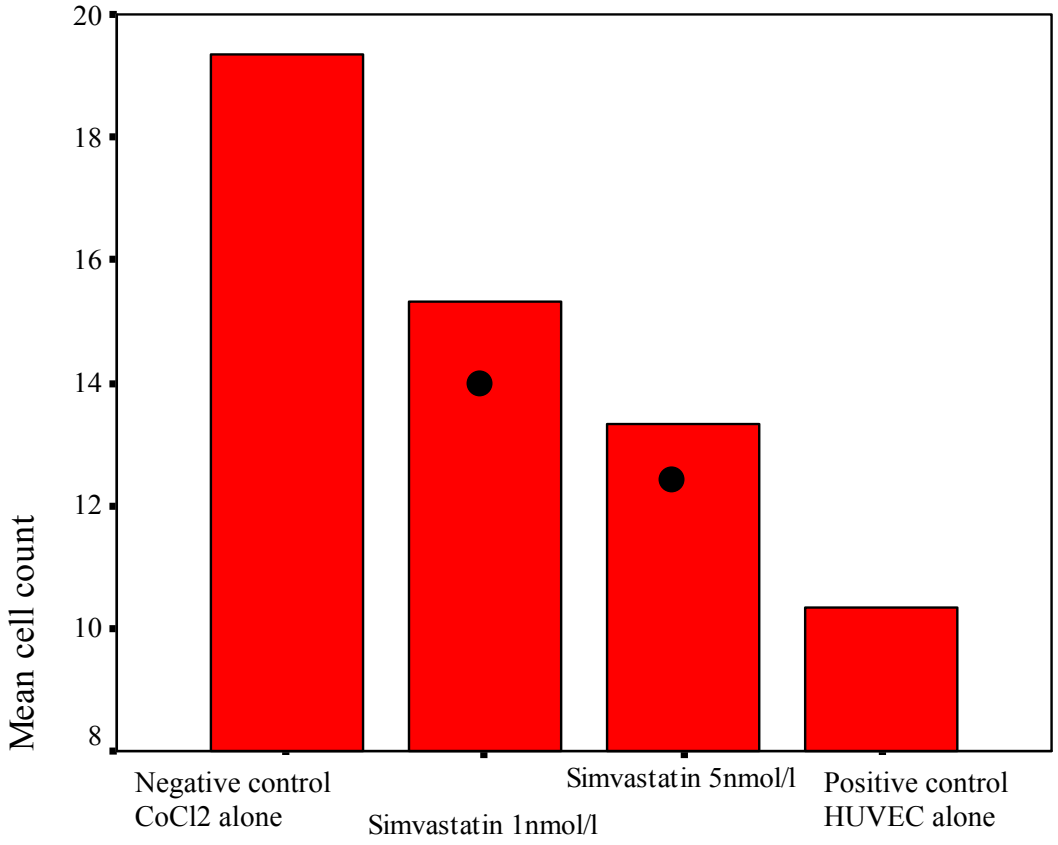
[Statin vs Negative control, * = $p<0.05$ Student's t- test, values expressed as mean (Standard Error of Mean)]

Figure 21. Apoptosis cell count after Cerivastatin and $CoCl_2$ (200µmol/l)



[Statin vs Negative control, ● = p < 0.05, paired t test]

Figure 22. Apoptosis cell count after Simvastain and $CoCl_2$ (200µmol/l)



[Statin vs Negative control, ● = p < 0.05, paired t test]

RT PCR

Cyclin D expression

There was an enhanced expression of cyclin D in HUVEC preincubated with a statin prior to induction of hypoxia with $CoCl_2$ 200μmol/l (Table 20). In addition, the Cyclin D expression in HUVEC exposed to statins, was abrogated by mevalonate (Figure 23 and 24)

Bax expression

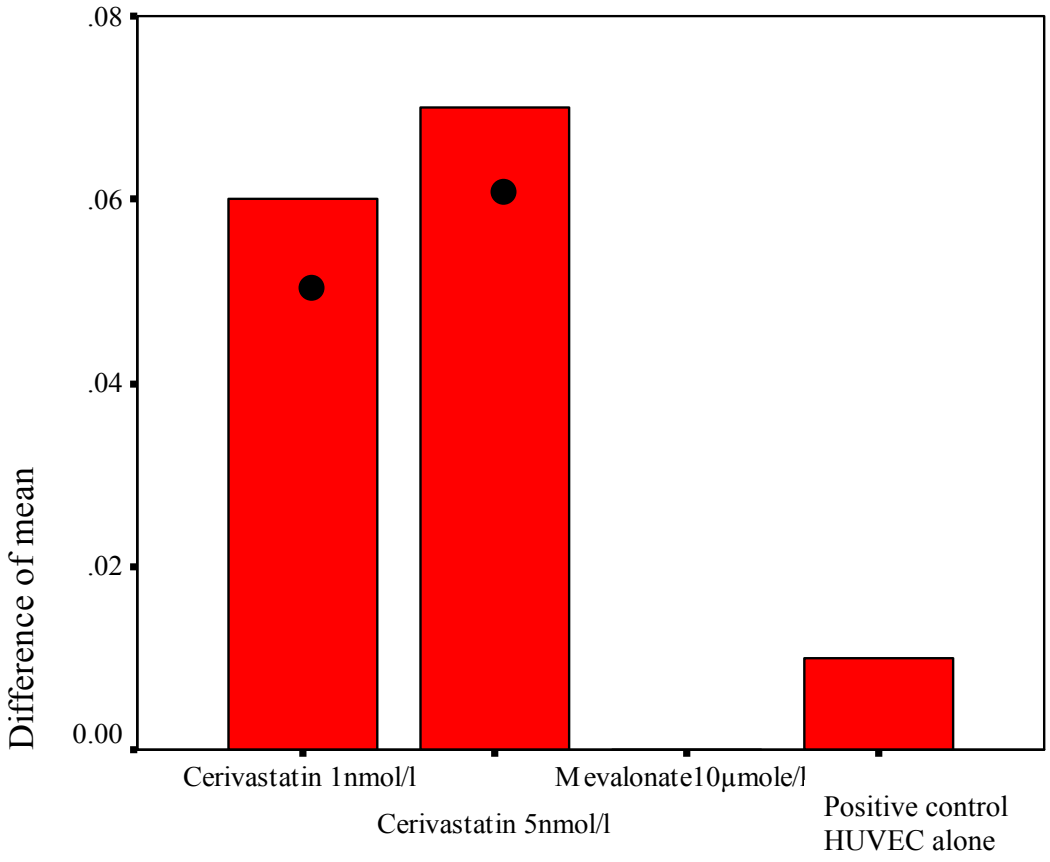
Although the expression of Bax is enhanced with CoCl2 treatment, preincubating the cells in statin significantly reduces the subsequent Bax expression by the hypoxic stimuli (Table 21). Mevalonate minimises these inhibitory effects of statin on Bax expression (Figure 25 and 26).

Table 20. Cyclin D expression after statin and $CoCl_2$ (200µmol/l) treatment

	Negative control (HUVEC + H_2O_2)	Statin 1nmol/l + $CoCl_2$	Statin 5nmol/l + $CoCl_2$	Statin 1nmol/l + $CoCl_2$ + Mevalonate 10µmol/l	Positive control (HUVEC Alone)
Cerivastatin Group	0.69(0.02)	0.75(0.02)*	0.76(0.01)*	0.69(0.02)	0.7(0.01)
Simvastatin Group	0.69(0.02)	0.72(0.01)	0.78(0)*	0.69(0.01)	0.7(0.01)

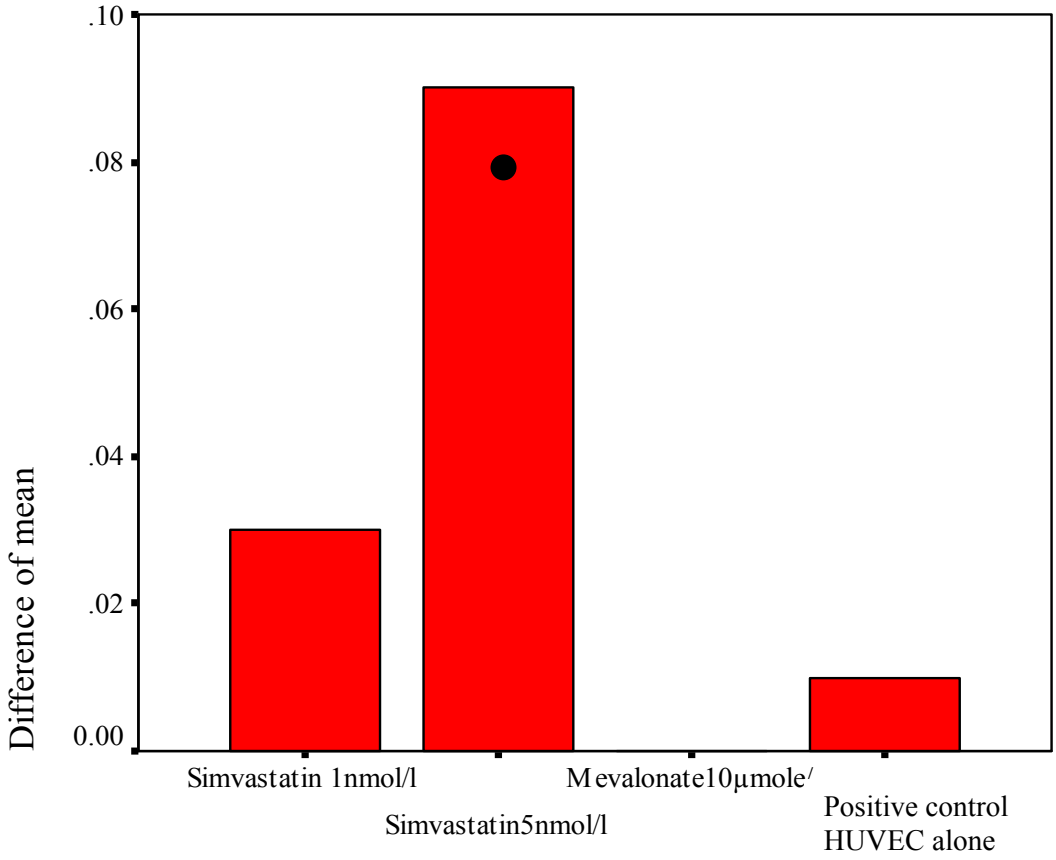
[Statin vs Negative control, * = $p<0.05$ Student's t- test, values expressed as mean (Standard Error of Mean)]

Figure 23. Difference in Cyclin D expression in comparison with the negative control ($CoCl_2$ 200µmol/l) after treatment with Cerivastatin and $CoCl_2$ 200µmol/l



[Statin vs Negative control, ● = p < 0.05, paired t test]

Figure 24. Difference in Cyclin D expression in comparison with the negative control ($CoCl_2$ 200µmol/l) after treatment with Simvastatin and $CoCl_2$ 200µmol/l



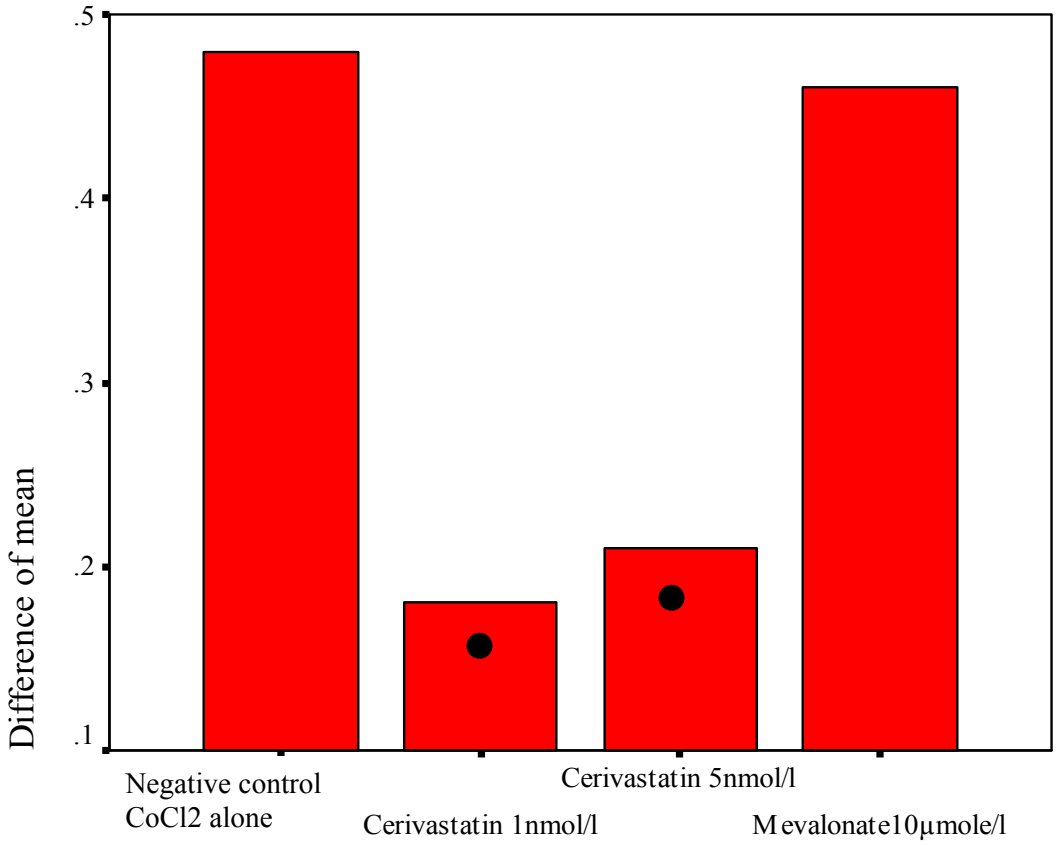
[Statin vs Negative control, ● = p < 0.05, paired t test]

Table 21. Bax expression after statin and $CoCl_2$ 200µmol/l treatment

	Negative control (HUVEC + $CoCl_2$)	Statin 1nmol/l + $CoCl_2$	Statin 5nmol/l + $CoCl_2$	Statin 1nmol/l + $CoCl_2$ + Mevalonate 10µmol/l	Positive control (HUVEC alone)
Cerivastatin Group	1.99(0.02)	1.69(0)*	1.72(0)*	1.97(0.03)	1.51(0.03)
Simvastatin Group	1.99(0.02)	1.82(0.01)*	1.85(0.03)	1.99(0.01)	1.51(0.03)

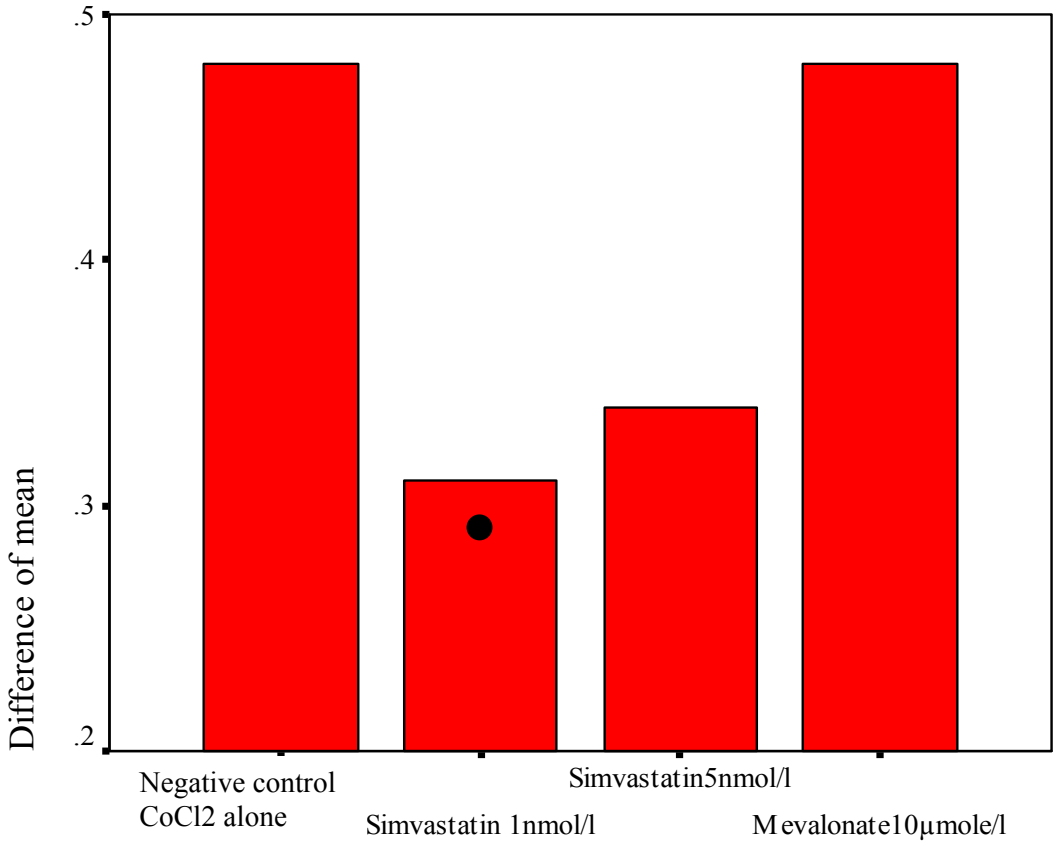
[Statin vs Negative control, * = $p<0.05$ Student's t- test, values expressed as mean (Standard Error of Mean)]

Figure 25. Difference in Bax expression after treatment with Cerivastatin and $CoCl_2$ 200µmol/l in comparison with positive control (healthy HUVEC)



[Statin vs Positive control, ● = p < 0.05 , paired t test]

Figure 26. Difference in Bax expression after treatment with Simvastatin and $CoCl_2$ 200µmol/ in comparison with positive control (healthy HUVEC)



[Statin vs Positive control, ● = p < 0.05, paired t test]

Conclusion

Statin treatment preserves the number of viable HUVEC in the context of hypoxia. The proliferative activity of the HUVEC preincubated with a statin before being subject to hypoxia have a significantly higher proliferative activity compared to the control group. However this activity was not to the level of the normal HUVEC. Similarly the apoptotic cell count by TUNEL assay showed a reduction in cell death confirming the above findings. Although hypoxia does not have a stimulatory effect on Cyclin D, statin treatment was noted to enhance their expression. HUVEC expression of Bax protein in response to hypoxia was reduced by statin preincubation and this may have a vital role in the protective effect of statins. The expression of Cyclin D and Bax by HUVEC in response to hypoxic conditions was reversed by mevalonate, confirming that these protective effects of statin were dependent on HMGCoA reductase inhibition.

Chapter 8. Effects of Acute Withdrawal of Statins on Cellular Proliferation and Apoptosis

Introduction

Statins are now recognised as a drug with pleiotropic effects which reduce the cardiovascular morbidity and mortality. However, the emphasis of statin therapy has recently shifted from treating hypercholesterolemia to atherogenesis prevention.

Rebound symptoms are well recognised with the withdrawal of many drugs and there have been recent concerns about similar rebound effects after withdrawal of statin therapy (273-276). Accumulation of the metabolite competitively inhibited by the drug can result in larger concentrations of the metabolite entering the rate limiting reaction, with consequent rebound effects following removal of the inhibitor. Statin withdrawal activates the mevalonate pathway and isoprenylation of the G proteins vital for the cellular signalling mechanisms. The Rac protein activation by geranylgeranylpyrophosphate following statin withdrawal enhances the NADPH oxidase activity and this enhances the generation of ROS. This results in attenuated NO bioavailability and consequent endothelial dysfunction.

However the effects of statin withdrawal on EC proliferative response and apoptosis are not clearly defined.

Aim

The aim of this experiment was to determine the effect of statin withdrawal on the HUVEC cell cycle. The effects on cellular proliferation and apoptosis were studied by assay of the cell cycle regulator Cyclin D and the apoptotic protein Bax.

Material and Methods

HUVEC were prepared as described in chapter 3. After allowing the cells to stabilise in wells for 48 hours they were treated with Simvastatin to obtain a final concentration of 5nmol/l. Cells were propagated for 7 days with replenishment of cell culture media and statin on day 3 and 5. On day 7 the plates were rinsed with cell culture media and fresh media devoid of statin was added. A negative control was included with HUVEC in statin free culture media and a positive control of HUVEC in culture media with statin. The experiments were performed in triplicates. Live cell counting, cellular proliferation assay using the CellTiter 96 Aqueous One Solution Cell Proliferation Assay kit (Promega UK) and cellular apoptosis assay using the DeadEnd^{TM} Fluorometric TUNEL System kit (Promega UK) were performed at termination of the experiment 24 hours after withdrawal of statin. RT-PCR was performed for Bax and Cyclin D assay in HUVEC propagated in 25cc culture flasks.

Data was expressed in means ± SEM. The paired samples t test was used to compare the groups. $P < 0.05$ was considered to be statistically significant.

Results

Live cell count

There was a significant reduction in the number of live cells after withdrawal of statin therapy suggesting reduced cell survival capacity upon withdrawal of statin (Figure 27).

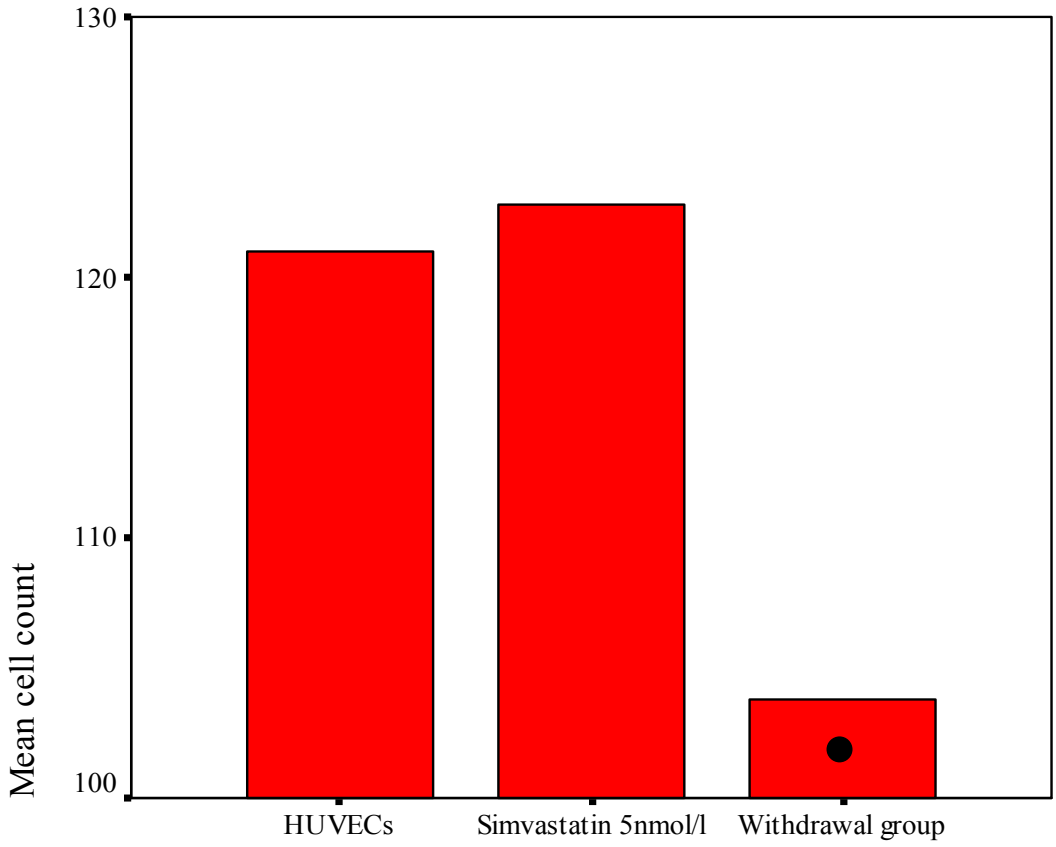
Cellular proliferation assay

The spectrophotometric absorption of HUVEC after withdrawal of statin therapy was significantly reduced. Withdrawal of statin reduces the proliferative activity of the HUVEC (Table 24).

Apoptosis cell count

There was a significant increase in the number of apoptotic cells after withdrawal of statin therapy in HUVEC when compared to the other two groups (Figure 28).

Figure 27. Live Cell Count after withdrawal of statin therapy



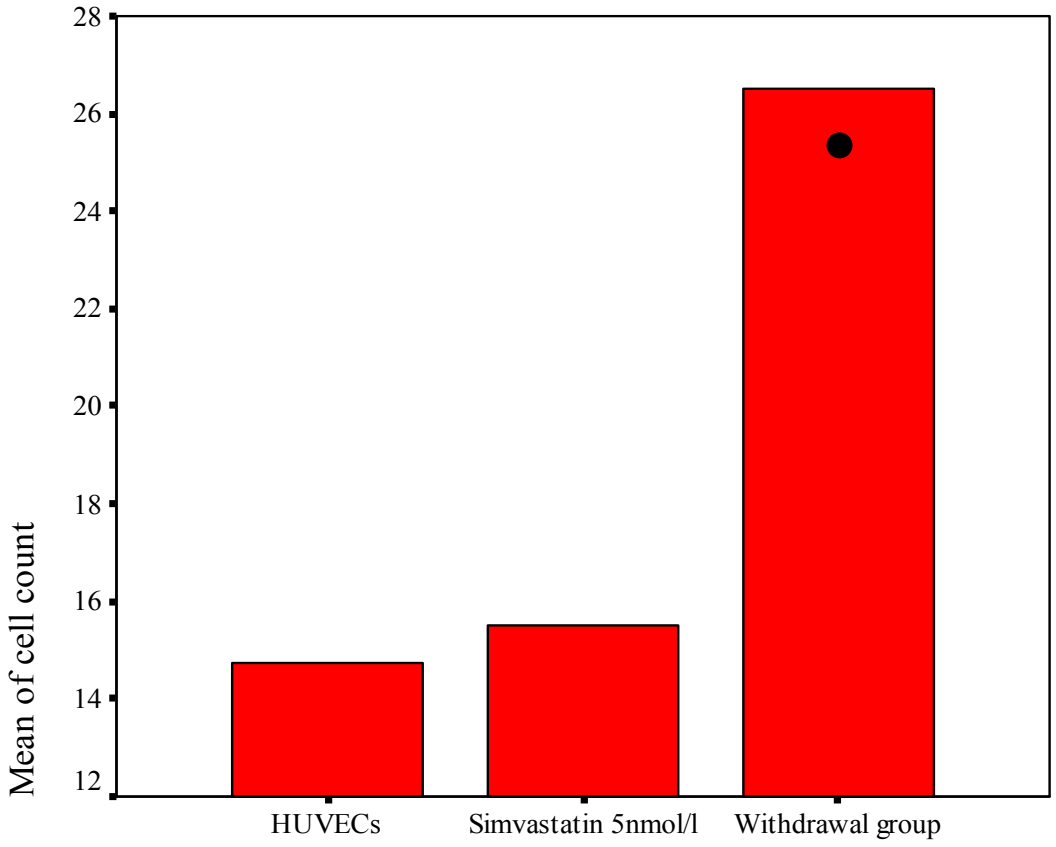
[Statin vs withdrawal group, ● = p < 0.05, paired t test]

Table 22. Spectrophotometric absorption 24 hours after statin withdrawal

	HUVEC	5nmol/l	Withdrawal group
Simvastatin	75(1.08)	79(1.47) *	51(1.88) *

[Statin vs withdrawal group, * = $p<0.05$ Student's t- test, values expressed as mean (Standard Error of Mean)]

Figure 28. Apoptosis cell count 24 hours after statin withdrawal



[Statin vs withdrawal group, ● = p < 0.05, paired t test]

RT PCR

Cyclin D expression

Withdrawal of statin therapy had no influence on Cyclin D expression in HUVEC in comparison to the control group (Table 23).

Bax expression

There was an enhanced expression of the pro-apoptotic protein Bax by HUVEC on withdrawal of statin therapy. This was statistically significant in comparison with the untreated cells (Table 24)**.**

Table 23. Cyclin D expression 24 hours after statin treatment

	HUVEC	5nmol/l	Withdrawal group
Simvastatin	0.7(0.01)	0.84(0.02) *	0.73(0.02)

[Statin vs withdrawal group * = $p<0.05$ Student's t- test, values expressed as mean (Standard Error of Mean)]

Table 24. Bax expression 24 hours after statin treatment

	HUVEC	5nmol/l	Withdrawal group
Simvastatin	1.5(0.01)	1.52(0.01)	1.86(0.03) *

[Statin vs withdrawal group * = $p<0.05$ Student's t- test, values expressed as mean (Standard Error of Mean)]

Conclusion

Withdrawal of statin therapy after preincubation of HUVEC in Simvastatin 5nmol/l for 7 days showed a significant reduction in the number of live cells in comparison to the control group which received uninterrupted treatment with a statin. Statin withdrawal was associated with a reduction in the proliferative response of the HUVEC as shown by a lower spectrophotometric absorption. The number of HUVEC undergoing apoptosis as a consequence of the withdrawal of statin therapy was significantly elevated and this was associated with a rise in Bax expression in comparison with the control group. The expression of the cell cycle promoter Cyclin D remained unaffected by withdrawal of statin therapy.

Chapter 9. General Discussion

The indications for the use of HMGCoA reductase inhibitors have evolved from its initial intended use in the early 1990s for hypercholesterolemia. Since then, with the evidence based on large clinical trials, the "pleiotropic effects" of statins were identified. These are the effects of statin independent of its lipid lowering property. Statins reduce cardiovascular morbidity and mortality by improving endothelial function and slowing the progression of atherosclerosis. This is achieved primarily by improving the NO bioavailability within the tissues by enhancing the synthesis of NO and by the antioxidant systems. Other important functions of statins are reduction in endothelial/leucocyte interaction, inhibition of platelet activation and VSMC proliferation.

However, there is a dearth of evidence relating to the effects of statins on endothelial cell proliferation and apoptosis. Statins have an inhibitory effect on vascular smooth muscle proliferation and the regulatory mechanisms involved in this effect are well elucidated (227). Statins block the activation of the Rho and Rac proteins which are critically involved in the translocation of the proteins from the cytosol to the cell membrane (277). This impedes cytoskeleton formation which affects cellular proliferation. However endothelial cells do not behave in a similar fashion on exposure to statins. Large population based clinical studies have demonstrated a survival benefit from statin therapy implying a beneficial effect on endothelial cell survival by favourably modulating its microenvironment. However, there is lack of cell culture based evidence on the response of EC exposed to statins. In addition, it is relevant to characterise any beneficial effects of statins on EC in an environment simulating ischemic insult with hypoxia and associated oxidative stress. Cellular proliferation and apoptosis markers were used to categorise the

degree of insult on the EC as they reflect the survival capability of the EC. An actively proliferating cell has effective counter mechanisms in place to balance the ischemic insults and hence is capable of responding to the natural stimulus to proliferate. However when the counter mechanisms are inadequate, then the cell fails to respond to proliferative stimulus and succumbs to the insult by undergoing apoptosis.

This study set out initially to characterise the cellular responses to statins in Human Saphenous Vein endothelial cells (HSVEC). HSVEC were harvested from long saphenous veins of patients during varicose vein surgery with appropriate consent and local ethics committee approval. However, attempts at obtaining a reliable line of actively growing cells failed due to slow rate of cell proliferation and contamination by vigorously growing smooth muscle cells and fibroblasts. Hence it was deemed inadequate as a source for cell culture studies for cellular proliferation and apoptosis.

At this juncture an alternative cell line was sought, which had rapid growth characteristics for proliferation and apoptosis studies. Human umbilical vein endothelial cells (HUVEC) were hence chosen for this study. They have been extensively studied and have morphological and physiological similarities to arterial EC. In addition, they have been characterised in cellular proliferation, apoptosis and statin related studies previously (278, 279). HUVEC are easily obtained through various commercial sources, can be stored by freezing and are thus a good source for propagating reliable stock for cell culture experiments.

In this study, Simvastatin was chosen as it is the most widely prescribed statin belonging to the Type 1 family of statins. Cerivastatin, although discontinued from clinical practise due

to the incidence of rhabdomyolysis, was the other statin used as it is the most potent statin with clinical effects at lower doses. It belongs to the Type 2 family of synthetic statins.

The concentrations of statins used in this study were based on published data from in-vitro studies on human and animal tissue (280, 281). In addition, the initial dose titration studies helped choose the final statin concentrations for cellular apoptosis and proliferation assay in the context of oxidative and hypoxic stress.

To characterise the response of HUVEC to statins on a time based scale, initial experiments were performed at 24, 48 and 72 hours after incubation with the statin. The best cellular response to proliferative and apoptotic markers were obtained at 24 hours with severe depletion of cell numbers when incubated for longer durations. Hence, 24 hours of incubation with the statin was used for all experiments.

Although pretreatment with statin has been explored in this study, there is a valid arguement to determine the effects of statin treatment after exposure to oxidative and hypoxic stress. There is clinical evidence to substantiate the use of statin after an acute vascular event (282, 283). However, it was beyond the scope of this project to look at this aspect of statins effect on EC.

Controls were used in this study to ensure all confounding effects were identified and discounted. Negative controls were selected such that they were devoid of the active ingredient which was being tested. The positive control with HUVEC alone, ensured that the cells were of a healthy cell line.

Throughout this study, induction of hypoxic and oxidative stress on cells was by the use of chemicals with properties to induce hypoxia or oxidative stress. H_2O_2 was used for inducing oxidative stress. H_2O_2 is a ROS readily available in the cellular microenvironment

and it degrades to H_2O and O_2. $CoCl_2$ has a high affinity for O_2 and renders the microenvironment hypoxic. The use of both these chemicals has been well documented in literature as discussed in Chapter 2. These chemicals allowed for easier and accurate titration of the oxidative and hypoxic stress exposure to the cells. In addition, these chemicals have high solubility in cell culture media which ensured contact of the chemicals to HUVEC.

Statins block the rate limiting step converting HMGCoA to Mevalonate. Mevalonate was used for the various experiments to ensure that the response noted with statin were due to blocking the rate limiting step. Its use in cell culture studies is well documented and the concentrations used were based on data from literature (284).

Chapter 3 examined the effect of statins on cellular proliferation and induction of apoptosis. Higher doses of statin resulted in reduction of live cell count with associated rise in Bax levels. This rise in Bax levels was only observed with high doses of statin which would have been toxic to the cell and likely to have accelerated apoptosis via Bax. The TUNEL assay confirms that the high statin dose related cell death is apoptosis. This is in sharp contrast to the response of statins on human smooth muscle cells where physiological levels of statin cause apoptosis and thus have a beneficial effect in atherosclerosis (285). This study concludes that physiological concentrations of statin have different effects on HUVEC and smooth muscle cells and this protects the integrity of the vasculature by controlling intimal hyperplasia without compromise to the endothelial layer. The study by Llevadot et al demonstrated proliferative response in endothelial progenitor cells exposed to statins (230). In addition, Assmus et al were able to demonstrate a similar effect with an associated rise in Cyclin D levels (249). However there is no evidence in the literature to

suggest that statins have a similar proliferative effect on HUVEC. This is the first study to demonstrate that statins induce a proliferative response in HUVEC. In addition, it has also demonstrated that the proliferative effect on EC is associated with an up regulation of the cell cycle promoter Cyclin D. This study was not designed to elucidate mechanism of action of statin on Cyclin D. Further targeted work is essential to determine the mechanism of interaction between statin and Cyclin D.

The cellular response to various strengths of statins also helped determine statin concentrations best suited for experiments on oxidative and hypoxic stress. Higher statin doses (1000nmol/l and 5000nmol/l) were toxic to HUVEC's with accelerated apoptosis and irreparably damaged cells. However 1nmol/l and 5nmol/l concentrations of simvastatin and cerivastatin produced apoptosis above the mean in the negative control and hence were ideal doses for inducing oxidative and hypoxic stress.

In chapter 4, the effect of oxidative stress on the EC was examined. This was essential to characterise the baseline response of EC to oxidative stress prior to conducting studies to determine the modulating effects of statin in oxidative stress. H_2O_2 is a potent ROS which activates the pro-apoptotic proteins and cytochrome c release from the mitochondria to effect apoptosis. Exposure of HUVEC's to H_2O_2 accelerated the apoptotic phenomena. This was evidenced by a decline in the number of live cells and an increase in the fluorescent uptake by the apoptotic cells. In addition, accelerated apoptosis was associated with a rise in the level of pro-apoptotic protein Bax. This experiment confirms that cell death from exposure to ROS is predominantly apoptosis related. It also confirms that

apoptosis plays a key role in ROS induced cell death in EC and this is mediated by Bax protein.

The proliferative capacity of HUVEC's exposed to oxidative stress was explored in this experiment. ROS have a deleterious effect on cellular proliferation on live cell counting. This study failed to demonstrate a significant reduction in Cyclin D in association with reduction in the proliferative capacity. There is a baseline level of Cyclin D within the cells and its activity is regulated by binding to CDK as discussed in the introduction. Hence, it is likely that Cyclin D levels do not drop in conjunction with reduced cellular proliferation which would be in keeping with the finding of this experiment. In addition, the pathways responsible for dampening the proliferative response are likely to be independent of Cyclin D.

This experiment is akin to pathophysiological states of excessive oxidative stress such as diabetes, hypertension and hypercholesterolemia where accelerated apoptosis and a reduced proliferative response of the lining endothelial cells may predispose to atherosclerosis by exposing the sub-endothelial layers to the cellular elements of the blood.

Chapter 5 explored the impact of statins on the EC subjected to oxidative stress. Although oxidative stress augmented Bax expression in EC, pretreating EC with statins reduced the expression of Bax and thus the oxidative stress related EC apoptosis. This experiment demonstrates that statins offer a defence mechanism against apoptosis induced by oxidative stress. Statins enhance NO synthesis as discussed in the introduction and it is likely that this NO helps to neutralise the oxidative stress and thus facilitate reduction in the apoptosis phenomenon. Cellular proliferation assay used in this study to quantify proliferation is

dependent on activation of the tetrazolium compound by NADP and NAD in actively proliferating cells. Augmented NO synthesis by statins ensure that these substrates are spared of oxidation and thus influence proliferation.

Although oxidative stress had minimal influence on Cyclin D synthesis as demonstrated in Chapter 4, statin exposure in Chapter 3 did show a rise in Cyclin D exposure. In EC pre-treated with statins before exposure to oxidative stress, active cellular proliferation persisted. This was accompanied by an increase in Cyclin D levels. Pre-treating EC cells with statins activate the Cyclin D pathway and this persists with addition of oxidative stress suggesting that statins are more efficient when administered before the oxidative insult.

There was a survival benefit in treating EC with a statin in the context of oxidative stress. The expression of Cyclin D was enhanced and Bax expression reduced, indicating that statin therapy has a normalising effect on the cellular proliferative mechanism. This experiment simulates the effects of statin at a cellular level in the context of oxidative stress prevalent in conditions such as hypertension, hypercholesterolemia and diabetes. This chapter, in addition provides some explanations for the clinical benefits observed in the cardiovascular risk reduction by statin therapy.

Chapter 6 was designed to characterise the baseline response of EC to hypoxic environment prior to pre-treatment with statins in chapter 7. Three doses of $CoCl_2$ were chosen for hypoxic stress induction and 200μmole/l $CoCl_2$ produced statistically significant results which were ideal to test the response of statin. However 100μmole/l was noted to have minimal effect on the EC and 500μmole/l produced significant toxicity and poor quality surviving cells for further experiments.

In addition, this experiment design helped obtain baseline information on EC proliferation and apoptosis response to hypoxic stress. Hypoxia resulted in a dose related increase in apoptosis which was associated with an increase in Bax expression. Although hypoxic stress can directly act on MOMP and cytochrome c to increase EC apoptosis, this experiment has demonstrated an increase in Bax levels. Previous studies on hypoxia in piglet neuronal tissue have demonstrated a similar increase in Bax protein and this study confirms that apoptosis due to hypoxia is mediated in EC through Bax (286). Bax is uncoupled from the antiapoptotic Bcl-2 protein by hypoxic stress and this renders Bax protein free to activate the caspase pathway.

Hypoxic stress produced a reduction in the proliferative function of EC. However this was not associated with any detectable changes in the levels of Cyclin D. As discussed earlier, Cyclin D levels are relatively static and its activity is determined by binding to CDK. Hence, these findings are in keeping with the conclusions drawn from chapter 3.

Atherosclerosis and thromboembolic events can result in acute and chronic hypoxia on the tissues and adversely affect the EC function. Hypoxia in the cellular environment has a negative effect on cellular proliferation and induces apoptosis by enhanced expression of Bax protein.

The modulating effects of statin therapy on the EC subjected to hypoxic stress were studied in chapter 7. Hypoxic stress has deleterious effect on EC proliferation and promotes apoptosis. The effect of pre-treatment of EC with statins is vital to understand how statins modify the effects of oxidative stress on the equilibrium between cell apoptosis and proliferation.

The apoptotic phenomenon was reduced by statin pre-treatment before exposure to hypoxic stress. Chapter 6 had shown that hypoxic stress without statin pre-treatment accelerated apoptosis with consequent rise in Bax protein levels. Here, Bax protein levels were noted to be reduced, offering EC protection against apoptosis. Hypoxia results in accelerated anaerobic metabolism which is an abundant source of ROS and pre-treatment with statins neutralise the effects of ROS by production of NO. This reduces the toxic effect of hypoxia on the EC and promotes cell survival by inhibiting Bax protein activation.

Statin exposure resulted in enhanced cellular proliferation as evidenced by cellular proliferation assay and cyclin D levels. Although a similar effect was not observed when EC was subjected solely to hypoxic stress, pre-treatment with statin helped maintain cellular proliferation which was associated with elevated cyclin D levels.

Hence statin pre-treatment offers survival benefit by reducing the apoptotic influence of hypoxia and promoting cellular proliferation. This experiment demonstrates the protective benefits of statin treatment in pathophysiological states of tissue ischemia causing cellular hypoxia. Pre-treatment with a statin helps abrogate the apoptotic effects of hypoxia and promote cellular proliferation ensuring that the integrity of the vascular endothelial lining is maintained.

Chapter 8 examines the effects of acute statin withdrawal on EC. Compliance to statin therapy is determined by various issues such as drug side-effects and also cost (287). Various trials have demonstrated that statin withdrawal is associated with higher incidence of cardiovascular morbidity and mortality (288-290). Hence withdrawal of statin is an issue of high clinical relevance. This experiment was aimed at determining how statin withdrawal affected the balance between cellular apoptosis and proliferation. Withdrawal

of statin results in accelerated apoptosis and this was accompanied by an increase in the levels of Bax protein expression. With removal of the rate limiting step in cholesterol synthesis, the activation of the messenger proteins G is accelerated via Rho and Ras proteins. This reaction is statin dependent and unblocking this rate limiting step results in a flux of substrates undergoing activation and thus influencing Bax protein activation (291). In addition, reversal of the beneficial effects of statin on EC by mevalonate suggests that metabolites of the mevalonate pathway mediate activation of Bax protein synthesis.

The expression of Cyclin D remained unchanged with statin withdrawal. This observation on Cyclin D was in keeping with the previous experiments where toxic effects on the cell failed to produce any change to Cyclin D levels. This phenomenon has been discussed in the earlier paragraphs.

Statin withdrawal disrupts the balance between cellular apoptosis and proliferation with more cells undergoing apoptosis. This reduces the capacity of the EC lining the vessel wall to repair damage and predisposes to atherosclerosis. This experimental model explains the cellular basis for clinical events observed in statin withdrawal trials.

This work has clearly shown that statins exert a beneficial effect on EC survival by favouring a proliferative response. This is however different to its effect on vascular smooth muscle cell where it favours apoptosis. This difference in its effect is critical to curtail the pathological changes of atherosclerosis on the vessel wall. In addition, this work has shown that statins have a protective effect on EC in the context of hypoxia and ischemia by modulating intracellular mechanisms such as Bax protein and Cyclin D.

These beneficial effects of statins can be extrapolated to the clinical scenario of ischemia reperfusion. The mechanisms involved here include an initial ischemic insult on the cell resulting in hypoxic damage to the cellular mechanisms. Subsequent reperfusion exposes the cell to ROS and oxidative stress. This causes EC damage and death with predisposition to a prothrombotic state.

Statins are well documented to have anti-inflammatory and anti-thrombotic effects as discussed in the introduction. In addition, the evidence from this work supports the hypothesis that statins offer EC a survival benefit by modulating the deleterious effects of hypoxia and oxidative stress. Animal studies on ischaemia reperfusion have demonstrated benefits from the use of a statin (292, 293). However the mechanisms involved at the cellular level remain unknown and future studies in this direction would be invaluable to understand the cellular responses to this complex phenomenon.

References

1. Pepper O. The History and Derivation of Medical Terms. Medical Etymology, Philadelphia, Pa: WB Saunders. 1949.

2. Jaffe EA. Physiologic functions of normal endothelial cells. Ann N Y Acad Sci. 1985;454:279-91.

3. EM Renkin CM, SR Geiger. Handbook of Physiology Section 2 The cardiovascular System Volume 4,Part 1. 1984:42.

4. LWeiss. Cell and Tissue Biology, A Textbook of Histology. Cell and Tissue Biology, A Textbook of Histology. 1988:357-62.

5. Furchgott RF, Zawadzki JV. The obligatory role of endothelial cells in the relaxation of arterial smooth muscle by acetylcholine. Nature. 1980 Nov 27;288(5789):373-6.

6. Nathan C, Xie QW. Nitric oxide synthases: roles, tolls, and controls. Cell. 1994 Sep 23;78(6):915-8.

7. Kilbourn RG, Belloni P. Endothelial cell production of nitrogen oxides in response to interferon gamma in combination with tumor necrosis factor, interleukin-1, or endotoxin. J Natl Cancer Inst. 1990 May 2;82(9):772-6.

8. Busse R, Mulsch A. Induction of nitric oxide synthase by cytokines in vascular smooth muscle cells. FEBS Lett. 1990 Nov 26;275(1-2):87-90.

9. Schini VB, Kim ND, Vanhoutte PM. The basal and stimulated release of EDRF inhibits the contractions evoked by endothelin-1 and endothelin-3 in aortae of normotensive and spontaneously hypertensive rats. J Cardiovasc Pharmacol. 1991;17 Suppl 7:S267-71.

10. Tayeh MA, Marletta MA. Macrophage oxidation of L-arginine to nitric oxide, nitrite, and nitrate. Tetrahydrobiopterin is required as a cofactor. J Biol Chem. 1989 Nov 25;264(33):19654-8.

11. Ratz PH, Gleason MM, Flaim SF. Simultaneous measurement of force and calcium uptake during acetylcholine-induced endothelium-dependent relaxation of rabbit thoracic aorta. Circ Res. 1987 Jan;60(1):31-8.

12. Popescu LM, Panoiu C, Hinescu M, Nutu O. The mechanism of cGMP-induced relaxation in vascular smooth muscle. Eur J Pharmacol. 1985 Jan 8;107(3):393-4.

13. Gryglewski RJ, Bunting S, Moncada S, Flower RJ, Vane JR. Arterial walls are protected against deposition of platelet thrombi by a substance (prostaglandin X) which they make from prostaglandin endoperoxides. Prostaglandins. 1976 Nov;12(5):685-713.

14. Eldor A, Falcone DJ, Hajjar DP, Minick CR, Weksler BB. Recovery of prostacyclin production by de-endothelialized rabbit aorta. Critical role of neointimal smooth muscle cells. J Clin Invest. 1981 Mar;67(3):735-41.

15. Salmon JA, Mullane KM, Dusting GJ, Moncada S, Vane JR. Elimination of prostacyclin (PGI2) and 6-oxo-PGF1 alpha in anaesthetized dogs. J Pharm Pharmacol. 1979 Aug;31(8):529-32.

16. Moncada S, Gryglewski R, Bunting S, Vane JR. An enzyme isolated from arteries transforms prostaglandin endoperoxides to an unstable substance that inhibits platelet aggregation. Nature. 1976 Oct 21;263(5579):663-5.

17. Gryglewski RJ, Szczeklik A, Nizankowski R. Anti-platelet action of intravenous infusion of prostacyclin in man. Thromb Res. 1978 Aug;13(2):153-63.

18. Shimokawa H, Flavahan NA, Lorenz RR, Vanhoutte PM. Prostacyclin releases endothelium-derived relaxing factor and potentiates its action in coronary arteries of the pig. Br J Pharmacol. 1988 Dec;95(4):1197-203.

19. Inoue A, Yanagisawa M, Kimura S, Kasuya Y, Miyauchi T, Goto K, et al. The human endothelin family: three structurally and pharmacologically distinct isopeptides predicted by three separate genes. Proc Natl Acad Sci U S A. 1989 Apr;86(8):2863-7.

20. Schini VB, Hendrickson H, Heublein DM, Burnett JC, Jr., Vanhoutte PM. Thrombin enhances the release of endothelin from cultured porcine aortic endothelial cells. Eur J Pharmacol. 1989 Jun 20;165(2-3):333-4.

21. Kurihara H, Yoshizumi M, Sugiyama T, Yamaoki K, Nagai R, Takaku F, et al. The possible role of endothelin-1 in the pathogenesis of coronary vasospasm. J Cardiovasc Pharmacol. 1989;13 Suppl 5:S132-7; discussion S42.

22. Hieda HS, Gomez-Sanchez CE. Hypoxia increases endothelin release in bovine endothelial cells in culture, but epinephrine, norepinephrine, serotonin, histamine and angiotensin II do not. Life Sci. 1990;47(3):247-51.

23. Yanagisawa M, Kurihara H, Kimura S, Tomobe Y, Kobayashi M, Mitsui Y, et al. A novel potent vasoconstrictor peptide produced by vascular endothelial cells. Nature. 1988 Mar 31;332(6163):411-5.

24. Resink TJ, Scott-Burden T, Buhler FR. Endothelin stimulates phospholipase C in cultured vascular smooth muscle cells. Biochem Biophys Res Commun. 1988 Dec 30;157(3):1360-8.

25. Yoshizumi M, Kurihara H, Sugiyama T, Takaku F, Yanagisawa M, Masaki T, et al. Hemodynamic shear stress stimulates endothelin production by cultured endothelial cells. Biochem Biophys Res Commun. 1989 Jun 15;161(2):859-64.

26. Shiba R, Yanagisawa M, Miyauchi T, Ishii Y, Kimura S, Uchiyama Y, et al. Elimination of intravenously injected endothelin-1 from the circulation of the rat. J Cardiovasc Pharmacol. 1989;13 Suppl 5:S98-101; discussion S2.

27. Hirata Y, Yoshimi H, Takaichi S, Yanagisawa M, Masaki T. Binding and receptor down-regulation of a novel vasoconstrictor endothelin in cultured rat vascular smooth muscle cells. FEBS Lett. 1988 Oct 24;239(1):13-7.

28. Boulanger C, Luscher TF. Release of endothelin from the porcine aorta. Inhibition by endothelium-derived nitric oxide. J Clin Invest. 1990 Feb;85(2):587-90.

29. de Nucci G, Thomas R, D'Orleans-Juste P, Antunes E, Walder C, Warner TD, et al. Pressor effects of circulating endothelin are limited by its removal in the pulmonary circulation and by the release of prostacyclin and endothelium-derived relaxing factor. Proc Natl Acad Sci U S A. 1988 Dec;85(24):9797-800.

30. Luscher TF, Aarhus LL, Vanhoutte PM. Indomethacin improves the impaired endothelium-dependent relaxations in small mesenteric arteries of the spontaneously hypertensive rat. Am J Hypertens. 1990 Jan;3(1):55-8.

31. Vaughan DE, Lazos SA, Tong K. Angiotensin II regulates the expression of plasminogen activator inhibitor-1 in cultured endothelial cells. A potential link between the renin-angiotensin system and thrombosis. J Clin Invest. 1995 Mar;95(3):995-1001.

32. Stoltz JF. Vascular potential and thrombosis. Thromb Res. 1983;Suppl 5:73-82.

33. Mellion BT, Ignarro LJ, Myers CB, Ohlstein EH, Ballot BA, Hyman AL, et al. Inhibition of human platelet aggregation by S-nitrosothiols. Heme-dependent activation of soluble guanylate cyclase and stimulation of cyclic GMP accumulation. Mol Pharmacol. 1983 May;23(3):653-64.

34. Kulkarni PS, Roberts R, Needleman P. Paradoxical endogenous synthesis of a coronary dilating substance from arachidonate. Prostaglandins. 1976 Sep;12(3):337-53.

35. Hong SL. Effect of bradykinin and thrombin on prostacyclin synthesis in endothelial cells from calf and pig aorta and human umbilical cord vein. Thromb Res. 1980 Jun 15;18(6):787-95.

36. Coughlin SR, Moskowitz MA, Zetter BR, Antoniades HN, Levine L. Platelet-dependent stimulation of prostacyclin synthesis by platelet-derived growth factor. Nature. 1980 Dec 11;288(5791):600-2.

37. Weksler BB, Ley CW, Jaffe EA. Stimulation of endothelial cell prostacyclin production by thrombin, trypsin, and the ionophore A 23187. J Clin Invest. 1978 Nov;62(5):923-30.

38. Baenziger NL, Fogerty FJ, Mertz LF, Chernuta LF. Regulation of histamine-mediated prostacyclin synthesis in cultured human vascular endothelial cells. Cell. 1981 Jun;24(3):915-23.

39. Pearson JD, Carleton JS, Gordon JL. Metabolism of adenine nucleotides by ectoenzymes of vascular endothelial and smooth-muscle cells in culture. Biochem J. 1980 Aug 15;190(2):421-9.

40. Owen WG, Esmon CT. Functional properties of an endothelial cell cofactor for thrombin-catalyzed activation of protein C. J Biol Chem. 1981 Jun 10;256(11):5532-5.

41. Esmon NL, Owen WG, Esmon CT. Isolation of a membrane-bound cofactor for thrombin-catalyzed activation of protein C. J Biol Chem. 1982 Jan 25;257(2):859-64.

42. Lammle B, Griffin JH. Formation of the fibrin clot: the balance of procoagulant and inhibitory factors. Clin Haematol. 1985 Jun;14(2):281-342.

43. Kisiel W, Canfield WM, Ericsson LH, Davie EW. Anticoagulant properties of bovine plasma protein C following activation by thrombin. Biochemistry. 1977 Dec 27;16(26):5824-31.

44. Yegneswaran S, Wood GM, Esmon CT, Johnson AE. Protein S alters the active site location of activated protein C above the membrane surface. A fluorescence resonance energy transfer study of topography. J Biol Chem. 1997 Oct 3;272(40):25013-21.

45. Busch C, Owen WG. Identification in vitro of an endothelial cell surface cofactor for antithrombin III. Parallel studies with isolated perfused rat hearts and microcarrier cultures of bovine endothelium. J Clin Invest. 1982 Mar;69(3):726-9.

46. Lollar P, Owen WG. Clearance of thrombin from circulation in rabbits by high-affinity binding sites on endothelium. Possible role in the inactivation of thrombin by antithrombin III. J Clin Invest. 1980 Dec;66(6):1222-30.

47. Lynch DC, Williams R, Zimmerman TS, Kirby EP, Livingston DM. Biosynthesis of the subunits of factor VIIIR by bovine aortic endothelial cells. Proc Natl Acad Sci U S A. 1983 May;80(9):2738-42.

48. Lynch DC, Zimmerman TS, Kirby EP, Livingston DM. Subunit composition of oligomeric human von Willebrand factor. J Biol Chem. 1983 Nov 10;258(21):12757-60.

49. Zimmerman GA, McIntyre TM, Prescott SM. Production of platelet-activating factor by human vascular endothelial cells: evidence for a requirement for specific agonists and modulation by prostacyclin. Circulation. 1985 Oct;72(4):718-27.

50. Prescott SM, McIntyre TM, Zimmerman GA. The role of platelet-activating factor in endothelial cells. Thromb Haemost. 1990 Aug 13;64(1):99-103.

51. Siminiak T, Flores NA, Sheridan DJ. Neutrophil interactions with endothelium and platelets: possible role in the development of cardiovascular injury. Eur Heart J. 1995 Feb;16(2):160-70.

52. Bernatchez PN, Allen BG, Gelinas DS, Guillemette G, Sirois MG. Regulation of VEGF-induced endothelial cell PAF synthesis: role of p42/44 MAPK, p38 MAPK and PI3K pathways. Br J Pharmacol. 2001 Nov;134(6):1253-62.

53. Hofmann B, Ostermann G, Hoffmann A, Klocking HP, Kertscher HP. Effect of specific antagonists on PAF-induced platelet aggregation and release of plasminogen activator. Biomed Biochim Acta. 1988;47(10-11):S157-60.

54. Booyse FM, Osikowicz G, Feder S, Scheinbuks J. Isolation and characterization of a urokinase-type plasminogen activator (Mr = 54,000) from cultured human endothelial cells indistinguishable from urinary urokinase. J Biol Chem. 1984 Jun 10;259(11):7198-205.

55. Tan P, Luscinskas FW, Homer-Vanniasinkam S. Cellular and molecular mechanisms of inflammation and thrombosis. Eur J Vasc Endovasc Surg. 1999 May;17(5):373-89.

56. Loppnow H, Libby P. Comparative analysis of cytokine induction in human vascular endothelial and smooth muscle cells. Lymphokine Res. 1989 Fall;8(3):293-9.

57. Madge LA, Pober JS. TNF signaling in vascular endothelial cells. Exp Mol Pathol. 2001 Jun;70(3):317-25.

58. Loyer P, Ilyin G, Abdel Razzak Z, Banchereau J, Dezier JF, Campion JP, et al. Interleukin 4 inhibits the production of some acute-phase proteins by human hepatocytes in primary culture. FEBS Lett. 1993 Dec 27;336(2):215-20.

59. Miossec P. [Anti-inflammatory properties of interleukin-4]. Rev Rhum Ed Fr. 1993 Feb;60(2):119-24.

60. Van Damme J, Rampart M, Conings R, Decock B, Van Osselaer N, Willems J, et al. The neutrophil-activating proteins interleukin 8 and beta-thromboglobulin: in vitro and in vivo comparison of NH2-terminally processed forms. Eur J Immunol. 1990 Sep;20(9):2113-8.

61. Dustin ML, Singer KH, Tuck DT, Springer TA. Adhesion of T lymphoblasts to epidermal keratinocytes is regulated by interferon gamma and is mediated by intercellular adhesion molecule 1 (ICAM-1). J Exp Med. 1988 Apr 1;167(4):1323-40.

62. Dustin ML, Springer TA. Lymphocyte function-associated antigen-1 (LFA-1) interaction with intercellular adhesion molecule-1 (ICAM-1) is one of at least three mechanisms for lymphocyte adhesion to cultured endothelial cells. J Cell Biol. 1988 Jul;107(1):321-31.

63. Springer TA. Adhesion receptors of the immune system. Nature. 1990 Aug 2;346(6283):425-34.

64. Thornhill MH, Kyan-Aung U, Haskard DO. IL-4 increases human endothelial cell adhesiveness for T cells but not for neutrophils. J Immunol. 1990 Apr 15;144(8):3060-5.

65. Castellot JJ, Jr., Addonizio ML, Rosenberg R, Karnovsky MJ. Cultured endothelial cells produce a heparinlike inhibitor of smooth muscle cell growth. J Cell Biol. 1981 Aug;90(2):372-9.

66. DiCorleto PE. Cultured endothelial cells produce multiple growth factors for connective tissue cells. Exp Cell Res. 1984 Jul;153(1):167-72.

67. Kusuhara M, Yamaguchi K, Ohnishi A, Abe K, Kimura S, Oono H, et al. Endothelin potentiates growth factor-stimulated DNA synthesis in Swiss 3T3 cells. Jpn J Cancer Res. 1989 Apr;80(4):302-5.

68. Suzuki Y, Ruiz-Ortega M, Lorenzo O, Ruperez M, Esteban V, Egido J. Inflammation and angiotensin II. Int J Biochem Cell Biol. 2003 Jun;35(6):881-900.

69. Zachary I. Vascular endothelial growth factor. Int J Biochem Cell Biol. 1998 Nov;30(11):1169-74.

70. Baker DP, Van Lenten BJ, Fogelman AM, Edwards PA, Kean C, Berliner JA. LDL, scavenger, and beta-VLDL receptors on aortic endothelial cells. Arteriosclerosis. 1984 May-Jun;4(3):248-55.

71. Fielding CJ, Vlodavsky I, Fielding PE, Gospodarowicz D. Characteristics of chylomicron binding and lipid uptake by endothelial cells in culture. J Biol Chem. 1979 Sep 25;254(18):8861-8.

72. Sage H, Bornstein P. Endothelial cells from umbilical vein and a hemangioendothelioma secrete basement membrane largely to the exclusion of interstitial procollagens. Arteriosclerosis. 1982 Jan-Feb;2(1):27-36.

73. Mecham RP, Madaras J, McDonald JA, Ryan U. Elastin production by cultured calf pulmonary artery endothelial cells. J Cell Physiol. 1983 Sep;116(3):282-8.

74. Kramer RH, Fuh GM, Bensch KG, Karasek MA. Synthesis of extracellular matrix glycoproteins by cultured microvascular endothelial cells isolated from the dermis of neonatal and adult skin. J Cell Physiol. 1985 Apr;123(1):1-9.

75. Gospodarowicz D, Greenburg G, Foidart JM, Savion N. The production and localization of laminin in cultured vascular and corneal endothelial cells. J Cell Physiol. 1981 May;107(2):171-83.

76. Bihari-Varga M, Csonka E, Jellinek H. Endothelial glycosaminoglycans-in vitro studies. Artery. 1980;8(4):355-61.

77. Oohira A, Wight TN, Bornstein P. Sulfated proteoglycans synthesized by vascular endothelial cells in culture. J Biol Chem. 1983 Feb 10;258(3):2014-21.

78. McPherson J, Sage H, Bornstein P. Isolation and characterization of a glycoprotein secreted by aortic endothelial cells in culture. Apparent identity with platelet thrombospondin. J Biol Chem. 1981 Nov 10;256(21):11330-6.

79. Birdwell CR, Gospodarowicz D, Nicolson GL. Identification, localization, and role of fibronectin in cultured bovine endothelial cells. Proc Natl Acad Sci U S A. 1978 Jul;75(7):3273-7.

80. Ross R. Atherosclerosis--an inflammatory disease. N Engl J Med. 1999 Jan 14;340(2):115-26.

81. Taddei S, Virdis A, Ghiadoni L, Salvetti G, Bernini G, Magagna A, et al. Age-related reduction of NO availability and oxidative stress in humans. Hypertension. 2001 Aug;38(2):274-9.

82. Barton M, Cosentino F, Brandes RP, Moreau P, Shaw S, Luscher TF. Anatomic heterogeneity of vascular aging: role of nitric oxide and endothelin. Hypertension. 1997 Oct;30(4):817-24.

83. Tschudi MR, Barton M, Bersinger NA, Moreau P, Cosentino F, Noll G, et al. Effect of age on kinetics of nitric oxide release in rat aorta and pulmonary artery. J Clin Invest. 1996 Aug 15;98(4):899-905.

84. Hamilton CA, Brosnan MJ, McIntyre M, Graham D, Dominiczak AF. Superoxide excess in hypertension and aging: a common cause of endothelial dysfunction. Hypertension. 2001 Feb;37(2 Part 2):529-34.

85. Barua RS, Ambrose JA, Eales-Reynolds LJ, DeVoe MC, Zervas JG, Saha DC. Heavy and light cigarette smokers have similar dysfunction of endothelial vasoregulatory activity: an in vivo and in vitro correlation. J Am Coll Cardiol. 2002 Jun 5;39(11):1758-63.

86. Mikhailidis DP, Barradas MA, Jeremy JY, Dandona P. Cigarette smoking inhibits prostacyclin formation. Lancet. 1983 Sep 10;2(8350):627-8.

87. Shen Y, Rattan V, Sultana C, Kalra VK. Cigarette smoke condensate-induced adhesion molecule expression and transendothelial migration of monocytes. Am J Physiol. 1996 May;270(5 Pt 2):H1624-33.

88. Carty CS, Huribal M, Marsan BU, Ricotta JJ, Dryjski M. Nicotine and its metabolite cotinine are mitogenic for human vascular smooth muscle cells. J Vasc Surg. 1997 Apr;25(4):682-8.

89. Woodman OL. Modulation of vasoconstriction by endothelium-derived nitric oxide: the influence of vascular disease. Clin Exp Pharmacol Physiol. 1995 Sep;22(9):585-93.

90. Kugiyama K, Kerns SA, Morrisett JD, Roberts R, Henry PD. Impairment of endothelium-dependent arterial relaxation by lysolecithin in modified low-density lipoproteins. Nature. 1990 Mar 8;344(6262):160-2.

91. Keaney JF, Jr., Guo Y, Cunningham D, Shwaery GT, Xu A, Vita JA. Vascular incorporation of alpha-tocopherol prevents endothelial dysfunction due to oxidized LDL by inhibiting protein kinase C stimulation. J Clin Invest. 1996 Jul 15;98(2):386-94.

92. Harrison DG. Endothelial control of vasomotion and nitric oxide production: a potential target for risk factor management. Cardiol Clin. 1996 Feb;14(1):1-15.

93. Salvemini D, Wang ZQ, Bourdon DM, Stern MK, Currie MG, Manning PT. Evidence of peroxynitrite involvement in the carrageenan-induced rat paw edema. Eur J Pharmacol. 1996 May 15;303(3):217-20.

94. Salvemini D, Riley DP, Lennon PJ, Wang ZQ, Currie MG, Macarthur H, et al. Protective effects of a superoxide dismutase mimetic and peroxynitrite decomposition catalysts in endotoxin-induced intestinal damage. Br J Pharmacol. 1999 Jun;127(3):685-92.

95. White CR BT, Chang LY,. Superoxide and peroxynitrite in atherosclerosis. Proc Natl Acad Sci USA. 1994;91:1044-8.

96. Baylis C, Mitruka B, Deng A. Chronic blockade of nitric oxide synthesis in the rat produces systemic hypertension and glomerular damage. J Clin Invest. 1992 Jul;90(1):278-81.

97. Griendling KK, Minieri CA, Ollerenshaw JD, Alexander RW. Angiotensin II stimulates NADH and NADPH oxidase activity in cultured vascular smooth muscle cells. Circ Res. 1994 Jun;74(6):1141-8.

98. Christ M, Bauersachs J, Liebetrau C, Heck M, Gunther A, Wehling M. Glucose increases endothelial-dependent superoxide formation in coronary arteries by NAD(P)H oxidase activation: attenuation by the 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitor atorvastatin. Diabetes. 2002 Aug;51(8):2648-52.

99. Podrez EA, Abu-Soud HM, Hazen SL. Myeloperoxidase-generated oxidants and atherosclerosis. Free Radic Biol Med. 2000 Jun 15;28(12):1717-25.

100. Daugherty A, Dunn JL, Rateri DL, Heinecke JW. Myeloperoxidase, a catalyst for lipoprotein oxidation, is expressed in human atherosclerotic lesions. J Clin Invest. 1994 Jul;94(1):437-44.

101. Tucker BJ. Early onset of increased transcapillary albumin escape in awake diabetic rats. Diabetes. 1990 Aug;39(8):919-23.

102. Williams B, Baker AQ, Gallacher B, Lodwick D. Angiotensin II increases vascular permeability factor gene expression by human vascular smooth muscle cells. Hypertension. 1995 May;25(5):913-7.

103. Williams B, Gallacher B, Patel H, Orme C. Glucose-induced protein kinase C activation regulates vascular permeability factor mRNA expression and peptide production by human vascular smooth muscle cells in vitro. Diabetes. 1997 Sep;46(9):1497-503.

104. Leung DW, Cachianes G, Kuang WJ, Goeddel DV, Ferrara N. Vascular endothelial growth factor is a secreted angiogenic mitogen. Science. 1989 Dec 8;246(4935):1306-9.

105. Shweiki D, Itin A, Neufeld G, Gitay-Goren H, Keshet E. Patterns of expression of vascular endothelial growth factor (VEGF) and VEGF receptors in mice suggest a role in hormonally regulated angiogenesis. J Clin Invest. 1993 May;91(5):2235-43.

106. Ohara Y, Sayegh HS, Yamin JJ, Harrison DG. Regulation of endothelial constitutive nitric oxide synthase by protein kinase C. Hypertension. 1995 Mar;25(3):415-20.

107. Rosenthal ET, Hunt T, Ruderman JV. Selective translation of mRNA controls the pattern of protein synthesis during early development of the surf clam, Spisula solidissima. Cell. 1980 Jun;20(2):487-94.

108. Matsushime H, Ewen ME, Strom DK, Kato JY, Hanks SK, Roussel MF, et al. Identification and properties of an atypical catalytic subunit (p34PSK-J3/cdk4) for mammalian D type G1 cyclins. Cell. 1992 Oct 16;71(2):323-34.

109. Matsushime H, Roussel MF, Sherr CJ. Novel mammalian cyclins (CYL genes) expressed during G1. Cold Spring Harb Symp Quant Biol. 1991;56:69-74.

110. Miyashita T, Reed JC. Tumor suppressor p53 is a direct transcriptional activator of the human bax gene. Cell. 1995 Jan 27;80(2):293-9.

111. Pagano M, Pepperkok R, Verde F, Ansorge W, Draetta G. Cyclin A is required at two points in the human cell cycle. Embo J. 1992 Mar;11(3):961-71.

112. Sherr CJ, Kato J, Quelle DE, Matsuoka M, Roussel MF. D-type cyclins and their cyclin-dependent kinases: G1 phase integrators of the mitogenic response. Cold Spring Harb Symp Quant Biol. 1994;59:11-9.

113. Mihara K, Cao XR, Yen A, Chandler S, Driscoll B, Murphree AL, et al. Cell cycle-dependent regulation of phosphorylation of the human retinoblastoma gene product. Science. 1989 Dec 8;246(4935):1300-3.

114. Buchkovich K, Duffy LA, Harlow E. The retinoblastoma protein is phosphorylated during specific phases of the cell cycle. Cell. 1989 Sep 22;58(6):1097-105.

115. Reynisdottir I, Polyak K, Iavarone A, Massague J. Kip/Cip and Ink4 Cdk inhibitors cooperate to induce cell cycle arrest in response to TGF-beta. Genes Dev. 1995 Aug 1;9(15):1831-45.

116. Isobe M, Emanuel BS, Givol D, Oren M, Croce CM. Localization of gene for human p53 tumour antigen to band 17p13. Nature. 1986 Mar 6-12;320(6057):84-5.

117. Farmer G, Bargonetti J, Zhu H, Friedman P, Prywes R, Prives C. Wild-type p53 activates transcription in vitro. Nature. 1992 Jul 2;358(6381):83-6.

118. Ababneh M, Gotz C, Montenarh M. Downregulation of the cdc2/cyclin B protein kinase activity by binding of p53 to p34(cdc2). Biochem Biophys Res Commun. 2001 May 4;283(2):507-12.

119. Kazantsev A, Sancar A. Does the p53 up-regulated Gadd45 protein have a role in excision repair? Science. 1995 Nov 10;270(5238):1003-4; author reply 5-6.

120. Kearsey JM, Shivji MK, Hall PA, Wood RD. Does the p53 up-regulated Gadd45 protein have a role in excision repair? Science. 1995 Nov 10;270(5238):1004-5; author reply 5-6.

121. Mummenbrauer T, Janus F, Muller B, Wiesmuller L, Deppert W, Grosse F. p53 Protein exhibits 3'-to-5' exonuclease activity. Cell. 1996 Jun 28;85(7):1089-99.

122. Kerr JF, Wyllie AH, Currie AR. Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics. Br J Cancer. 1972 Aug;26(4):239-57.

123. Debatin KM, Krammer PH. Death receptors in chemotherapy and cancer. Oncogene. 2004 Apr 12;23(16):2950-66.

124. Green DR, Kroemer G. The pathophysiology of mitochondrial cell death. Science. 2004 Jul 30;305(5684):626-9.

125. Kuwana T, Mackey MR, Perkins G, Ellisman MH, Latterich M, Schneiter R, et al. Bid, Bax, and lipids cooperate to form supramolecular openings in the outer mitochondrial membrane. Cell. 2002 Nov 1;111(3):331-42.

126. Slee EA, Harte MT, Kluck RM, Wolf BB, Casiano CA, Newmeyer DD, et al. Ordering the cytochrome c-initiated caspase cascade: hierarchical activation of caspases-2, -3, -6, -7, -8, and -10 in a caspase-9-dependent manner. J Cell Biol. 1999 Jan 25;144(2):281-92.

127. Bouillet P, Strasser A. BH3-only proteins - evolutionarily conserved proapoptotic Bcl-2 family members essential for initiating programmed cell death. J Cell Sci. 2002 Apr 15;115(Pt 8):1567-74.

128. Donovan M, Cotter TG. Control of mitochondrial integrity by Bcl-2 family members and caspase-independent cell death. Biochim Biophys Acta. 2004 Mar 1;1644(2-3):133-47.

129. Zimmermann KC, Bonzon C, Green DR. The machinery of programmed cell death. Pharmacol Ther. 2001 Oct;92(1):57-70.

130. Ichas F, Mazat JP. From calcium signaling to cell death: two conformations for the mitochondrial permeability transition pore. Switching from low- to high-conductance state. Biochim Biophys Acta. 1998 Aug 10;1366(1-2):33-50.

131. Crompton M. The mitochondrial permeability transition pore and its role in cell death. Biochem J. 1999 Jul 15;341 (Pt 2):233-49.

132. Salvesen GS, Duckett CS. IAP proteins: blocking the road to death's door. Nat Rev Mol Cell Biol. 2002 Jun;3(6):401-10.

133. Beere HM, Wolf BB, Cain K, Mosser DD, Mahboubi A, Kuwana T, et al. Heat-shock protein 70 inhibits apoptosis by preventing recruitment of procaspase-9 to the Apaf-1 apoptosome. Nat Cell Biol. 2000 Aug;2(8):469-75.

134. Mosser DD, Caron AW, Bourget L, Meriin AB, Sherman MY, Morimoto RI, et al. The chaperone function of hsp70 is required for protection against stress-induced apoptosis. Mol Cell Biol. 2000 Oct;20(19):7146-59.

135. Schwartz D, Rotter V. p53-dependent cell cycle control: response to genotoxic stress. Semin Cancer Biol. 1998;8(5):325-36.

136. Gottlieb TM, Oren M. p53 and apoptosis. Semin Cancer Biol. 1998;8(5):359-68.

137. MacLachlan TK, El-Deiry WS. Apoptotic threshold is lowered by p53 transactivation of caspase-6. Proc Natl Acad Sci U S A. 2002 Jul 9;99(14):9492-7.

138. Gupta S, Radha V, Sudhakar C, Swarup G. A nuclear protein tyrosine phosphatase activates p53 and induces caspase-1-dependent apoptosis. FEBS Lett. 2002 Dec 4;532(1-2):61-6.

139. Chiarugi P, Cirri P. Redox regulation of protein tyrosine phosphatases during receptor tyrosine kinase signal transduction. Trends Biochem Sci. 2003 Sep;28(9):509-14.

140. Reth M. Hydrogen peroxide as second messenger in lymphocyte activation. Nat Immunol. 2002 Dec;3(12):1129-34.

141. Sauer H, Wartenberg M, Hescheler J. Reactive oxygen species as intracellular messengers during cell growth and differentiation. Cell Physiol Biochem. 2001;11(4):173-86.

142. Darley-Usmar V, Wiseman H, Halliwell B. Nitric oxide and oxygen radicals: a question of balance. FEBS Lett. 1995 Aug 7;369(2-3):131-5.

143. Fridovich I. Superoxide anion radical (O2-.), superoxide dismutases, and related matters. J Biol Chem. 1997 Jul 25;272(30):18515-7.

144. Babior BM. NADPH oxidase: an update. Blood. 1999 Mar 1;93(5):1464-76.

145. Griendling KK, Sorescu D, Ushio-Fukai M. NAD(P)H oxidase: role in cardiovascular biology and disease. Circ Res. 2000 Mar 17;86(5):494-501.

146. Harrison DG. Endothelial function and oxidant stress. Clin Cardiol. 1997 Nov;20(11 Suppl 2):II-11-7.

147. Lassegue B, Clempus RE. Vascular NAD(P)H oxidases: specific features, expression, and regulation. Am J Physiol Regul Integr Comp Physiol. 2003 Aug;285(2):R277-97.

148. Grote K, Flach I, Luchtefeld M, Akin E, Holland SM, Drexler H, et al. Mechanical stretch enhances mRNA expression and proenzyme release of matrix metalloproteinase-2 (MMP-2) via NAD(P)H oxidase-derived reactive oxygen species. Circ Res. 2003 Jun 13;92(11):e80-6.

149. Nishikawa T, Edelstein D, Du XL, Yamagishi S, Matsumura T, Kaneda Y, et al. Normalizing mitochondrial superoxide production blocks three pathways of hyperglycaemic damage. Nature. 2000 Apr 13;404(6779):787-90.

150. Hink U, Li H, Mollnau H, Oelze M, Matheis E, Hartmann M, et al. Mechanisms underlying endothelial dysfunction in diabetes mellitus. Circ Res. 2001 Feb 2;88(2):E14-22.

151. White CR, Darley-Usmar V, Berrington WR, McAdams M, Gore JZ, Thompson JA, et al. Circulating plasma xanthine oxidase contributes to vascular dysfunction in hypercholesterolemic rabbits. Proc Natl Acad Sci U S A. 1996 Aug 6;93(16):8745-9.

152. Milstien S, Katusic Z. Oxidation of tetrahydrobiopterin by peroxynitrite: implications for vascular endothelial function. Biochem Biophys Res Commun. 1999 Oct 5;263(3):681-4.

153. Vasquez-Vivar J, Duquaine D, Whitsett J, Kalyanaraman B, Rajagopalan S. Altered tetrahydrobiopterin metabolism in atherosclerosis: implications for use of oxidized tetrahydrobiopterin analogues and thiol antioxidants. Arterioscler Thromb Vasc Biol. 2002 Oct 1;22(10):1655-61.

154. Bagi Z, Koller A. Lack of nitric oxide mediation of flow-dependent arteriolar dilation in type I diabetes is restored by sepiapterin. J Vasc Res. 2003 Jan-Feb;40(1):47-57.

155. Landmesser U, Dikalov S, Price SR, McCann L, Fukai T, Holland SM, et al. Oxidation of tetrahydrobiopterin leads to uncoupling of endothelial cell nitric oxide synthase in hypertension. J Clin Invest. 2003 Apr;111(8):1201-9.

156. Beckman JS, Koppenol WH. Nitric oxide, superoxide, and peroxynitrite: the good, the bad, and ugly. Am J Physiol. 1996 Nov;271(5 Pt 1):C1424-37.

157. Griendling KK, Sorescu D, Lassegue B, Ushio-Fukai M. Modulation of protein kinase activity and gene expression by reactive oxygen species and their role in vascular physiology and pathophysiology. Arterioscler Thromb Vasc Biol. 2000 Oct;20(10):2175-83.

158. Zalba G, San Jose G, Moreno MU, Fortuno MA, Fortuno A, Beaumont FJ, et al. Oxidative stress in arterial hypertension: role of NAD(P)H oxidase. Hypertension. 2001 Dec 1;38(6):1395-9.

159. Cai H, Harrison DG. Endothelial dysfunction in cardiovascular diseases: the role of oxidant stress. Circ Res. 2000 Nov 10;87(10):840-4.

160. Garcia Soriano F, Virag L, Jagtap P, Szabo E, Mabley JG, Liaudet L, et al. Diabetic endothelial dysfunction: the role of poly(ADP-ribose) polymerase activation. Nat Med. 2001 Jan;7(1):108-13.

161. Kamoshima W, Kitamura Y, Nomura Y, Taniguchi T. Possible involvement of ADP-ribosylation of particular enzymes in cell death induced by nitric oxide-donors in human neuroblastoma cells. Neurochem Int. 1997 Mar;30(3):305-11.

162. Nagase M, Ando K, Nagase T, Kaname S, Sawamura T, Fujita T. Redox-sensitive regulation of lox-1 gene expression in vascular endothelium. Biochem Biophys Res Commun. 2001 Mar 2;281(3):720-5.

163. Pueyo ME, Gonzalez W, Nicoletti A, Savoie F, Arnal JF, Michel JB. Angiotensin II stimulates endothelial vascular cell adhesion molecule-1 via nuclear factor-kappaB activation induced by intracellular oxidative stress. Arterioscler Thromb Vasc Biol. 2000 Mar;20(3):645-51.

164. Schieffer B, Luchtefeld M, Braun S, Hilfiker A, Hilfiker-Kleiner D, Drexler H. Role of NAD(P)H oxidase in angiotensin II-induced JAK/STAT signaling and cytokine induction. Circ Res. 2000 Dec 8;87(12):1195-201.

165. Willam C, Schindler R, Frei U, Eckardt KU. Increases in oxygen tension stimulate expression of ICAM-1 and VCAM-1 on human endothelial cells. Am J Physiol. 1999 Jun;276(6 Pt 2):H2044-52.

166. Mueller C, Baudler S, Welzel H, Bohm M, Nickenig G. Identification of a novel redox-sensitive gene, Id3, which mediates angiotensin II-induced cell growth. Circulation. 2002 May 21;105(20):2423-8.

167. DiGiacomo JE, Pane CR, Gwiazdowski S, Mishra OP, Delivoria-Papadopoulos M. Effect of graded hypoxia on brain cell membrane injury in newborn piglets. Biol Neonate. 1992;61(1):25-32.

168. McCord JM. Oxygen-derived free radicals in postischemic tissue injury. N Engl J Med. 1985 Jan 17;312(3):159-63.

169. Numagami Y, Zubrow AB, Mishra OP, Delivoria-Papadopoulos M. Lipid free radical generation and brain cell membrane alteration following nitric oxide synthase inhibition during cerebral hypoxia in the newborn piglet. J Neurochem. 1997 Oct;69(4):1542-7.

170. Kontos HA, Wei EP, Ellis EF, Jenkins LW, Povlishock JT, Rowe GT, et al. Appearance of superoxide anion radical in cerebral extracellular space during increased prostaglandin synthesis in cats. Circ Res. 1985 Jul;57(1):142-51.

171. Kourembanas S, McQuillan LP, Leung GK, Faller DV. Nitric oxide regulates the expression of vasoconstrictors and growth factors by vascular endothelium under both normoxia and hypoxia. J Clin Invest. 1993 Jul;92(1):99-104.

172. Ashraf QM, Zanelli SA, Mishra OP, Delivoria-Papadopoulos M. Phosphorylation of Bcl-2 and Bax proteins during hypoxia in newborn piglets. Neurochem Res. 2001 Jan;26(1):1-9.

173. Wei H, Wei W, Bredesen DE, Perry DC. Bcl-2 protects against apoptosis in neuronal cell line caused by thapsigargin-induced depletion of intracellular calcium stores. J Neurochem. 1998 Jun;70(6):2305-14.

174. Eskes R, Desagher S, Antonsson B, Martinou JC. Bid induces the oligomerization and insertion of Bax into the outer mitochondrial membrane. Mol Cell Biol. 2000 Feb;20(3):929-35.

175. Nouraini S, Six E, Matsuyama S, Krajewski S, Reed JC. The putative pore-forming domain of Bax regulates mitochondrial localization and interaction with Bcl-X(L). Mol Cell Biol. 2000 Mar;20(5):1604-15.

176. Boger RH, Bode-Boger SM, Thiele W, Creutzig A, Alexander K, Frolich JC. Restoring vascular nitric oxide formation by L-arginine improves the symptoms of intermittent claudication in patients with peripheral arterial occlusive disease. J Am Coll Cardiol. 1998 Nov;32(5):1336-44.

177. Ceremuzynski L, Chamiec T, Herbaczynska-Cedro K. Effect of supplemental oral L-arginine on exercise capacity in patients with stable angina pectoris. Am J Cardiol. 1997 Aug 1;80(3):331-3.

178. Maxwell AJ, Zapien MP, Pearce GL, MacCallum G, Stone PH. Randomized trial of a medical food for the dietary management of chronic, stable angina. J Am Coll Cardiol. 2002 Jan 2;39(1):37-45.

179. Thannickal VJ, Fanburg BL. Reactive oxygen species in cell signaling. Am J Physiol Lung Cell Mol Physiol. 2000 Dec;279(6):L1005-28.

180. Crapo JD, Tierney DF. Superoxide dismutase and pulmonary oxygen toxicity. Am J Physiol. 1974 Jun;226(6):1401-7.

181. Schafer FQ, Buettner GR. Redox environment of the cell as viewed through the redox state of the glutathione disulfide/glutathione couple. Free Radic Biol Med. 2001 Jun 1;30(11):1191-212.

182. Semenza GL. Angiogenesis in ischemic and neoplastic disorders. Annu Rev Med. 2003;54:17-28.

183. Sharp FR, Bernaudin M. HIF1 and oxygen sensing in the brain. Nat Rev Neurosci. 2004 Jun;5(6):437-48.

184. Ratcliffe PJ. New insights into an enigmatic tumour suppressor. Nat Cell Biol. 2003 Jan;5(1):7-8.

185. Huang LE, Bunn HF. Hypoxia-inducible factor and its biomedical relevance. J Biol Chem. 2003 May 30;278(22):19575-8.

186. O'Rourke JF, Dachs GU, Gleadle JM, Maxwell PH, Pugh CW, Stratford IJ, et al. Hypoxia response elements. Oncol Res. 1997;9(6-7):327-32.

187. Ratcliffe PJ, O'Rourke JF, Maxwell PH, Pugh CW. Oxygen sensing, hypoxia-inducible factor-1 and the regulation of mammalian gene expression. J Exp Biol. 1998 Apr;201(Pt 8):1153-62.

188. Semenza GL, Jiang BH, Leung SW, Passantino R, Concordet JP, Maire P, et al. Hypoxia response elements in the aldolase A, enolase 1, and lactate dehydrogenase A gene promoters contain essential binding sites for hypoxia-inducible factor 1. J Biol Chem. 1996 Dec 20;271(51):32529-37.

189. Tzung SP, Kim KM, Basanez G, Giedt CD, Simon J, Zimmerberg J, et al. Antimycin A mimics a cell-death-inducing Bcl-2 homology domain 3. Nat Cell Biol. 2001 Feb;3(2):183-91.

190. Hasan MK, Yaguchi T, Sugihara T, Kumar PK, Taira K, Reddel RR, et al. CARF is a novel protein that cooperates with mouse p19ARF (human p14ARF) in activating p53. J Biol Chem. 2002 Oct 4;277(40):37765-70.

191. Chen D, Li M, Luo J, Gu W. Direct interactions between HIF-1 alpha and Mdm2 modulate p53 function. J Biol Chem. 2003 Apr 18;278(16):13595-8.

192. Pedersen T, Kjekshus J, Berg K, Haghfelt T, al e. Randomised trial of cholesterol lowering in 4444 patients with coronary heart disease: The Scandinavian Simvastatin Survival Study (4S). The Lancet. 1994 Nov 19, 1994;344(8934):1383.

193. Shepherd J, Cobbe SM, Ford I, Isles CG, Lorimer AR, MacFarlane PW, et al. Prevention of coronary heart disease with pravastatin in men with hypercholesterolemia. West of Scotland Coronary Prevention Study Group. N Engl J Med. 1995 Nov 16;333(20):1301-7.

194. Prevention of cardiovascular events and death with pravastatin in patients with coronary heart disease and a broad range of initial cholesterol levels. The Long-Term Intervention with Pravastatin in Ischaemic Disease (LIPID) Study Group. N Engl J Med. 1998 Nov 5;339(19):1349-57.

195. Plehn JF, Davis BR, Sacks FM, Rouleau JL, Pfeffer MA, Bernstein V, et al. Reduction of stroke incidence after myocardial infarction with pravastatin: the Cholesterol and Recurrent Events (CARE) study. The Care Investigators. Circulation. 1999 Jan 19;99(2):216-23.

196. Byington RP, Davis BR, Plehn JF, White HD, Baker J, Cobbe SM, et al. Reduction of stroke events with pravastatin: the Prospective Pravastatin Pooling (PPP) Project. Circulation. 2001 Jan 23;103(3):387-92.

197. Shepherd J, Blauw GJ, Murphy MB, Bollen EL, Buckley BM, Cobbe SM, et al. Pravastatin in elderly individuals at risk of vascular disease (PROSPER): a randomised controlled trial. Lancet. 2002 Nov 23;360(9346):1623-30.

198. Prueksaritanont T, Zhao JJ, Ma B, Roadcap BA, Tang C, Qiu Y, et al. Mechanistic studies on metabolic interactions between gemfibrozil and statins. J Pharmacol Exp Ther. 2002 Jun;301(3):1042-51.

199. Istvan ES, Deisenhofer J. Structural mechanism for statin inhibition of HMG-CoA reductase. Science. 2001 May 11;292(5519):1160-4.

200. Nelson DR, Koymans L, Kamataki T, Stegeman JJ, Feyereisen R, Waxman DJ, et al. P450 superfamily: update on new sequences, gene mapping, accession numbers and nomenclature. Pharmacogenetics. 1996 Feb;6(1):1-42.

201. Lennernas H, Fager G. Pharmacodynamics and pharmacokinetics of the HMG-CoA reductase inhibitors. Similarities and differences. Clin Pharmacokinet. 1997 May;32(5):403-25.

202. Boberg M, Angerbauer R, Fey P, Kanhai WK, Karl W, Kern A, et al. Metabolism of cerivastatin by human liver microsomes in vitro. Characterization of primary metabolic pathways and of cytochrome P450 isozymes involved. Drug Metab Dispos. 1997 Mar;25(3):321-31.

203. Transon C, Leemann T, Vogt N, Dayer P. In vivo inhibition profile of cytochrome P450TB (CYP2C9) by (+/-)-fluvastatin. Clin Pharmacol Ther. 1995 Oct;58(4):412-7.

204. Pasternak RC, Smith SC, Jr., Bairey-Merz CN, Grundy SM, Cleeman JI, Lenfant C. ACC/AHA/NHLBI Clinical Advisory on the Use and Safety of Statins. Circulation. 2002 Aug 20;106(8):1024-8.

205. Staffa JA, Chang J, Green L. Cerivastatin and reports of fatal rhabdomyolysis. N Engl J Med. 2002 Feb 14;346(7):539-40.

206. Siegel-Axel DI. Cerivastatin: a cellular and molecular drug for the future? Cell Mol Life Sci. 2003 Jan;60(1):144-64.

207. Coleman ML, Olson MF. Rho GTPase signalling pathways in the morphological changes associated with apoptosis. Cell Death Differ. 2002 May;9(5):493-504.

208. Olson MF, Ashworth A, Hall A. An essential role for Rho, Rac, and Cdc42 GTPases in cell cycle progression through G1. Science. 1995 Sep 1;269(5228):1270-2.

209. Macaluso M, Russo G, Cinti C, Bazan V, Gebbia N, Russo A. Ras family genes: an interesting link between cell cycle and cancer. J Cell Physiol. 2002 Aug;192(2):125-30.

210. Knapp AC, Huang J, Starling G, Kiener PA. Inhibitors of HMG-CoA reductase sensitize human smooth muscle cells to Fas-ligand and cytokine-induced cell death. Atherosclerosis. 2000 Sep;152(1):217-27.

211. Laufs U, La Fata V, Plutzky J, Liao JK. Upregulation of endothelial nitric oxide synthase by HMG CoA reductase inhibitors. Circulation. 1998 Mar 31;97(12):1129-35.

212. Edwards CJ, Hart DJ, Spector TD. Oral statins and increased bone-mineral density in postmenopausal women. Lancet. 2000 Jun 24;355(9222):2218-9.

213. Kureishi Y, Luo Z, Shiojima I, Bialik A, Fulton D, Lefer DJ, et al. The HMG-CoA reductase inhibitor simvastatin activates the protein kinase Akt and promotes angiogenesis in normocholesterolemic animals. Nat Med. 2000 Sep;6(9):1004-10.

214. Feron O, Dessy C, Desager JP, Balligand JL. Hydroxy-methylglutaryl-coenzyme A reductase inhibition promotes endothelial nitric oxide synthase activation through a decrease in caveolin abundance. Circulation. 2001 Jan 2;103(1):113-8.

215. Wassmann S, Laufs U, Baumer AT, Muller K, Konkol C, Sauer H, et al. Inhibition of geranylgeranylation reduces angiotensin II-mediated free radical production in vascular smooth muscle cells: involvement of angiotensin AT1 receptor expression and Rac1 GTPase. Mol Pharmacol. 2001 Mar;59(3):646-54.

216. Wassmann S, Laufs U, Baumer AT, Muller K, Ahlbory K, Linz W, et al. HMG-CoA reductase inhibitors improve endothelial dysfunction in normocholesterolemic hypertension via reduced production of reactive oxygen species. Hypertension. 2001 Jun;37(6):1450-7.

217. Wagner AH, Kohler T, Ruckschloss U, Just I, Hecker M. Improvement of nitric oxide-dependent vasodilatation by HMG-CoA reductase inhibitors through attenuation of endothelial superoxide anion formation. Arterioscler Thromb Vasc Biol. 2000 Jan;20(1):61-9.

218. Eto M, Kozai T, Cosentino F, Joch H, Luscher TF. Statin prevents tissue factor expression in human endothelial cells: role of Rho/Rho-kinase and Akt pathways. Circulation. 2002 Apr 16;105(15):1756-9.

219. Vaughan CJ, Gotto AM, Jr., Basson CT. The evolving role of statins in the management of atherosclerosis. J Am Coll Cardiol. 2000 Jan;35(1):1-10.

220. Bellosta S, Via D, Canavesi M, Pfister P, Fumagalli R, Paoletti R, et al. HMG-CoA reductase inhibitors reduce MMP-9 secretion by macrophages. Arterioscler Thromb Vasc Biol. 1998 Nov;18(11):1671-8.

221. Bourcier T, Libby P. HMG CoA reductase inhibitors reduce plasminogen activator inhibitor-1 expression by human vascular smooth muscle and endothelial cells. Arterioscler Thromb Vasc Biol. 2000 Feb;20(2):556-62.

222. Rezaie-Majd A, Maca T, Bucek RA, Valent P, Muller MR, Husslein P, et al. Simvastatin reduces expression of cytokines interleukin-6, interleukin-8, and monocyte chemoattractant protein-1 in circulating monocytes from hypercholesterolemic patients. Arterioscler Thromb Vasc Biol. 2002 Jul 1;22(7):1194-9.

223. Sukhova GK, Williams JK, Libby P. Statins reduce inflammation in atheroma of nonhuman primates independent of effects on serum cholesterol. Arterioscler Thromb Vasc Biol. 2002 Sep 1;22(9):1452-8.

224. Weitz-Schmidt G, Welzenbach K, Brinkmann V, Kamata T, Kallen J, Bruns C, et al. Statins selectively inhibit leukocyte function antigen-1 by binding to a novel regulatory integrin site. Nat Med. 2001 Jun;7(6):687-92.

225. Ridker PM, Rifai N, Clearfield M, Downs JR, Weis SE, Miles JS, et al. Measurement of C-reactive protein for the targeting of statin therapy in the primary prevention of acute coronary events. N Engl J Med. 2001 Jun 28;344(26):1959-65.

226. Ridker PM, Rifai N, Pfeffer MA, Sacks FM, Moye LA, Goldman S, et al. Inflammation, pravastatin, and the risk of coronary events after myocardial infarction in patients with average cholesterol levels. Cholesterol and Recurrent Events (CARE) Investigators. Circulation. 1998 Sep 1;98(9):839-44.

227. Yang Z, Kozai T, van der Loo B, Viswambharan H, Lachat M, Turina MI, et al. HMG-CoA reductase inhibition improves endothelial cell function and inhibits smooth muscle cell proliferation in human saphenous veins. J Am Coll Cardiol. 2000 Nov 1;36(5):1691-7.

228. Hengst L, Reed SI. Translational control of p27Kip1 accumulation during the cell cycle. Science. 1996 Mar 29;271(5257):1861-4.

229. Hughes DA. Control of signal transduction and morphogenesis by Ras. Semin Cell Biol. 1995 Apr;6(2):89-94.

230. Llevadot J, Murasawa S, Kureishi Y, Uchida S, Masuda H, Kawamoto A, et al. HMG-CoA reductase inhibitor mobilizes bone marrow--derived endothelial progenitor cells. J Clin Invest. 2001 Aug;108(3):399-405.

231. Sindermann JR, Schmidt A, Breithardt G, Buddecke E. Lovastatin controls signal transduction in vascular smooth muscle cells by modulating phosphorylation levels of mevalonate-independent pathways. Basic Res Cardiol. 2001 May-Jun;96(3):283-9.

232. Takemoto M, Liao JK. Pleiotropic effects of 3-hydroxy-3-methylglutaryl coenzyme a reductase inhibitors. Arterioscler Thromb Vasc Biol. 2001 Nov;21(11):1712-9.

233. Wassmann S, Laufs U, Muller K, Konkol C, Ahlbory K, Baumer AT, et al. Cellular antioxidant effects of atorvastatin in vitro and in vivo. Arterioscler Thromb Vasc Biol. 2002 Feb 1;22(2):300-5.

234. Gahtan V, Wang XJ, Willis AI, Tuszynski GP, Sumpio BE. Thrombospondin-1 regulation of smooth muscle cell chemotaxis is extracellular signal-regulated protein kinases 1/2 dependent. Surgery. 1999 Aug;126(2):203-7.

235. Girona J, La Ville AE, Sola R, Plana N, Masana L. Simvastatin decreases aldehyde production derived from lipoprotein oxidation. Am J Cardiol. 1999 Mar 15;83(6):846-51.

236. Sadeghi MM, Collinge M, Pardi R, Bender JR. Simvastatin modulates cytokine-mediated endothelial cell adhesion molecule induction: involvement of an inhibitory G protein. J Immunol. 2000 Sep 1;165(5):2712-8.

237. Decastel M, Leborgne-Samuel Y, Alexandre L, Merault G, Berchel C. Morphological features of the human umbilical vein in normal, sickle cell trait, and sickle cell disease pregnancies. Hum Pathol. 1999 Jan;30(1):13-20.

238. Tan PH, Chan C, Xue SA, Dong R, Ananthesayanan B, Manunta M, et al. Phenotypic and functional differences between human saphenous vein (HSVEC) and umbilical vein (HUVEC) endothelial cells. Atherosclerosis. 2004 Apr;173(2):171-83.

239. Zahler S, Kupatt C, Mobert J, Becker BF, Gerlach E. Effects of ACE-inhibition on redox status and expression of P-selectin of endothelial cells subjected to oxidative stress. J Mol Cell Cardiol. 1997 Nov;29(11):2953-60.

240. Cuda G, Paterno R, Ceravolo R, Candigliota M, Perrotti N, Perticone F, et al. Protection of human endothelial cells from oxidative stress: role of Ras-ERK1/2 signaling. Circulation. 2002 Feb 26;105(8):968-74.

241. Canty TG, Jr., Boyle EM, Jr., Farr A, Morgan EN, Verrier ED, Pohlman TH. Oxidative stress induces NF-kappaB nuclear translocation without degradation of IkappaBalpha. Circulation. 1999 Nov 9;100(19 Suppl):II361-4.

242. Wang GL, Jiang BH, Rue EA, Semenza GL. Hypoxia-inducible factor 1 is a basic-helix-loop-helix-PAS heterodimer regulated by cellular O2 tension. Proc Natl Acad Sci U S A. 1995 Jun 6;92(12):5510-4.

243. Liu SF, Ye X, Malik AB. Inhibition of NF-kappaB activation by pyrrolidine dithiocarbamate prevents In vivo expression of proinflammatory genes. Circulation. 1999 Sep 21;100(12):1330-7.

244. Mandriota SJ, Pyke C, Di Sanza C, Quinodoz P, Pittet B, Pepper MS. Hypoxia-inducible angiopoietin-2 expression is mimicked by iodonium compounds and occurs in the rat brain and skin in response to systemic hypoxia and tissue ischemia. Am J Pathol. 2000 Jun;156(6):2077-89.

245. Chachami G, Simos G, Hatziefthimiou A, Bonanou S, Molyvdas PA, Paraskeva E. Cobalt induces hypoxia-inducible factor-1alpha expression in airway smooth muscle cells by a reactive oxygen species- and PI3K-dependent mechanism. Am J Respir Cell Mol Biol. 2004 Nov;31(5):544-51.

246. McCarthy NJ, Evan GI. Methods for detecting and quantifying apoptosis. Curr Top Dev Biol. 1998;36:259-78.

247. Gavrieli Y, Sherman Y, Ben-Sasson SA. Identification of programmed cell death in situ via specific labeling of nuclear DNA fragmentation. J Cell Biol. 1992 Nov;119(3):493-501.

248. Alberts B, Bray, D., Lewis, J., Raff, M., Roberts, K. & Watson, J. D. Molecular Biology of the Cell (Garland, New York)]. 1994 1994.

249. Assmus B, Urbich C, Aicher A, Hofmann WK, Haendeler J, Rossig L, et al. HMG-CoA reductase inhibitors reduce senescence and increase proliferation of endothelial progenitor cells via regulation of cell cycle regulatory genes. Circ Res. 2003 May 16;92(9):1049-55.

250. Landmesser U, Harrison DG. Oxidative stress and vascular damage in hypertension. Coron Artery Dis. 2001 Sep;12(6):455-61.

251. Zalba G, Beaumont FJ, San Jose G, Fortuno A, Fortuno MA, Etayo JC, et al. Vascular NADH/NADPH oxidase is involved in enhanced superoxide production in spontaneously hypertensive rats. Hypertension. 2000 May;35(5):1055-61.

252. Davies KJ. Oxidative stress: the paradox of aerobic life. Biochem Soc Symp. 1995;61:1-31.

253. Miwa S, Brand MD. Mitochondrial matrix reactive oxygen species production is very sensitive to mild uncoupling. Biochem Soc Trans. 2003 Dec;31(Pt 6):1300-1.

254. Genova ML, Pich MM, Biondi A, Bernacchia A, Falasca A, Bovina C, et al. Mitochondrial production of oxygen radical species and the role of Coenzyme Q as an antioxidant. Exp Biol Med (Maywood). 2003 May;228(5):506-13.

255. Seshiah PN, Weber DS, Rocic P, Valppu L, Taniyama Y, Griendling KK. Angiotensin II stimulation of NAD(P)H oxidase activity: upstream mediators. Circ Res. 2002 Sep 6;91(5):406-13.

256. Xia Y, Tsai AL, Berka V, Zweier JL. Superoxide generation from endothelial nitric-oxide synthase. A Ca2+/calmodulin-dependent and tetrahydrobiopterin regulatory process. J Biol Chem. 1998 Oct 2;273(40):25804-8.

257. Xia Y, Roman LJ, Masters BS, Zweier JL. Inducible nitric-oxide synthase generates superoxide from the reductase domain. J Biol Chem. 1998 Aug 28;273(35):22635-9.

258. Shimokawa H, Flavahan NA, Vanhoutte PM. Loss of endothelial pertussis toxin-sensitive G protein function in atherosclerotic porcine coronary arteries. Circulation. 1991 Feb;83(2):652-60.

259. Gauthier TW, Scalia R, Murohara T, Guo JP, Lefer AM. Nitric oxide protects against leukocyte-endothelium interactions in the early stages of hypercholesterolemia. Arterioscler Thromb Vasc Biol. 1995 Oct;15(10):1652-9.

260. Garg UC, Hassid A. Nitric oxide-generating vasodilators and 8-bromo-cyclic guanosine monophosphate inhibit mitogenesis and proliferation of cultured rat vascular smooth muscle cells. J Clin Invest. 1989 May;83(5):1774-7.

261. Radomski MW, Rees DD, Dutra A, Moncada S. S-nitroso-glutathione inhibits platelet activation in vitro and in vivo. Br J Pharmacol. 1992 Nov;107(3):745-9.

262. Caplan BA, Schwartz CJ. Increased endothelial cell turnover in areas of in vivo Evans Blue uptake in the pig aorta. Atherosclerosis. 1973 May-Jun;17(3):401-17.

263. Graham E, Mishra OP, Delivoria-Papadopoulos M. Brain cell membrane Na+,K(+)-ATPase modification following hypoxia in the guinea pig fetus. Neurosci Lett. 1993 Apr 16;153(1):93-7.

264. Mishra OP, Delivoria-Papadopoulos M. Lipid peroxidation in developing fetal guinea pig brain during normoxia and hypoxia. Brain Res Dev Brain Res. 1989 Jan 1;45(1):129-35.

265. Kourembanas S, Marsden PA, McQuillan LP, Faller DV. Hypoxia induces endothelin gene expression and secretion in cultured human endothelium. J Clin Invest. 1991 Sep;88(3):1054-7.

266. Liao JK, Zulueta JJ, Yu FS, Peng HB, Cote CG, Hassoun PM. Regulation of bovine endothelial constitutive nitric oxide synthase by oxygen. J Clin Invest. 1995 Dec;96(6):2661-6.

267. Maulik D, Numagami Y, Ohnishi ST, Mishra OP, Delivoria-Papadopoulos M. Direct measurement of oxygen free radicals during in utero hypoxia in the fetal guinea pig brain. Brain Res. 1998 Jul 6;798(1-2):166-72.

268. Kroemer G, Dallaporta B, Resche-Rigon M. The mitochondrial death/life regulator in apoptosis and necrosis. Annu Rev Physiol. 1998;60:619-42.

269. Lemasters JJ. The mitochondrial permeability transition: from biochemical curiosity to pathophysiological mechanism. Gastroenterology. 1998 Sep;115(3):783-6.

270. Smith WL, Eling TE, Kulmacz RJ, Marnett LJ, Tsai A. Tyrosyl radicals and their role in hydroperoxide-dependent activation and inactivation of prostaglandin endoperoxide synthase. Biochemistry. 1992 Jan 14;31(1):3-7.

271. Rosenbaum DM, Michaelson M, Batter DK, Doshi P, Kessler JA. Evidence for hypoxia-induced, programmed cell death of cultured neurons. Ann Neurol. 1994 Dec;36(6):864-70.

272. Wenger RH. Cellular adaptation to hypoxia: O2-sensing protein hydroxylases, hypoxia-inducible transcription factors, and O2-regulated gene expression. Faseb J. 2002 Aug;16(10):1151-62.

273. Thomas M, Mann J. Increased thrombotic vascular events after change of statin. Lancet. 1998 Dec 5;352(9143):1830-1.

274. Heeschen C, Hamm CW, Laufs U, Snapinn S, Bohm M, White HD. Withdrawal of statins increases event rates in patients with acute coronary syndromes. Circulation. 2002 Mar 26;105(12):1446-52.

275. Heeschen C, Hamm CW, Laufs U, Bohm M, Snapinn S, White HD. Withdrawal of statins in patients with acute coronary syndromes. Circulation. 2003 Jan 28;107(3):e27.

276. Laufs U, Wassmann S, Hilgers S, Ribaudo N, Bohm M, Nickenig G. Rapid effects on vascular function after initiation and withdrawal of atorvastatin in healthy, normocholesterolemic men. Am J Cardiol. 2001 Dec 1;88(11):1306-7.

277. Wang CY, Liu PY, Liao JK. Pleiotropic effects of statin therapy: molecular mechanisms and clinical results. Trends Mol Med. 2008 Jan;14(1):37-44.

278. Piconi L, Corgnali M, Da Ros R, Assaloni R, Piliego T, Ceriello A. The protective effect of rosuvastatin in human umbilical endothelial cells exposed to constant or intermittent high glucose. J Diabetes Complications. 2008 Jan-Feb;22(1):38-45.

279. Schaefer CA, Kuhlmann CR, Weiterer S, Fehsecke A, Abdallah Y, Schaefer C, et al. Statins inhibit hypoxia-induced endothelial proliferation by preventing calcium-induced ROS formation. Atherosclerosis. 2006 Apr;185(2):290-6.

280. Prueksaritanont T, Ma B, Tang C, Meng Y, Assang C, Lu P, et al. Metabolic interactions between mibefradil and HMG-CoA reductase inhibitors: an in vitro investigation with human liver preparations. Br J Clin Pharmacol. 1999 Mar;47(3):291-8.

281. Bischoff H, Heller AH. Preclinical and clinical pharmacology of cerivastatin. Am J Cardiol. 1998 Aug 27;82(4B):18J-25J.

282. Sakamoto T, Kojima S, Ogawa H, Shimomura H, Kimura K, Ogata Y, et al. Effects of early statin treatment on symptomatic heart failure and ischemic events after acute myocardial infarction in Japanese. Am J Cardiol. 2006 Apr 15;97(8):1165-71.

283. Rodes-Cabau J, Tardif JC, Cossette M, Bertrand OF, Ibrahim R, Larose E, et al. Acute effects of statin therapy on coronary atherosclerosis following an acute coronary syndrome. Am J Cardiol. 2009 Sep 15;104(6):750-7.

284. Kato T, Hashikabe H, Iwata C, Akimoto K, Hattori Y. Statin blocks Rho/Rho-kinase signalling and disrupts the actin cytoskeleton: relationship to enhancement of LPS-mediated nitric oxide synthesis in vascular smooth muscle cells. Biochim Biophys Acta. 2004 Aug 4;1689(3):267-72.

285. Erl W, Hristov M, Neureuter M, Yan ZQ, Hansson GK, Weber PC. HMG-CoA reductase inhibitors induce apoptosis in neointima-derived vascular smooth muscle cells. Atherosclerosis. 2003 Aug;169(2):251-8.

286. Ravishankar S, Ashraf QM, Fritz K, Mishra OP, Delivoria-Papadopoulos M. Expression of Bax and Bcl-2 proteins during hypoxia in cerebral cortical neuronal nuclei of newborn piglets: effect of administration of magnesium sulfate. Brain Res. 2001 May 18;901(1-2):23-9.

287. Caspard H, Chan AK, Walker AM. Compliance with a statin treatment in a usual-care setting: retrospective database analysis over 3 years after treatment initiation in health maintenance organization enrollees with dyslipidemia. Clin Ther. 2005 Oct;27(10):1639-46.

288. Schouten O, Hoeks SE, Welten GM, Davignon J, Kastelein JJ, Vidakovic R, et al. Effect of statin withdrawal on frequency of cardiac events after vascular surgery. Am J Cardiol. 2007 Jul 15;100(2):316-20.

289. van der Harst P, Asselbergs FW, Hillege HL, Bakker SJ, Voors AA, van Veldhuisen DJ, et al. Effect of withdrawal of pravastatin therapy on C-reactive protein and low-density lipoprotein cholesterol. Am J Cardiol. 2007 Nov 15;100(10):1548-51.

290. Lee KT, Lai WT, Chu CS, Tsai LY, Yen HW, Voon WC, et al. Effect of withdrawal of statin on C-reactive protein. Cardiology. 2004;102(3):166-70.

291. Lai WT, Lee KT, Chu CS, Voon WC, Yen HW, Tsai LY, et al. Influence of withdrawal of statin treatment on proinflammatory response and fibrinolytic activity in

humans: an effect independent on cholesterol elevation. Int J Cardiol. 2005 Feb 28;98(3):459-64.

292. Nouri M, Pourabbasi A, Ebrahimnejad M, Abolhassani F, Jahanzad I. Pravastatin prevents ischemia-reperfusion injury in rat sciatic nerve. Surg Neurol. 2006 Sep;66(3):337-8; discussion 8-40.

293. Naidu BV, Woolley SM, Farivar AS, Thomas R, Fraga C, Mulligan MS. Simvastatin ameliorates injury in an experimental model of lung ischemia-reperfusion. J Thorac Cardiovasc Surg. 2003 Aug;126(2):482-9.