Endothelial Cell Annexins and Oxidative Stress

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Abstract

Annexins are a family of calcium-binding proteins that bind membranes containing negatively charged phospholipids in a calcium-dependent manner. To date 12 human annexins have been identified, many of which are expressed in vascular endothelial cells. Annexins 1 and 5 have been suggested to play a role in the inhibition of endothelial cell cPLA₂, but this may be by substrate sequestration rather than a direct interaction between the proteins. Annexin 6 has been shown in vitro to produce a less thrombogenic surface when bound to phospholipids on the cell surface of endothelial cell membranes. The most interesting suggestion is that annexin 2 is a receptor for plasminogen and tissue plasminogen activator on the cell surface of human umbilical vascular endothelial cells (HUVECs), acting as a catalyst for the conversion of plasminogen to plasmin. When the vascular endothelium is damaged by oxidative stress this can lead to diseases such as atherosclerosis and hypertension. This damage is accompanied by changes in calcium responses and transcriptional regulation.

This thesis reports an investigation into how the behaviour and expression of certain annexins is changed as a consequence of oxidative injury to vascular endothelial cells. HUVECs were cultured in 40% oxygen in a custom-built chamber to induce chronic oxidative stress over a period of 12 days. Immunostaining of annexins revealed altered patterns of translocation following stimulation with a range of different agonists, in cells cultured under normal and hyperoxic conditions. To investigate the underlying causes of these phenomena, changes in cellular levels of calcium, protein phosphorylation, MAP kinase activation and tyrosine phosphorylation were examined in oxidatively stressed cells. Levels of annexin mRNA and protein expression were also examined in HUVECs.

The major findings of this work showed that annexin protein expression was unchanged, although perhaps with modest changes in mRNA expression for some annexins, in oxidatively stressed HUVECs. Changes in calcium mobilisation and other signalling events induced by oxidative stress were observed in conjunction with changes in annexin localisation. Annexin 5 showed the most dramatic change by translocating to the nuclei of oxidatively stressed cells, when it would not normally be seen in the nucleus. Studies in this thesis together with published work, indicate protein phosphorylation as being a major event leading to the changes observed in calcium signalling and annexin localisation in oxidative stressed HUVECs.
# Table of Contents

ABSTRACT..................................................................................................................2

LIST OF FIGURES........................................................................................................5

LIST OF TABLES.........................................................................................................7

LIST OF ABBREVIATIONS...........................................................................................8

CHAPTER 1: INTRODUCTION ....................................................................................10

1.1 ENDOTHELIAL CELLS ........................................................................................11
   1.1.1 Coagulation/fibrinolysis.............................................................................12

1.2 CARDIOVASCULAR DISEASE/ENDOTHELIAL DYSFUNCTION.......................14
   1.2.1 Hypercholesterolaemia ...........................................................................16
   1.2.2 Homocysteine ..........................................................................................17
   1.2.3 Hypertension and endothelial derived relaxation.......................................18

1.3 OXIDATIVE STRESS ........................................................................................19
   1.3.1 Production of reactive oxygen species ......................................................20
   1.3.2 Antioxidant defence mechanisms ...............................................................21
   1.3.3 Protein kinases/phosphatases ...................................................................23
   1.3.4 Diabetes ....................................................................................................23
   1.3.5 Experimental investigation of oxidative stress ...........................................24
   1.3.6 Oxidative stress and apoptosis .................................................................25

1.4 CALCIUM SIGNALLING ...................................................................................26
   1.4.1 Calcium regulation in the vascular endothelium ......................................27
   1.4.2 Calcium signalling under oxidative stress ...............................................28
   1.4.3 Atherosclerosis and calcium signalling ....................................................30

1.5 ANNEXINS .....................................................................................................30
   1.5.1 Structure ...................................................................................................31
   1.5.2 Annexin protein ligands ............................................................................33
   1.5.3 Tissue and cellular localisation .................................................................35

1.6 PROPOSED FUNCTIONS OF ANNEXINS .......................................................39
   1.6.1 Exocytosis ................................................................................................39
   1.6.2 Endocytosis ...............................................................................................40
   1.6.3 Ca\(^{2+}\) signalling and homeostasis .........................................................42
   1.6.4 Annexins in disease ..................................................................................46

CHAPTER 2: MATERIALS AND METHODS ...............................................................53

2.1 CELL CULTURE ...............................................................................................54
2.1.2 Cell culture under hyperoxic conditions .............................................. 55

2.2 CELL STIMULATION ............................................................................ 56

2.3 EXTRACTION OF CELLULAR PROTEIN ................................................. 56

2.4 POLYACRYLAMIDE GEL ELECTROPHORESIS ........................................ 57

2.5 WESTERN BLOTTING ........................................................................... 57

2.6 IMMUNOBLOTTING .............................................................................. 57

2.7 IMMUNOPRECIPITATION ..................................................................... 60

2.8 IMMUNOFLUORESCENCE ................................................................... 61

2.9 FLUORIMETRIC ANALYSIS OF CALCIUM MOBILISATION ....................... 62

2.10 PCR BASED ANALYSIS OF ANNEXIN GENE EXPRESSION .................... 62

2.11 OLIGONUCLEOTIDE SEQUENCES ...................................................... 63

CHAPTER 3: ENDOTHELIAL MORPHOLOGY AND ANNEXIN EXPRESSION .......... 65

3.1 VALIDATION OF THE EXPERIMENTAL MODEL ...................................... 66

3.2 ENDOTHELIAL CELL MORPHOLOGY .................................................... 67

3.2.1 Actin cytoskeleton ........................................................................... 68

3.3 OXIDATIVE DAMAGE ........................................................................... 69

3.4 CHANGES IN GENE EXPRESSION ....................................................... 71

3.4.1 Annexin protein expression .............................................................. 72

3.4.2 Annexin mRNA expression .............................................................. 73

CHAPTER 4: ANNEXIN LOCALISATION ....................................................... 75

4.1 CELL STIMULATION ............................................................................ 76

4.2 METHOD FOR ASSESSING CELL POPULATIONS WITH ANNEXINS EITHER IN THE CYTOSOL OR THE NUCLEUS ................................................................. 77

4.3 ANNEXIN 1 AND cPLA$_2$ .................................................................... 78

4.3.1 Annexin 1 and cPLA$_2$ in cells stimulated with thapsigargin and ionomycin .. 80

4.3.2 Annexin 1 and cPLA$_2$ response to ATP and EGF .............................. 83

4.3.3 Inhibition of tyrosine phosphorylation ............................................. 87

4.4 ANNEXIN 2 LOCALISATION ................................................................. 88

4.5 CELLULAR DISTRIBUTION OF ANNEXIN 4 AFTER OXIDATIVE STRESS ........................ 89

4.6 TRANSLOCATION OF ANNEXIN 5 UNDER OXIDATIVE STRESS ................. 91

4.6.1 HUVEC annexin 5 does not respond to thapsigargin but does translocate in response to ionomycin ............................................................. 92

4.6.2 The response of HUVEC annexin 5 to ATP and EGF ......................... 95

4.7 RESPONSE OF ANNEXIN 6 TO OXIDATIVE STRESS .............................. 97

4.7.1 Effect of thapsigargin and ionomycin on annexin 6 localisation ............ 99

4.7.2 Response of annexin 6 to ATP and EGF stimulation .......................... 101

4.8 LOCALISATION OF ANNEXIN 7 IN HUVECs ........................................ 103
CHAPTER 5: CELL SIGNALLING ............................................................................. 105
5.1 CALCIUM SIGNALLING .................................................................................. 106
5.2 SIGNALLING PROTEINS ................................................................................. 109
5.3 PROTEIN TYROSINE PHOSPHORYLATION ..................................................... 111

CHAPTER 6: DISCUSSION ....................................................................................... 114
6.1 ENDOTHELIAL MORPHOLOGY ......................................................................... 115
6.2 ANNEXIN EXPRESSION .................................................................................. 118
6.3 ANNEXIN LOCALISATION ................................................................................. 119
6.4 CELL SIGNALLING .......................................................................................... 123
6.5 SUMMARY ...................................................................................................... 127

ACKNOWLEDGEMENTS.......................................................................................... 129

REFERENCES ........................................................................................................... 130

List of figures

FIGURE 1. INTRINSIC PATHWAY OF CLOT FORMATION WITHIN BLOOD VESSELS .......... 13
FIGURE 2. ATHEROSCLEROTIC PATHWAY .............................................................. 14
FIGURE 3. AN AVERAGED RIBBON STRUCTURE OF THE ANNEXINS ......................... 32
FIGURE 5. MEMBRANE SPANNING α-HELIX. .......................................................... 43
FIGURE 4. MODELS FOR ION CHANNELS / REGULATION BY ANNEXINS .................. 44
FIGURE 6. MODEL FOR BINDING OF PLASMINOGEN AND TPA TO ANNEXIN 2 ON THE ENDOTHELIAL CELL SURFACE ................................................................. 47
FIGURE 8. HYPEROXIA HAS NO EFFECT ON LOCALISATION OF VON WILLEBRAND FACTOR IN HUVECS ................................................................. 67
FIGURE 9. CHANGES IN ENDOTHELIAL CELL MORPHOLOGY GENERATED BY HYPEROXIA ... 68
FIGURE 10. ACTIN FILAMENT ARRANGEMENT IN HUVECS .................................... 69
FIGURE 11. EXPRESSION OF APOPTOTIC PROTEINS .............................................. 70
FIGURE 12. COOMASSIE STAINED GEL OF WHOLE CELL LYSATES FROM OXIDATIVELY STRESSED CELLS ............................................................... 71
FIGURE 13. EXPRESSION OF ANNEXINS DURING INCUBATION IN HYPEROXIC CONDITIONS.. 72
FIGURE 14. RT-PCR ANALYSIS OF ANNEXIN GENE EXPRESSION IN CONTROL AND OXIDATIVELY STRESSED HUVECS AND A431 CELLS ........................................ 74
FIGURE 15. SCHEMATIC REPRESENTATION OF CELL PATCHES ASSOCIATED WITH TRANSLOCATION OF ANNEXINS INTO HUVEC NUCLEI ................................. 78
FIGURE 16. OXIDANT STRESS INCREASES TRANSLOCATION OF ANNEXIN 1 TO THE PLASMA MEMBRANE REGION .............................................................. 79
List of tables

**TABLE 1 SOURCES OF CELLULAR ROS PRODUCTION** ................................................................. 21
**TABLE 2 REACTIONS CATALYSED BY THE ANTIOXIDANT DEFENCE MECHANISMS** .......... 22
**TABLE 3 CONCENTRATIONS AGONISTS, GROWTH FACTORS AND PHARMACOLOGICAL**
MEDIATORS USED TO ACTIVATE HUVECS ............................................................................. 56
**TABLE 4 SECONDARY ANTIBODIES USED FOR IMMUNOFLUORESCENCE** ....................... 62
List of Abbreviations

AGEs advanced glycation end products
AP alkaline phosphatase
β-ME β-mercaptoethanol
cAMP cyclic adenosine monophosphate
cPLA2 cytosolic phospholipase A2
DMEM Dulbecco's modified Eagle medium
DMSO dimethyl sulphoxide
DTT dithiothreitol
EC endothelial cells
ECL enhanced chemiluminescence
EDTA ethylenediaminetetraacetic acid
EGF epidermal growth factor
EGFr epidermal growth factor receptor
EGTA ethylene glycol-bis(β-aminoethyl ether) N,N,N',N'-tetraacetic acid
eNOS endothelial nitric oxide synthase
ER endoplasmic reticulum
FCS foetal calf serum
FITC fluorescein isothiocyanate
GSH oxidised glutathione
GSSH reduced glutathione
HBSS Hanks' balanced salts solution
H2O2 hydrogen peroxide
HRP horseradish peroxidase
HUVEC human umbilical vein endothelial cells
ICAM-1 intercellular adhesion molecule 1
IgG immunoglobulin G
IP3 inositol 1,4,5-trisphosphate
LDL low density lipoprotein
LPC lysophosphatidylcholine
MAPK mitogen activated protein kinase
MCP-1 monocyte chemoattractant protein-1
NADPH nicotinamide-adenine dinucleotide phosphate (reduced form)
O2•− superoxide
ox-LDL oxidised low density lipoprotein
NO nitric oxide
•OH hydroxyl radical
ONOO$^-$ peroxynitrite
PBS phosphate buffered saline
PBS-T PBS containing 0.05% Tween-20
PDGF platelet derived growth factor
PGI$_2$ prostacyclin
PKC protein kinase C
PMSF phenyl-methyl-sulfonyl-flouride
PVDF polyvinyl difluoride
ROS reactive oxygen species
SDS sodium dodecylsulphate
SDS-PAGE SDS-polyacrylamide gel electrophoresis
SOD superoxide dismutase
SPI small peptide inhibitors
SR sarcoplasmic reticulum
t-BOOH tert-butyl-hydroperoxide
tPA tissue plasminogen activator
Tris tris(hydroxymethyl)methylamine
TRITC tetramethylrhodamine isothiocyanate
uPA urokinase plasminogen activator
VCAM-1 vascular adhesion molecule 1
vWF von Willebrand factor
CHAPTER 1: INTRODUCTION
1.1 Endothelial Cells

During the 1800s von Reckingausen discovered that the blood vessels of the body were lined with cells (Fishman, 1982), endothelial cells (ECs). ECs are approximately 15 \( \mu \text{M} \) at their widest point, with their length being between 25 - 50 \( \mu \text{M} \). The thickness of the cells varies greatly from one region to another. The area around the nucleus is the thickest at around a few \( \mu \text{M} \) with the cell periphery being so thin that it would not embody two adjacent plasmalemmal vesicles (Pittilo, 1988). Scanning electron microscopy shows them to overlap the luminal membranes of adjacent cells (Thomsen and Kjeldsen, 1978). In an adult human the endothelial lining consists of about 1-6x \( 10^{13} \) cells, covering an area of 1-7 \( \text{m}^2 \), weighing approximately 1 kg (Augustin et al., 1994). Experimentation on these cells has now led to the view that they are an integral part of the cardiovascular system providing many vital functions to the vasculature. They line all of the blood vessels of the body, placing them in a unique position between blood and tissues where they can perform various regulatory functions. Their most vital functions are to stop platelets binding and to thus prevent blood from clotting in the vessels, along with modulating the immune system, regulating transport of macromolecules and solutes between the vessel wall and the blood, and control of both the tone and growth of the vasculature. When the endothelium is healthy it presents itself as a non-thrombogenic surface (Vane, 1993).

The first detailed studies of ECs became possible in the 1970s when techniques were developed to culture these cells \textit{in vitro} (Jaffe et al., 1973). Cultured ECs are clearly an important resource in the study of this cell type, but caution should be exercised when making observations about ECs in culture, as ECs are normally quiescent \textit{in vivo}, but \textit{in vitro} they grow in an activated proliferative state. Proliferation can be stimulated with endothelial cell growth factor or fibroblast growth factor, both of which activate pathways suggested to involve protein kinase C (PKC) (Friedlander et al., 1995; Kent et al., 1995). One of the PKC family, PKC\( \theta \) has been indicated to have an important role in progression of ECs through the cell cycle, as inhibition causes a delay at \( G_2/M \). PKC\( \theta \) also appears to be required for maintenance of the cytoskeleton, as cells exposed to a PKC\( \theta \) inhibitor are unable to form actin stress fibres (Tang et al., 1997).

ECs vary in phenotype depending on the blood vessels of the body from which they originate. In the kidneys they are selectively permeable to allow absorption and secretion, the liver and the spleen have discontinuous ECs to allow cellular trafficking, whereas in the brain and the retina they exhibit tight junctions, this being essential for the maintenance of the blood brain barrier (Dejana, 1996).
ECs are very active, producing many different physiological mediators, including nitric oxide (NO), eicosanoids, cytokines, growth factors, vasoactive substances, procoagulant and anticoagulant factors as well as fibrinolytic factors and inhibitors. Many of these substances act in pairs in agonist/antagonist relationships. ECs also synthesise and release various factors, for instance, NO, endothelin and prostacyclin (PGI$_2$), endothelium derived hyperpolarising factor, platelet derived growth factor (PDGF), interleukin-1, platelet activating factor, tissue plasminogen activator (tPA), urokinase plasminogen activator (uPA), von Willebrand factor (v-WF), fibrinogen, thrombodulin, tissue factor, plasminogen activator inhibitors and thrombin. Many of these factors are involved in either coagulation or fibrinolysis (see section 1.1.1).

The major vasoconstrictors produced by ECs are platelet activating factor and endothelin, with endothelin being the most potent. The production of endothelin is stimulated by interleukin 1 but is inhibited by NO (Masaki, 1995). It exerts its effects locally, as the circulating concentration is too low to be pharmacologically active (Battistini et al., 1993). The other effect of endothelin is to encourage leukocyte adhesion. Working in opposition are the vasodilators, PGI$_2$, NO and the controversial endothelium derived hyperpolarising factor (Feletou and Vanhoutte, 1996). PGI$_2$ is the main product of arachidonic acid metabolism in ECs (Moncada and Vane, 1979), and causes vasodilation of vascular smooth muscle by stimulating an increase in cyclic adenosine mono-phosphate (cAMP) (Kukovetz et al., 1979), which in turn leads to changes in Ca$^{2+}$ levels. PGI$_2$ also facilitates the release of NO (Shimokawa et al., 1988), prevents platelets from aggregating (Bunting et al., 1981), and is able to suppress growth of cultured smooth muscle cells taken from atherosclerotic plaques (Akopov et al., 1988), although smooth muscle cells are thought to be able to develop a tolerance to the antiproliferative effects of PGI$_2$ (Weber et al., 1998). NO is constitutively synthesised from L-arginine by membrane-bound endothelial nitric oxide synthase (eNOS), with its activity controlled by the level of Ca$^{2+}$ (Palmer et al., 1988). NO decreases binding of platelets and monocytes to ECs, inhibits growth and migration of vascular smooth muscle, and can control other vascular functions by causing changes in protein expression (Zeiher et al., 1995). It may also be able to reduce the amount of oxidised low density lipoprotein (ox-LDL) by decreasing lipoxygenase activity (Rubbo et al., 1995). Like PGI$_2$, NO causes relaxation of vascular smooth muscle by increasing the levels of cAMP (for review see Kannan et al., 1998).

### 1.1.1 Coagulation/fibrinolysis

ECs maintain their antithrombotic surface via a complex system. They express thrombomodulin, which aids in suppressing thrombin activity (Esmon and Fukudome, 12
1995), along with the expression of a tissue factor inhibitor, which binds to factor Xa (Broze, 1995). Thrombomodulin can bind factor Xa causing an indirect inhibition of prothrombinase activity (Thompson and Salem, 1986). When thrombin binds thrombomodulin it increases thrombomodulin's ability to stimulate the anticoagulant protein C. If then combined with protein S (also synthesised by ECs) there is further enhancement of anticoagulation (Fair et al., 1986). The vascular endothelium also has some procoagulant mechanisms. Induction of tissue factor signifies the change of the endothelial surface from being anticoagulant to being procoagulant (Figure 1). It causes accelerated activation of factors X and XI. Healthy ECs do not express tissue factor (Drake et al., 1989), but can be stimulated to do so by oxidised lipoprotein; thrombin, shear stress and other damaging conditions (Rapaport and Rao, 1995).

**Figure 1. Intrinsic pathway of clot formation within blood vessels.**

Pathway of activation of factors involved in the blood clotting cascade, (a) = activated. Tissue factor acts to accelerate activation of factors IX and X.

The contribution of ECs to fibrinolysis depends on their metabolic state, where they are derived from and the concentration in the surrounding plasma of other molecules involved in this process. It is widely believed that all ECs produce tPA, though this view has been questioned. The other plasminogen activator, uPA, is expressed by
ECs involved in wound repair (Bacharach et al., 1992), but not by most quiescent cells (Wojta et al., 1989). Mice with a targeted disruption of the uPA gene develop inflammation-induced thrombi (Carmeliet et al., 1994), indicating the importance of uPA in vascular haemostasis. Fibrinolytic activity (Hajjar and Hamel, 1990) and stimulation of cell proliferation (Weling et al., 1996) are reported to be increased when tPA binds via a receptor to ECs. Annexin 2 has been identified as being a receptor for tPA and plasminogen on the cell surface (Hajjar et al., 1996), although expression of annexin 2 on the endothelial surface in vivo has not yet been unequivocally demonstrated.

1.2 Cardiovascular Disease/Endothelial dysfunction

Although the last 25 years have brought reductions in the death rate from the initial clinical complications of cardiovascular disease, it remains the biggest threat to life and health in Britain and the U.S.A., due to an ever-increasing ageing population (Kannel, 1998). EC dysfunction is an important factor in the development of heart disease, especially atherosclerosis, which is a slow developing chronic inflammatory disease that can take decades before it exhibits itself with clinical symptoms (Munro and Cotran, 1988). ECs were first recognised as being involved in an early part of the disease process, after the observation that removal of ECs led to the development of atherosclerotic lesions (Ross et al., 1977; Ross, 1993). It was first thought that physical damage to the endothelium was the initiating cause of atherosclerosis, but this idea was later dispelled by the observation that young human adults show signs of atherosclerotic lesions without any sign of endothelial disruption (Babaev et al., 1993).

Figure 2. Atherosclerotic pathway.
Endothelial dysfunction plays a pivotal role in the initiation of atherosclerosis, often leading to endothelial denudation. This step can be bypassed leading straight to generation of atherosclerotic tissue, with denudation occurring at a later stage.
Some patients with atherosclerotic risk factors show signs of endothelial dysfunction in angiographically normal arteries long before there are any lesions or thickening of the vessel wall (Reddy et al., 1994; Werns et al., 1989). Irregularities in EC organisation were found overlying fatty streaks, with denudation being seen only in the late stages of disease (Ross and Glomset, 1976). The current thinking is that endothelial dysfunction is systemic and one of the events that initiates atherosclerosis, with the early stages occurring before any changes in morphology are visible.

The mechanical force of blood flowing through the vessels is thought to be causal in the development of atherosclerotic lesions. Most lesions are found focused around bifurcations, branches and narrow regions of the blood vessels (Comhill and Roach, 1976; Yoshida et al., 1990), where the flow would change from being laminar to more turbulent. Hydrostatic pressure, cyclic strain and shear wall stress are all classed as haemodynamic forces. As more research is conducted into these effects on ECs, the accumulated evidence suggests they can cause changes in endothelial structure and function. In the early 1980s Dewey et al. were able to show ECs changing their shape and alignment in response to a unidirectional laminar shear stress. On removal of this stress the cells gradually returned to their original state (Dewey et al., 1981).

It is also clear that biomechanical forces created during blood flow can alter the expression of endothelial genes (Resnick and Gimbrone, 1995). This is suggested to be via mechanotransduction activation of focal adhesion molecules within focal adhesion plaques (Davies, 1995). Some of these molecules have been reported to become phosphorylated upon cyclic strain (Yano et al., 1996a; Yano et al., 1996b). Mechanosensitive Ca^{2+} channels (Lansman et al., 1987) can allow Ca^{2+} influx leading to transcription of early response genes (see section 1.4, calcium signalling), and cyclic stress can activate PKC (Rosales and Sumpio, 1992). It has been suggested that a similar sequence to the shear stress response elements in the promoter region of platelet derived growth factor (PDGF) (Resnick et al., 1993) may play a role in the up-regulation of some endothelial genes. For example, cyclic stress has been reported to increase mRNA levels of eNOS (Awolesi et al., 1995) and endothelin in ECs (Sumpio and Widmann, 1990) along with c-jun, c-fos and fos B in human umbilical vein endothelial cells (HUVECs) (Oluwole et al., 1997).

There are various risk factors associated with endothelial dysfunction; these include hypercholesterolaemia, oxidised lipoproteins, advanced glycation end products in diabetes (Johnstone et al., 1993), hypertension (Panza et al., 1990; Anthony et al., 1995), smoking (Celemajer et al., 1993), hyperhomocyst(e)inaemia (Hankey and
Eikelboom, 1999), some cytokines and bacterial products. Gender also has some influence. Thus, premenopausal women obtain some protection from oestrogen (Grady et al., 1992). For many years the main focus has been on the damaging effect of cholesterol in the vascular wall. This was due to the clinical manifestation of atherosclerosis involving cholesterol build up under the epithelium. This first visible sign presents itself as fatty streaks, which are sub-endothelial aggregates of foam cells. These cells (mostly macrophages) have lipid droplets in their cytoplasm, which consist of cholesterol or cholesterol esters. Streaks start developing in early life, eventually disappearing later on or turning into atherosclerotic plaques in middle age (Wissler et al., 1984). Plaques are raised lesions that can be up to 1 cm in length (Wissler, 1984). The core of the lesion contains lipid droplets and cellular debris along with calcium deposits, leading to a sticky porridge like texture, termed an atheroma (from the Greek athere = gruel). Platelets and other blood cells can stick to this atheroma eventually building up to a point where the vessel becomes occluded.

1.2.1 Hypercholesterolaemia

Hypercholesterolaemia is classified as a long-lasting moderate increase in serum cholesterol and cholesterol LDL. In this disease EC dysfunction usually appears in the second decade of life (Sorensen et al., 1994). Endothelium-dependent relaxation of the coronary microcirculation in patients with this condition is reduced compared with control subjects (Drexler et al., 1991), while endothelium-independent vasodilators work to the same extent (Kuo et al., 1992). Hypercholesterolaemic monkeys have been reported to possess ECs that no longer orientate themselves to the direction of blood flow, and show less overlap between adjacent cells (Taylor et al., 1989). ECs exposed to mildly ox-LDL (as found in hypercholesterolaemia) form actin stress fibres that cause EC contraction, which in turn creates intercellular gaps that lead to increased permeability of the endothelial lining. This is not caused by a change in calcium concentration ([Ca^{2+}]) in the cells but is thought to be by activation of Rho and Rho kinase (Essler et al., 1999). The same effect has been observed in ECs stimulated with TNF-α (Wojciak-Stothard et al., 1998).

LDL is the primary carrier of cholesterol in the blood and is prone to changes caused by oxidative reactions, producing ox-LDL, which is collected by scavenger receptors on macrophages (for review see Kroon, (1997)). Ox-LDL and its peroxide derivative lyso-phosphatidylcholine (LPC) have various effects on ECs. These include activation of PKC, release of Ca^{2+} from internal stores, phosphoinisitol turnover, inhibition of endothelial repair and angiogenesis, and induction of apoptosis (Henry, 1994). The importance of PKC is further illustrated by the observation that PKC inhibitors block the attenuation of endothelial-dependent vasodilation by ox-LDL (Ohgushi et al., 1993),
and PKC activation by phorbol esters has been shown to inhibit NO release from aortic ECs (Hirata et al., 1995). Reductions in plasma cholesterol have led to a regression of atherosclerotic lesions (Benzuly et al., 1994), suggesting endothelial dysfunction in this condition is reversible once the primary insult is removed. Treatment with statins and the antioxidant probucol have shown advantageous results on endothelial-dependent relaxation of the vasculature (Anderson et al., 1995).

Models using hypercholesterolaemic animals report an increase in superoxide (O$_2^-$) (Ohara et al., 1993; Mugge et al., 1994). Reactive oxygen species (ROS) and ox-LDL are reported to inactivate NO, and decreased NO activity can increase the rate of progression of atherosclerosis by allowing an increase in leukocyte adhesion and migration into the endothelium (Naruse et al., 1994). Monocyte chemoattractant protein-1 (MCP-1) which is normally suppressed by NO (Zeiher et al., 1995) increases, compounding the problem. Dietary use of reduced glutathione (GSH) or vitamin C can help combat the decrease in NO activity (Kugiyama et al., 1998; Solzbach et al., 1997; Hornig et al., 1998). Vitamin E has also been reported to be beneficial in hypercholesterolaemia, but the results are not conclusive as some studies have shown no beneficial effect in these patients (McDowell et al., 1994; Elliot et al., 1995b), but this may have been due to the trials being held for too short a period of time to allow the appearance of beneficial effects.

### 1.2.2 Homocysteine

For the last 20 years it has been recognised that increased levels of homocysteine are associated with an increased risk of atherosclerosis (Cornhill and Roach, 1976). Homocysteine is a metabolic breakdown product produced by demethylation of dietary methionine (Hankey and Eikelboom, 1999). The route by which increased levels of homocysteine lead to atherosclerotic risk are still unclear, but are thought to be via oxidative damage to the endothelium (Dewey et al., 1981; Loscalzo, 1996). This may be through auto-oxidation, inhibition of glutathione peroxidase or oxidation of LDL (Upchurch et al., 1997; Loscalzo, 1996; Starkebaum and Harlan, 1993). In one study a partial impairment of endothelial vasodilatation of the brachial artery was produced by raised concentrations of homocysteine, addition of vitamin C was able to prevent this EC dysfunction (Kanani et al., 1999). This protective function could be due to prevention of a direct interaction of ROS and NO, or by increasing cellular levels of glutathione (Gryglewski et al., 1986; Winkler et al., 1994).

Another link to atherosclerosis is by modulation of annexin 2, an EC cell surface receptor for tPA and plasminogen. Addition of homocysteine to cultured ECs has been
reported to decrease tPA binding sites by 65% correlating with a decrease in tPA activity of 60% (Hajjar, 1993), presenting a more thrombogenic endothelial surface. Most studies have used concentrations of homocysteine ten times higher than those seen in patients suffering from moderate of hyperhomocysteinaemia, so results should be viewed with some caution. At present there are no data available for controlled trials showing effects of lowering plasma homocysteine on the incidence of atherosclerotic events.

1.2.3 Hypertension and endothelial derived relaxation

Contraction and relaxation of the vasculature is extremely important in maintaining blood pressure. A loss of vascular reactivity results in hypertension, and this is often seen as a consequence of EC dysfunction. Due to this fact, this condition is linked to many of the risk factors involved in atherosclerosis, and EC dysfunction can develop before hypertension exhibits itself clinically (Taddei et al., 1992). Resulting hypertension can lead to greater mechanical forces on the vessel walls which in turn may be a contributing factor to the atherosclerotic process.

Since the discovery of the essential role of the endothelium in causing relaxation of an isolated rabbit aorta stimulated with acetylcholine (Furchgott and Zawadzki, 1980), vascular relaxation has been used to assess the health of the endothelium. ECs release factors that cause vasodilatation (as mentioned in 1.1) in response to agonists released by the autonomic nervous system, hormones and autocoids produced by vascular smooth muscle or the endothelium itself (Bassenge and Heuch, 1990). In atherosclerosis, diabetes mellitus, smoking, hypertension and heart failure, endothelium-dependent relaxation of the vessels is diminished. Atherosclerotic vessels no longer dilate in response to increased blood flow (Nabel et al., 1990), and when stimulated with acetylcholine they no longer dilate but instead constrict (Ludmer et al., 1986). Endothelial control of vascular tone involves many factors including NO, PGI$_2$, endothelin and endothelium derived hyperpolarising factor (Drexler, 1997). Several mechanisms have been proposed to explain the loss of this response. Altered expression of eNOS, an increase in breakdown of NO by scavenging oxygen free radicals, modified signal transduction within cells, decreased release of endothelial derived relaxing factors, and a reduced responsiveness of smooth muscle to NO have all been suggested (Harrison, 1997). However NO production is enhanced in hypercholesterolaemic rabbit aorta, suggesting that there is no shortage of its substrate L-arginine, or decrease in endothelial eNOS activity (Minor et al., 1990).

Various experimental strategies have been developed to investigate the events involved in atherosclerosis. Mechanical denudation is a widely used method of study.
but is unlikely to be the cause of pathogenesis in spontaneous human atherosclerosis (Clowes et al., 1986). Other groups use models involving endotoxins, anoxia, hyperoxia, carbon dioxide, vasoactive amines, hypercholesterolaemia and viral injury, with model generated hypercholesterolaemia being the most extensively studied method.

1.3 Oxidative stress

Reactive oxygen species are implicated in various diseases, including Alzheimer's disease, cancer, and atherosclerosis, which is now seen as a disease with an abnormality in redox-mediated signals (Offermann and Medford, 1994). It has long been unclear how the disease atherosclerosis could be common to so many different risk factors. Many of the conditions that lead to atherosclerosis (hyperglycaemia, hypertension, hyperlipidaemia, hyperhomocysteinaemia and haemodynamic stress) show elevated levels of ROS in the vasculature. Lipid peroxides and ROS are thought to be causal in the formation of atheromatous plaques and are known to alter the synthesis of PGI\textsubscript{2} (Vane and Botting, 1994). The levels of ROS are elevated in the plasma of atherosclerotic tissue, and ROS activity has been reported in the cytoplasm of ECs (Lerman and Burnett, 1992; Winkles et al., 1993). It is thought that in the later stages of atherosclerosis, inflammatory cells may contribute to this production. Much current research therefore now concentrates on oxidative stress, as the production of ROS appears to be a common link.

Although it is known that ROS can be damaging to cells it is now also thought that they may play a second messenger role, regulating certain signal transduction pathways that ultimately lead to downstream events such as control of gene expression and post-translational modification of proteins. In this way ROS may modulate certain sets of vascular inflammatory genes, such as vascular cell adhesion molecule (VCAM-1) and MCP-1, whose expression is stimulated through a redox-sensitive pathway involving nuclear factor-\kappaB (Satriano et al., 1993; Larrick and Wright, 1990; Marui et al., 1993). VCAM-1 binds to its late antigen-4, which is expressed on monocytes (Elices et al., 1990) that later become the macrophage foam cells that form fatty streaks (Libby et al., 1996), and leukocytes, which accumulate in experimental atherosclerosis. This increase in VCAM-1 expression occurs along with an increase in intercellular adhesion molecule (ICAM-1) in ECs cultured under hyperoxic conditions. The mRNA and protein levels are both increased (William et al., 1999), but the change in ICAM-1 is reported to be blocked by the PKC inhibitor H-7 (Suzuki et al., 1997a). Changes of expression could be due either to ECs releasing
factors that stimulate ICAM-1 and VCAM-1 expression in response to exposure to ROS, or through ROS having a direct effect on gene transcription.

### 1.3.1 Production of reactive oxygen species

Many cell types are capable of producing ROS. Neutrophils and macrophages produce hydrogen peroxide (H$_2$O$_2$) and O$_2^-$ as a defence mechanism against invading organisms. Cells such as ECs, fibroblasts and smooth muscle cells have been shown to release ROS as a response to certain activation signals. ROS can also have an effect on Ca$^{2+}$ signalling, protein kinases and protein phosphatases, with exposure to O$_2^-$ causing damage to DNA, protein and lipids.

There are many sources of ROS (see Table 1), these include NOS (Pritchard et al., 1995), lipoxigenases, nicotinamide-adenine dinucleotide/phosphate (NADH/NADPH) oxidase (Harrison, 1997), xanthine oxidase, cyclo-oxygenase (Holland et al., 1988), cytochrome P-450 (Pritchard et al., 1990) and mono-oxygenase (Freeman and Crapo, 1982). Mitochondria are the most prolific generators of ROS in cells, generating them as by-products during electron transfer from NADH to FADH to molecular oxygen. Through these mediators, cells constantly produce ROS such as H$_2$O$_2$, but not all of these sources are well established in the vasculature. ECs produce free radicals and H$_2$O$_2$ in response to shear stress and certain agonists (Shimizu et al., 1994), and the NADPH oxidase pathway has been shown to generate O$_2^-$. In ECs, this pathway can be activated by cytokines and growth factors, such as TNF-α, angiotensin II and interferon-γ (Mohazzab et al., 1994; Griendling et al., 1994; De Keulenaer et al., 1998). LPC has also been shown to enhance the vascular production of O$_2^-$ by a mechanism involving the activation of PKC (Ohara et al., 1994).
Table 1. Sources of cellular ROS production.

<table>
<thead>
<tr>
<th>Source</th>
<th>Localisation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Electron transport system</td>
<td>Mitochondria</td>
</tr>
<tr>
<td>Cyclooxygenases</td>
<td>Plasma membrane</td>
</tr>
<tr>
<td>Lipoxygenases</td>
<td></td>
</tr>
<tr>
<td>NADPH oxidase</td>
<td></td>
</tr>
<tr>
<td>Cytochromes P-450 and b₅</td>
<td>Endoplasmic reticulum</td>
</tr>
<tr>
<td>Xanthine oxidase</td>
<td>Cytosol</td>
</tr>
<tr>
<td>Transition metals</td>
<td></td>
</tr>
<tr>
<td>Catecholamines</td>
<td></td>
</tr>
<tr>
<td>Haemoglobin</td>
<td></td>
</tr>
<tr>
<td>Riboflavin</td>
<td></td>
</tr>
<tr>
<td>Oxidases</td>
<td>Peroxisome</td>
</tr>
<tr>
<td>Flavoproteins</td>
<td></td>
</tr>
</tbody>
</table>

Reduction of oxygen at the molecular level yields $O_2^-$, $H_2O_2$ and hydroxyl radicals (·OH). Oxygen tends to gain electrons due to its electronic configuration, making it a powerful oxidant. NO can also be oxidised into reactive species that act in a similar way to ROS, NO and $O_2^-$ together forming peroxynitrite ($ONOO^-$) (Murphy et al., 1998), this reaction is extremely rapid ($6.7x10^9$ moles/sec) (Thomson et al., 1995), three times faster than superoxide dismutase (SOD) can scavenge $O_2^-$ (Beckman et al., 1994).

1.3.2 Antioxidant defence mechanisms

Mammalian cells have antioxidant defence mechanisms in the form of the enzymes SOD, catalase and the glutathione-dependent system which consists of glutathione peroxidase (GSH) and reduced glutathione (GSSH), which together function as a homeostatic redox buffer. Oxidative stress occurs when the rate of formation of ROS exceeds the capability of these defence systems.
SOD converts $O_2^-$ to oxygen and $H_2O_2$. Through this reaction SOD is able to maintain the concentration of $O_2^-$ at a level far lower than would be achieved by spontaneous breakdown. Inhibiting SOD with diethyldithiocarbamate can increase levels of $O_2^-$ (Pagano et al., 1993). In this way SOD protects NO that would normally be destroyed by $O_2^-$ (Rubanyi and Vanhoutte, 1986). Eukaryotic cells have two types of SOD, a cytosolic form that requires Cu$^{2+}$/Zn$^{2+}$ and a Mn$^{2+}$-dependent form, found in mitochondria. The latter shows an increase in activity in response to oxidative stress (Fridovich, 1995).

The $H_2O_2$ produced by this reaction is then metabolised by either catalase or the glutathione-dependent system, and is broken down into oxygen and water (Michiels et al., 1994). Glutathione is a tripeptide that contains thiol moieties that act as reducing agents. It is mainly expressed in organs exposed to oxidative stress with very little in body fluids (DeLeve and Kaplowitz, 1991). During oxidative stress the levels of GSH increase. In one illustrative study, ECs grown in 95% $O_2$ for 3 days showed the following increases; 60% in glutathione peroxidase transcription rate, 394% in mRNA levels and 81% in activity (Jornot and Junod, 1995).

Another component of the antioxidant defence mechanism is catalase. Catalase has a binding site for NADPH, and when bound it is able to function at similar levels of $H_2O_2$ to that of the glutathione system (Gaetani et al., 1996). Previously it had been thought to work only at high $H_2O_2$ concentrations, acting as a back-up system to glutathione. A few low molecular weight compounds also act as antioxidants, examples are vitamins C and E, carotenoids, bilirubin and uric acid. Consistent with this, use of antioxidants has been shown to have beneficial effects on EC function (Hennig et al., 1994). They can decrease adhesion molecule expression and release of MCP-1, by increasing NO activity through a decrease in cellular production of ROS (Frei, 1999).

<table>
<thead>
<tr>
<th>Antioxidant</th>
<th>Reaction</th>
</tr>
</thead>
<tbody>
<tr>
<td>Superoxide dismutase</td>
<td>$2O_2^- + 2H^+ \rightarrow H_2O_2 + O_2$</td>
</tr>
<tr>
<td>Catalase</td>
<td>$2H_2O_2 \rightarrow 2H_2O + O_2$</td>
</tr>
<tr>
<td>Glutathione peroxidase</td>
<td>$2GSH + H_2O_2 \rightarrow GSSG + 2H_2O$</td>
</tr>
<tr>
<td>Glutathione reductase</td>
<td>$GSSG + NADPH + H^+ \rightarrow 2GSH + NADP^+$</td>
</tr>
</tbody>
</table>

Table 2. Reactions catalysed by antioxidant defence mechanisms.
1.3.3 Protein kinases/phosphatases

Experimental evidence is now emerging to suggest that some protein kinase/phosphatase pathways are involved in redox mediated-signalling. Oxidant-mediated changes in gene transcription are thought to require changes in both tyrosine and serine/threonine phosphorylations (Barchowsky et al., 1995). Tyrosine kinases such as Ick are activated by oxidative stress induced by UVB and H₂O₂ (Nakamura et al., 1993). It is not clear whether ROS directly affect tyrosine kinases or if their action is due to increased inhibition of tyrosine phosphatase activity. There are suggestions that it is the latter, as all tyrosine phosphatases have reactive cysteine residues in their active sites (Suzuki et al., 1997b). Oxidation of a cysteine residue or formation of a disulphide bridge may alter the conformation or function of the protein. Inhibition of tyrosine phosphatases using phenylarsine oxide leads to endothelium-dependent relaxation of the vasculature due to NO and endothelium derived hyperpolarising factor release, and also produces increased [Ca²⁺], and accumulation of 6-ketoprostaglandin F₁α. Inhibition of tyrosine kinases prevented all of these effects (Flemming et al., 1996), supporting the idea that tyrosine phosphorylation is involved in redox mediated signalling and that this is caused by decreased tyrosine phosphatase activity.

It is widely believed that changes in cellular redox state are capable of activating members of the mitogen-activated protein kinase (MAPK) family (Laderoute and Webster, 1997; Rao et al., 1995). The MAPK family comprises the ERK subgroup, the p38 MAPK subgroup and the N-terminal kinase (SAPK/JNK) subgroup. Physiological ox-LDL that contributes to EC dysfunction has been shown to increase MAPK activity in smooth muscle cells (Auge et al., 1998). The effect of redox changes on this set of proteins may be due to modulation of the signalling processes that converge on the MAPKs. In smooth muscle O₂⁻ production from stimulation of NADPH oxidase led to activation of c-fos (Bhunia et al., 1997), and HUVECs show activation of p38 MAPK when stimulated with H₂O₂ (Huot et al., 1997). Proteins may react in different ways to oxidative stress according to their conformation, cysteine residues and the magnitude of oxidative stress applied.

1.3.4 Diabetes

A study of diabetic patients found that expression of E-selectin and ICAM-1 in blood samples was increased (Elhadd et al., 1999) along with the concentration of SOD. These and other observations have led to speculation that free radicals may contribute to EC dysfunction in diabetes. In agreement with this idea are studies that show that high glucose concentrations lead to O₂⁻ production (Graier et al., 1996),
suggesting that hyperglycaemia is a cause of oxidant stress (Baynes, 1991). Advanced glycation end products (AGEs) have been shown to interact with EC surface receptors, yielding ROS whilst decreasing glutathione levels (Yan et al., 1994). A soluble receptor for AGEs can block many of the pathological effects in diabetes (Lalla et al., 2000), by preventing direct interaction of AGEs with their receptor, which is known to cause vascular disorders (Schmidt et al., 1999).

1.3.5 Experimental investigation of oxidative stress

Various models are used for studying the effects of oxidative stress, typically based on the application of peroxides such as $\text{H}_2\text{O}_2$ and tert-butylhydroperoxide (t-BOOH). When added to cells in culture these agents give a short-term but potent exposure as they are quickly broken down by the glutathione system. One effect of t-BOOH is to activate a non-selective cation channel in calf pulmonary artery ECs, causing depolarisation of the plasma membrane (Koliwad et al., 1996b), and increasing $\text{Ca}^{2+}$ permeability which may in turn inhibit agonist activated $\text{Ca}^{2+}$ channels. It is suggested that in these cells oxidised glutathione produced during oxidant stress, is able to mediate channel activation and depolarisation (Koliwad et al., 1996a). t-BOOH selectively inhibits glutathione peroxidase activity without any effect on GSH activity (Ochi and Miyaura, 1989). From these results it is suggested that t-BOOH has an increased cytotoxic effect by also decreasing cellular glutathione levels. Depletion of glutathione has been reported to cause a G2/M arrest (Russo et al., 1995). Another method for inducing oxidative stress is extracellular exposure to $\text{O}_2^-$, which can be achieved using potassium superoxide, and again this produces a fast but transient effect. This form of oxidant stress stimulates Na$^+$/K$^+$ pump activity leading to a Na$^+$ influx (Rounds et al., 1998) that is also observed with use of t-BOOH (Elliott and Schilling, 1992).

Hyperoxia is a form of oxidant stress used to generate endogenous free radicals. Most primary cells have an optimum atmospheric oxygen concentration of between 2-5 % $\text{O}_2$ (Balin et al., 1984), which is classed as normoxic; higher percentages than this are hyperoxic. Most cell cultures are gassed at 20% $\text{O}_2$, which is a hyperoxic condition, compared with their physiological environment, the exception being lung tissue. Under hyperoxic conditions $\text{O}_2^-$ and $\text{H}_2\text{O}_2$ are presumed to be produced in increased amounts compared with cells grown in normoxic conditions. The conditions for inducing hyperoxia are usually 98% $\text{O}_2$ for a period of a few days, though smaller percentages can be used over longer periods of time. It is known the effects produced result from a direct interaction of $\text{O}_2$ with the cells rather than an interaction with the medium, which could then have an indirect effect on the cells (Spitz et al., 1990). Cells grown in 95% $\text{O}_2$ for 3 days become more permeable to albumin (Phillips
and Tsan, 1988) and have been reported to release more PGI$_2$ metabolite, 6-ketoprostaglandin F$_1$α and prostaglandin F$_2$ alpha than control cells (Jackson et al., 1986).

### 1.3.6 Oxidative stress and apoptosis

The mechanisms that link oxidative stress to cardiovascular disease and cell death are poorly understood. Some groups have suggested the mechanism to be apoptosis, a genetically controlled and energy-dependent process. One group reported that following treatment with H$_2$O$_2$ or exposure to hyperoxia, ECs show a reduced proliferation rate and an increased incidence of apoptosis (Hogg et al., 1999). ECs that do undergo apoptosis after addition of staurosporin (a non-specific protein kinase inhibitor), have been demonstrated to become procoagulant due to a swift redistribution of phosphatidylserine accompanied by a decrease in anticoagulant surface proteins such as thrombomodulin and heparin sulphate (Bombeli et al., 1997). Other groups have examined cells from atherosclerotic plaques and shown that many cells appeared to be undergoing apoptosis, although this may have been accompanied by necrotic cell death (Han et al., 1995; Isner et al., 1995). Generation of EC apoptosis by oxidative stress can be inhibited by NO \textit{in vitro}, perhaps by stress-induced modification of gene expression which occurs before cell death (DeMeester et al., 1998). \textit{In vivo} many other modulators such as growth factors and some cytokines are also able to inhibit apoptosis, suggesting that NO does not act alone but instead makes a contribution to this effect.

Some groups classify cell death caused by hyperoxia as necrosis, and although this description is vague it suggests that cell death by hyperoxia and low dose oxidants is not apoptotic (Kazzaz et al., 1996). Experiments on bovine aortic ECs have shown by in situ end labelling, both hyperoxia and treatment of cells with H$_2$O$_2$ to increase the level of apoptosis compared with control cells (Hogg et al., 1999). ECs treated with t-BOOH have been found to produce membrane blebs, nuclear condensation and rearrangement of the actin cytoskeleton that is not associated with apoptosis, as judged by an absence of cell surface phosphatidylserine or fragmentation of DNA (van Gorp et al., 1999). The difference in opinion as to whether death is by apoptosis or necrosis may arise due to the different types of stress used to damage the cells, and that death may occur by a mixture of the two.
1.4 Calcium signalling

Cells have signalling mechanisms that enable them to transmit information from the cell surface to particular destinations within the cell. This process is often mediated through the use of messengers, with Ca\(^{2+}\) being an important example. Ca\(^{2+}\) regulates a variety of processes including gene expression, neurotransmission, cell growth, secretion, metabolism, contraction and motility. Although Ca\(^{2+}\) signals are usually short lived, because of the inherent cytotoxicity of Ca\(^{2+}\), they are able to effect transcription of early response genes such as fos and jun (van Haasteren et al., 1999). Within the cell Ca\(^{2+}\) operates in a global sense, penetrating the nucleus and mitochondria and/or small cellular compartments. Cells exert tight control over intracellular calcium concentration ([Ca\(^{2+}\)]), by sequestering it into intracellular stores and via extrusion through the plasma membrane.

A wide variety of external stimuli cause intracellular levels of calcium to rise, these include hormones, growth factors and neurotransmitters, as well as nutrient and metabolic activators. A rise in intracellular Ca\(^{2+}\) usually consists of two phases; a transient peak due to release from internal stores and a maintained plateau due to influx from the extracellular space. This rise can elicit activation of Ca\(^{2+}\)-dependent proteins such as PKC, annexins, calcineurin and calmodulin-dependent protein kinases. The increase in [Ca\(^{2+}\)] is formed by release from internal stores, opening of Ca\(^{2+}\) channels and by activity of Ca\(^{2+}\) transporters. There are at least four types of plasma membrane Ca\(^{2+}\) channel; voltage operated-channels, receptor-operated channels, mechanically operated channels and store-operated channels, which are opened by depletion of internal stores. Internal stores release Ca\(^{2+}\) via channels; the three main ones are; inositol 1,4,5-trisphosphate (IP\(_3\)) receptors, ryanodine receptors and SCaMPER (sphingolipid Ca\(^{2+}\)-release-mediated protein of endoplasmic reticulum) channels.

Global signalling is initiated by local Ca\(^{2+}\) signals in the form of puffs, waves and oscillations, which are normally co-ordinated through Ca\(^{2+}\)-induced Ca\(^{2+}\)-release in non-excitable cells (Putney and Bird, 1993). If cells are connected by gap junctions a Ca\(^{2+}\) wave can spread from one cell to the next. Local Ca\(^{2+}\) signals control release of secretory vesicles, activation of ion channels and formation of nuclear Ca\(^{2+}\) signals. The [Ca\(^{2+}\)] rise generated by these signals can be removed from the cytoplasm promptly compared with global signals and requires a minimal use of ATP.

In acting as a second messenger Ca\(^{2+}\) mediates many of its pleiotropic activities through interaction with Ca\(^{2+}\)-binding proteins, which in turn impinge on signalling pathways by their modulation of protein interactions or their own enzymatic activity.
Ca²⁺-binding proteins can roughly be split into three categories; EF hand proteins, Ca²⁺/phospholipid binding proteins and Ca²⁺ storage proteins. EF hand proteins, which encompass calmodulin, troponins and the S100 family, are mainly located within the cytoplasm and the nucleus, with only a few forming direct interactions with membrane lipids. The Ca²⁺ / phospholipid binding proteins can be split into two groups; the annexin family and the C2 domain proteins. These proteins generally respond to increased [Ca²⁺], by translocating and binding to membranes, whereupon they become active. Calcium storage proteins such as calsequestrin and careticulin act to take up and hold Ca²⁺ in the internal stores, the ER and SR, until required. This Ca²⁺ buffering effect is important in the fine control of Ca²⁺ fluxes within cells. These proteins have a low affinity for Ca²⁺, but a high binding capacity.

1.4.1 Calcium regulation in the vascular endothelium

Calcium signalling in the vascular endothelium is important for autacoid release. A PLC-dependent rise in [Ca²⁺], leads to production of NO and PGI₂ (Kruse et al., 1994) mediated by the Ca²⁺/calmodulin-dependent NOS (Blatter et al., 1995). This release has been shown to be critically dependent on influx of external Ca²⁺ (Long and Stone, 1985). Factors within serum, in which the endothelium is constantly bathed, exert some effect over Ca²⁺ signalling. In HUVECs serum albumin has been reported to increase [Ca²⁺], both by release from internal stores and influx across the plasma membrane. In contrast, plasma albumin caused a sustained decrease in [Ca²⁺], due to uptake into internal stores (Fuentes et al., 1997), which potentiates the Ca²⁺ signal when cells are stimulated with agonists such as histamine. Plasma albumin is converted to serum albumin during blood coagulation, suggesting that coagulation may have an effect in regulating vascular tone. Addition of unstimulated platelets to cultured ECs has been shown to produce a transient increase in cytosolic Ca²⁺ involving PLC (van Ijzendoom et al., 1996). This was not due to platelet secretion as the supernatant was found to be inactive, but is thought to be through mechanical stimulation.

ATP stimulation of ECs is reported to activate Ca²⁺-dependent K⁺ channels (Sauve et al., 1988) thought to be distinct from those found in other cell types (Laskey et al., 1990). These channels are proposed to hyperpolarise the membrane increasing the force driving Ca²⁺ into the cell during agonist stimulation (Luckhoff and Busse, 1990). In other experiments where cells were depolarised Ca²⁺ influx was decreased (He and Curry, 1991).

In ECs calcium entry is not via voltage-gated channels, channels activated by increased [Ca²⁺], or by a Na⁺/Ca²⁺ exchange mechanism. It is known that following
agonist binding inositol hydrolysis and/or release of Ca\(^{2+}\) from IP\(_3\) sensitive stores is required (Schilling and Elliott, 1996). Non-specific cation channels have been suggested as a possible route for Ca\(^{2+}\) entry (Nilius, 1990; Fichtner et al., 1987). The mechanism of agonist activated Ca\(^{2+}\) entry in ECs is unclear, although it is known to increase membrane permeability of both K\(^+\) and Ca\(^{2+}\) (Adams et al., 1989).

Release of Ca\(^{2+}\) from internal stores generates a Ca\(^{2+}\) influx into the cell which is termed capacitative Ca\(^{2+}\) entry. The generation of this influx of Ca\(^{2+}\) has been suggested to be activated via two distinct mechanisms, the first involves the release of a diffusible message when intracellular stores are depleted. In ECs activation of capacitative Ca\(^{2+}\) entry is thought to involve cytochrome P450 (Graier et al., 1995) or tyrosine kinases (Sharma and Davis, 1996). Thus, depletion of intracellular Ca\(^{2+}\) stores has been shown to lead to activation of P450 mono-oxygenase which in turn induces production of 5,6-epoxyeicosatrienoic acid, which is proposed to be a second messenger activating Ca\(^{2+}\) influx (Graier et al., 1995). Experiments using genistein or tyrphostin (both tyrosine kinase inhibitors) on ECs, are reported to decrease the Ca\(^{2+}\) influx stimulated by substance P. Similar results were reported when using 2,5-di-tert-butylhydroquinone, which induces release of intracellular Ca\(^{2+}\) (Sharma and Davis, 1996).

The other mechanism of Ca\(^{2+}\) entry is via a direct interaction of IP\(_3\) receptors on the ER with the plasma membrane termed conformational coupling (for a review see Putney, 1999). This process has been suggested to involve members of the TRP family of proteins that act as Ca\(^{2+}\) channels in the plasma membrane, and that come into direct contact with IP\(_3\) receptors during conformational coupling.

1.4.2 Calcium signalling under oxidative stress

An early event in endothelial dysfunction is the modification of transmembrane signalling mechanisms. Growing evidence suggests that oxidative stress causes changes in Ca\(^{2+}\) signalling and homeostasis, with some oxidants reported to modulate Ca\(^{2+}\) signalling in cells (Suzuki et al., 1997b), but the mechanisms by which they do this are not known. One possibility is the modification of Ca\(^{2+}\) pumps, as a variety of oxidants have been shown to block ATP-dependent Ca\(^{2+}\) pump activity (Grover and Samson, 1988; Grover et al., 1992). Another idea is enhanced influx of Ca\(^{2+}\) through Ca\(^{2+}\) channels, as the Ca\(^{2+}\) channel blocker Ni\(^{2+}\), partially inhibits increased [Ca\(^{2+}\)], when ECs are stimulated with H\(_2\)O\(_2\) (Doan et al., 1994).

Experiments using t-BOOH have shown a time-dependent inhibition of agonist stimulated changes in [Ca\(^{2+}\)]\(_i\) within ECs (Elliott et al., 1989). Initially there is inhibition of
agonist stimulated Ca\(^{2+}\) influx without any effect on basal [Ca\(^{2+}\)]. After a longer incubation there was inhibition of release from internal stores with a raised basal [Ca\(^{2+}\)]. Eventually the basal level continued rising and the cells became unresponsive to agonist stimulation. This type of oxidative stress results in the inhibition of Na\(^+-K^+\)-Cl\(^-\) co-transporter activity, but increases the activation of Na\(^+/K^+\) ATPases (Elliot and Schilling, 1992). During incubation of ECs with t-BOOH, release of Ca\(^{2+}\) from internal stores by ionomycin and Ca\(^{2+}\)-ATPase inhibitors is maintained (Elliott and Doan, 1993). This indicates that the lack of agonist-dependent store release may be due to oxidant stress inhibiting one or more of the steps between the receptor binding and activation of IP\(_3\) receptors, rather than a depletion in releasable Ca\(^{2+}\). Certain parts of the Ca\(^{2+}\) signalling pathway including the IP\(_3\) receptor (Yang and Lee, 1989) and the plasmalemma Ca\(^{2+}\) pump (Nicotera et al., 1985) contain sulfhydryl groups which can be influenced by the redox status of the cell.

H\(_2\)O\(_2\) also produces an increase in [Ca\(^{2+}\)], (Vercellotti et al., 1991) in ECs. It initiates a biphasic signal with the maximum amount of [Ca\(^{2+}\)] seen within the first minute, followed by a lasting elevation of [Ca\(^{2+}\)], above the basal level. One group have reported that both O\(_2^\cdot\) and H\(_2\)O\(_2\) are needed to produce a maximal Ca\(^{2+}\) response (Dreher and Junod, 1995) comparable to that produced by inhibitors of ER Ca\(^{2+}\)-ATPase (Gericke et al., 1993) such as thapsigargin, or by receptor-agonists (Wickham et al., 1988) such as bradykinin. The ability to produce this response may be due to an interaction between O\(_2^\cdot\) and H\(_2\)O\(_2\) producing -OH.

In one case the basal increase in [Ca\(^{2+}\)] seen on addition of H\(_2\)O\(_2\) was attributed to the release of annexin 6 normally bound to the inner surface of the plasma membrane (Hoyal et al., 1996). This study used macrophages to show that translocation of annexin 6 during exposure to t-BOOH, matched the increase in [Ca\(^{2+}\)] in a time-dependent manner. The authors suggested that in the initial stages of oxidant stress there is inhibition of the plasma membrane Ca\(^{2+}\)-ATPase in conjunction with a release of Ca\(^{2+}\) from annexin 6 that is no longer bound to the membrane. It is known that the increase in Ca\(^{2+}\) is not from internal stores but it is possible that it may be released from mitochondria damaged by exposure to t-BOOH (Masaki et al., 1989). This implies that membrane-bound annexin 6 is a major Ca\(^{2+}\) store although in many cell types annexin 6 would not be bound to the membrane under resting conditions. It is suggested that an increase in [Ca\(^{2+}\)], similar to that observed, would require 3-7% release of Ca\(^{2+}\)-bound annexin 6 from the plasma membrane (Hoyal et al., 1996). However, annexin 6 is able to bind the membrane in a Ca\(^{2+}\)-independent manner, so some of the annexin 6 released from the membrane by H\(_2\)O\(_2\) may not be bound to Ca\(^{2+}\).
The antioxidant glutathione system has an important role in oxidant-induced changes in Ca\(^{2+}\) signalling. Decreased GSH potentiates the action of H\(_2\)O\(_2\) on Ca\(^{2+}\) signalling (Elliot et al., 1995a). Experiments have also been performed using t-BOOH and investigating Ca\(^{2+}\) levels in the lumen of IP\(_3\) sensitive stores. A decrease in reduced glutathione had no effect on changes in Ca\(^{2+}\) signalling produced by t-BOOH but an increase in GSSH caused a decrease in the IP\(_3\) sensitive Ca\(^{2+}\) stores (Henschke and Elliott, 1995). The levels required to produce a reduction can be achieved when cytosolic GSH is inhibited by oxidative stress.

1.4.3 **Atherosclerosis and calcium signalling**

There is evidence for Ca\(^{2+}\) having a central role in vascular function and the initiation and progression of atherosclerosis. A new way of treating disease states involving altered Ca\(^{2+}\) signalling in ECs, is by use of Ca\(^{2+}\) antagonists. Most act on L-type channels to regenerate normal physiological regulation of Ca\(^{2+}\) required for normal cellular function. So far not all clinical trials have shown encouraging results (Haller and Elliott, 1996), but experiments using Ca\(^{2+}\) channel blockers have led to reduced endothelial permeability (Strohschneider and Betz, 1989) and improved endothelium-derived relaxation of the vasculature (Habib et al., 1986) in atherosclerotic conditions.

1.5 **Annexins**

Annexins are a family of proteins which bind phospholipids and most are able to bind Ca\(^{2+}\). The first annexin discovered was in 1978 by Creutz et al., who identified a 55 kDa protein they named synexin (annexin 7), a protein that aggregates isolated chromaffin granules. Many other annexins emerged over the years, often being discovered serendipitously in biochemical assays. Annexins 1 and 2 were initially described as substrates for tyrosine phosphorylation by the epidermal growth factor receptor (EGFr) (Fava and Cohen, 1984) and pp60\(^{src}\) (Huang et al., 1986) respectively. Before the suggestion that annexins may share a common structure based on shared biochemical properties, they were given unrelated names derived from their particular biochemical properties or proposed functions (lipocortin, calelectrin, calpactin, calphobindins, calcyclin-associated protein-50, calcimedins, synexin, anchorin, endonexins, placental anticoagulant proteins p35, p36 and p38). Geisow and colleagues suggested the name “annexin”, as they had the property of “annexation” with membranes and proteins in response to Ca\(^{2+}\) (Geisow et al., 1987). In 1990 Crumpton and Dedman simplified the nomenclature (Crumpton and Dedman, 1990), and at present the nomenclature is again under review. It has been proposed that annexins should be numbered within their own species and that the numbers
should follow a sequence, to eliminate the gaps that appear in the numbering at present. This system is waiting for approval by the Human Genome Mapping Project and is expected to come into use soon.

Annexins have been found in all eukaryotic phyla except for yeasts (Morgan and Fernandez, 1997a). Organisms that do not have annexins or annexin-like sequences in their genomes include viruses and bacteria, and those yeasts (S.Cerevisiae) that have been fully sequenced. To date there are 12 known human annexins, and attempts to resolve the functions of these proteins are made more complicated by the fact that cells usually express a combination of these proteins. Last year saw the discovery of two new human annexins, annexin 31 (Morgan et al., 1999a) that shows similarities with annexin 2, and annexin A10 (Morgan et al., 1999b) (named using the system soon to be introduced). Neither of these proteins have functional Ca$^{2+}$-binding sites. Annexins have always been described as Ca$^{2+}$ and phospholipid binding proteins, but with the discovery of these new family members this is no longer a valid generalisation.

### 1.5.1 Structure

Annexins comprise four conserved repeats that show both intramolecular and intermolecular sequence homology (Morgan and Fernandez, 1995). Each is about 70-80 amino acids in length, with the exception being annexin 6 which has eight repeats folded as two similar halves. The tetrad structure has been conserved since the most primary eukaryotes (Morgan and Fernandez, 1997b), with the only real variation being in repeat 3 which incorporates single codon insertions or deletions. The repeats within a single annexin share 25-35% sequence homology to each other (Haigler et al., 1989), and 45-55% between annexin paralogues (Smith and Moss, 1994). Each repeat contains five α-helices folded as less than two turns of a right handed superhelix, connected by loops or turns, with loops from the convex to the curved side being involved in Ca$^{2+}$ binding (Huber et al., 1990b). Four of the helices lie almost antiparallel to each other with the fifth helix almost perpendicular to the others.
Together the repeats make up a core domain usually around 300 residues in length, that has a flat but slightly concave shape, containing a central hydrophobic pore (see Figure 3). The domains are arranged with repeats 1 and 4, and 2 and 3 forming tight hydrophobic side chain interactions (Swairjo and Seaton, 1994; Liemann and Lewit-Bentley, 1995). The core was originally characterised by its resistance to proteolysis (Glenney, 1986b; Glenney and Tack, 1985).

From X-ray crystallographic studies of core domains, it is apparent that there is considerable structural conservation between annexins 1, 2, 3, 5, 6 and 12 (Burger et al., 1996; Favier-Perron et al., 1996; Huber et al., 1990b; Luecke et al., 1995; Weng et al., 1993). This is not true of the N-termini, where there is very little homology, except between direct paralogues. As this region of the protein varies the most, it may underlie the differential activities of individual family members.

In an annexin with four repeats the convex surface would be positioned towards the membrane with the Ca\(^{2+}\)-binding sites to the convex side. Production of annexin crystals has allowed the identification of two types of Ca\(^{2+}\)-binding sites, which distinguish annexins from EF hand proteins (Weng et al., 1993). Location and exact numbers appears to differ between the annexins (Weng et al., 1993; Burger et al., 1996; Benz et al., 1996; Luecke et al., 1995; Favier-Perron et al., 1996), with human annexin 31 (Morgan and Fernandez, 1998) and A10 (Morgan et al., 1999b) predicted to have no functional Ca\(^{2+}\)-binding sites. These differences could help explain the varied observations in Ca\(^{2+}\) and phospholipid sensitivities. Upon Ca\(^{2+}\) binding this

**Figure 3. An averaged ribbon structure of the annexins.**

A ribbon structure created from an average of annexin structures. The Ca\(^{2+}\)-binding sites are depicted as red spheres, with the four domains indicated with different colours. Purple represents domain I, green domain II, blue domain III and yellow domain IV. (A) View of annexin structure from the side, and (B) from the convex Ca\(^{2+}\)-binding side. Illustration provided by Dr Anita Lewit-Bentley (Orsay, France).
structural arrangement, in marked contrast to EF hand proteins, is not vastly changed (Brisson et al., 1991; Voges et al., 1994; Driessen et al., 1992). In the case of annexin 6, to allow membrane binding, the two halves are rotated at right angles to each other via a 49 amino acid α-helical domain, permitting both to be planar to the membrane (Benz et al., 1996).

The N-terminal domain ranges in length between 11 and 19 amino acids for annexins 3, 4, 5, 6, 12 and 13, about 40 amino acids for annexins 1 and 2, to over 100 for annexins 7 and 11, the latter being especially rich in glycine, tyrosine and proline residues (for a review see Raynal and Pollard, 1994). For annexins with short N-termini, these appear to be positioned on the concave side of the protein (Favier-Perron et al., 1996), in close apposition with the C-termini. Longer N-termini are more complex and are thought able to affect the Ca\(^{2+}\) and phospholipid binding sites on the convex side of the protein through direct intramolecular interactions. The N-terminal domains are often sites for phosphorylation and binding of protein ligands, providing further evidence of likely functional importance. Annexins 1 and 2 have N-termini that contain both tyrosine and PKC serine phosphorylation sites (De et al., 1986; Glenney and Tack, 1985; Porte et al., 1996; Sarafian et al., 1991). This domain also encompasses sites for post-translational modification, annexin 1 at position 18 has a glutamine that allows transglutaminase crosslinking of two annexin 1 monomers (Ando et al., 1991; Pepinsky et al., 1989), forming a covalent annexin 1 homodimer. Annexins 13a and b associate with the plasma membrane through myristoylation of the N-terminals, allowing Ca\(^{2+}\)-independent interaction to occur (Wice and Gordon, 1992; Fiedler et al., 1995). It is unclear whether other annexins are modified in this way.

1.5.2 Annexin protein ligands

Several annexins have unique protein ligands, through association with constituents of the cytoskeleton, extracellular matrix or enzymes. Most binding sites are located within the N-terminal domains, which can modulate the core domain when ligand-bound. Four members of the S100 family have been shown to bind annexins. The S100 family of proteins all contain two EF hand motifs, which selectively bind Ca\(^{2+}\) with high affinity. The first interaction to be discovered was the annexin 2\(_2\)-p11\(_2\) heterotetramer, which comprises two annexin 2 molecules linked by a dimer of the S100 protein p11 (S100A10) (Gerke and Weber, 1985b). When formed as this complex annexin 2 is able to attach to the sub plasma membrane cytoskeleton (Thiel et al., 1992), neither monomeric annexin 2 with no p11 binding site, or a mutant with some Ca\(^{2+}\) binding sites removed, were able to share this localisation. The complex is stabilised by hydrophobic forces through an interaction between the annexin 2 N-
terminus and the C-terminal extension of the p11 subunit (Johnsson et al., 1988; Kube et al., 1992). This complex is unusual in that it forms without the presence of Ca\(^{2+}\), as p11 has deletions and substitution of residues responsible for Ca\(^{2+}\)-binding that render the EF hands non-functional, resulting in the protein being in a conformation that resembles the Ca\(^{2+}\) bound state and is suitable for binding annexin 2 (Gerke and Weber, 1985a).

Annexins 1 and 11 also bind S100 proteins. S100C (S100A11) can bind to the N-terminal domain of annexin 1 and annexin 11 can bind calcyclin (S100A6) (Mailliard et al., 1996; Naka et al., 1994; Seemann et al., 1996a; Tokumitsu et al., 1992; Sudo and Hidaka, 1999). Formation of these complexes is Ca\(^{2+}\)-dependent. Annexin 11 has two isoforms, 11a and 11b which are created by alternative splicing within the N-terminus and it is only annexin 11a that binds calcyclin (Sudo and Hidaka, 1998). Recently the crystal structure of the complex formed by annexin 1 and S100C was resolved (Rety et al., 2000). Not surprisingly, it was found to be very similar to that of the annexin 2 heterotetramer, though here the two S100C molecules are linked by a disulphide bridge in the dimer conformation. A member of the S100 family with four EF hand motifs, namely sorcin, is able to bind the N-terminal domain of annexin 7 (Brownawell and Creutz, 1997). Annexin 6 binds two S100 proteins, S100A1 and S100B, although this has only been demonstrated in vitro, and these complexes have not been shown to have any physiological relevance. Homodimers of these S100 proteins are speculated to be able to crosslink two annexin 6 molecules (Garbuglia et al., 1998).

Several annexins can bind to cytoskeletal proteins in vitro, for example annexins 1, 2 and 5 have been shown to bind filamentous actin in a Ca\(^{2+}\) controlled manner (Glenney, 1986b; Gerke and Weber, 1984; Glenney, 1986a; Tanaka et al., 1994). Of these, annexin 2 has been studied in the most detail. The binding site has been localised to a 9 amino acid sequence in repeat 3 (Jones et al., 1992). Binding, along with the ability of the annexin 2 heterotetramer to bundle actin filaments, can be blocked by tyrosine phosphorylation of annexin 2 by pp60\(^{v-scr}\) (Hubaishy et al., 1995). Associated with the actin filaments to which annexin 2 is proposed to bind, is another part of the submembraneous cytoskeleton, fodrin, that can link together sections of actin filaments and which has also been shown to be an in vitro ligand for annexin 2 (Thiel et al., 1992).

Extracellular ligands have also been suggested for annexins but there is no clear evidence to explain how annexins become secreted from cells, although annexins 1 and 5 have been found in extracellular fluid (Christmas et al., 1991; Ishitsuka et al., 1998). Receptors for annexins on the cell surface have not been formally identified.
but it is worth mentioning that annexins 1 and 2 are thought to be capable of binding choline, which is a constituent of phospholipids (Zimmerman et al., 1995) and annexins 4, 5 and 6 can bind carbohydrates in vitro (Kojima et al., 1996; Ishitsuka et al., 1998), which are components of the extracellular matrix and are also located on the cell surface and with secretory granules (Iozzo and Murdoch, 1996). These interactions may be particularly important on the surfaces of endothelial cells, where annexin 2 has been shown to bind tPA and enhance the activation of plasminogen (see 1.1.1 coagulation/fibrinolysis) (Hajjar et al., 1994). Annexin 2 on the surface of a human glioma cell line has been reported to interact with an alternatively spliced segment of tenascin C (Chung and Erickson, 1994). Another plasma protein noted to bind annexins is apolipoprotein A1 that binds annexins 1 and 7 (Brownawell and Creutz, 1996).

Another type of protein interaction involving annexins is self-association. Several members of the annexin family have been shown to do this both in solution and when attached to membrane surfaces. Annexin 7 has been shown to self-associate in solution (Creutz et al., 1979), in addition to Hydra annexin 12, which can form trimers and hexamers (Mailliard et al., 1997). Annexins 5, 6 and 12 are also suggested to self associate when bound to membranes (Mailliard et al., 1997; Mosser et al., 1991; Newman et al., 1989). Annexin 5 is reported to form hexamers and higher aggregates in the presence of Ca\(^{2+}\) and anionic phospholipids (Concha et al., 1992). During vesicle aggregation annexin 2\(_{p112}\) has been demonstrated by immunoelectron microscopy to form bridges between membranes (Lambert et al., 1997). These interactions form homomultimers but a heteromultimer has been suggested to occur between annexin 2 and annexin 1 (Lee et al., 1999). The protein ligands of the annexins discussed here are not a complete list but illustrate some of the interactions that can occur.

### 1.5.3 Tissue and cellular localisation

The annexins are frequently described as ubiquitous with regard to their tissue and cellular distribution. There have been many studies of annexin localisation that reveal great variability of expression especially during development. Most of the literature has focused on individual annexins in particular tissues or cell types, making it difficult to gain a general impression of annexin behaviour. In vertebrates the majority of reports focus on annexins 1 to 6 which have been found to be expressed in most organs (Dreier et al., 1998; Jans et al., 1995). Studies of isolated cells have shown that all of annexins 1 to 6 tend to be expressed to some degree (Barwise and Walker, 1996; Diakonova et al., 1997).
Annexin 1 is one of the most ubiquitous family members, and in most cell types is found in the cytoplasm, and in many instances the nucleus (Barwise and Walker, 1996; Doubell et al., 1993; Blanchard et al., 1996; Sun et al., 1992; Srivastava et al., 1996; Fernandez et al., 1996; Katoh et al., 1995; Vishwanatha et al., 1992) where it has been found to remain on treatment of purified nuclei with EDTA (Raynal et al., 1992). It can also associate with the plasma membrane, or membranes (Kaufman et al., 1996) of structures such as endosomes and phagosome, as seen in macrophages (Diakonova et al., 1997). In baby hamster kidney cells and Madin-Darby canine kidney cells it is localised with both the plasma membrane and early endosomes (Seemann et al., 1996b), and has been visualised on late endosomes (Futter et al., 1993). Annexin 1 associates with insulin-containing granules of pancreatic beta cells (Ohnishi et al., 1995), and can interact with cytoskeletal structures in human keratinocytes (Fava et al., 1993).

Annexin 2 demonstrates a very similar distribution to annexin 1 in its pattern of expression. It is also one of the most studied of the annexins with respect to localisation. It has been identified on early endosomes (Emans et al., 1993) and bound to the submembraneous cytoskeleton (Gerke, 1992), with uneven distribution in both cases. In polarised cells annexin 2 has a tendency to concentrate under the apical membrane (Gerke and Weber, 1984; Handel et al., 1991), whilst in macrophages (J774) the protein is clustered in areas on the membrane that show ruffles, microvilli and membrane folds (Diakonova et al., 1997). Annexin 2 can also be found in phagosome preparations from this cell type (Desjardins et al., 1994). Similar clustered patterns appear on early endosomes but in this case they are sometimes associated with actin filaments (Harder et al., 1997). Another internal structure with which annexin 2 can associate is the secretory vesicle. In adrenal chromaffin cells, annexin 2 is a substantial constituent of the secretory granule membrane. This interaction with secretory granules is Ca^{2+}-dependent (Creutz et al., 1987), whereas association with endosomes is reported to be Ca^{2+}-independent (Jost et al., 1997).

Translocation of annexin 2 is regulated by [Ca^{2+}]. Thus culture of Madin-Darby canine kidney cells in a low Ca^{2+} medium leads to translocation from the plasma membrane to the cytosol (Vaaraniemi et al., 1994; Harder and Gerke, 1993), whereas other examples of stimuli that can lead to translocation of annexin 2 are cell differentiation, transcytosis (Wilton et al., 1994) and secretion (Chasserot-Golaz et al., 1996). These stimuli may either change [Ca^{2+}], or cause phosphorylation of one of the sites in the annexin 2 N-terminal domain. Phosphorylation by PKC causes a conflict with p11 binding (Johnsson et al., 1988; Jost and Gerke, 1996) which is needed to allow annexin 2 to bind the submembraneous cytoskeleton. Annexin 2p11, is associated
with the cortical cytoskeleton whilst monomeric annexin 2 is found in the cytosol (Thiel et al., 1992). External association of annexin 2 with the plasma membrane has been reported in ECs (Hajjar et al., 1994; van Ijzendoom et al., 1996), on skin keratinocytes (Ma et al., 1994) and on some tumour cells (Yeatman et al., 1993).

Annexin 3 is predominantly expressed in neutrophils, monocytes and macrophages. At the subcellular level annexin 3 is found localised to the plasma membrane and intracellular granules and phagosome membranes of J774 macrophages (Diakonova et al., 1997). Annexin 4 also localises to the phagosomal membrane of this cell type (Diakonova et al., 1997; Jost et al., 1997). In polarised cells annexin 4 can show preference for a particular membrane, for example in lung epithelial cells annexin 4 concentrates on the basolateral membrane (Mayran et al., 1996), where it has been suggested to function as a chloride channel regulator.

Annexin 5 has been reported in the cytoplasm (Sun et al., 1992; Kawaminami et al., 1998), nuclei (Altieri et al., 1996; Mohiti et al., 1997; Sun et al., 1992), plasma membrane, late endosomes (Diakonova et al., 1997) and endocytic membranes of many different cells. Glial cells and skeletal muscle cells show annexin 5 localisation with the SR and ER respectively (Spreca et al., 1992), as well as t-tubule invaginations, cardiac intercalated discs (Doubell et al., 1993; Spreca et al., 1992), and mitochondria (Diakonova et al., 1997). Translocation of this protein to membranes can be induced by addition of thrombin to platelets (Trotter et al., 1994), or by depolarisation of neuroblastoma cells (Blanchard et al., 1996). In human dendritic cells endocytosis of albumin coincides with the movement of annexin 5 to these vesicles (Larsson et al., 1995). The phosphatase inhibitor okadaic acid has been reported to cause annexin 5 to bind to platelet membranes (Trotter et al., 1997). Interestingly reports of annexin 5 translocation from the cytoplasm to the nucleus have been indicated as being controlled by signalling pathways involving tyrosine kinases and serum factors (Mohiti et al., 1997). The pathway involved in translocation of annexin 5 thus appears to involve phosphorylation events.

Annexin 6 is associated with the plasma membrane in many cell types, with mitochondria in the liver (Rainteau et al., 1995), the SR of skeletal muscle (Luckcuck et al., 1997), possibly the ER of non-muscle cells (Hazarika et al., 1991), and phagosomes of J774 macrophages (Diakonova et al., 1997). There have also been reports of annexin 6 on rat liver endosomes (Jackle et al., 1994) but this finding may be specific for that cell type, as baby hamster kidney cells do not show this localisation (Seemann et al., 1996b).
Annexin 7 has a cytosolic distribution with a concentration around granules in adrenal chromaffin cells (Kuijpers et al., 1992). In striated muscle it is found on the plasma membrane and transverse tubules whereas in cultured muscle cells it resides in the cytosol (Selbert et al., 1995) and with mitochondria (Srivastava et al., 1996). Annexin 8 is expressed in lung (epithelia), skin, liver and kidney tissue, but is not found in ECs or human arterial smooth muscle (Reutelingsperger et al., 1994).

Annexin 11 localises to the nucleus in fibroblasts, a property that requires the presence of the N-terminal domain (Mizutani et al., 1995). Neutrophils accommodate a 42 kDa fragment of annexin 11 that translocates to granule membranes upon increases in $[\text{Ca}^{2+}]$. (Sjolin and Dahlgren, 1996). This annexin has also been found in macrophages where on particle ingestion it relocates to phagosomes (Pittis and Garcia, 1999). Annexin 13 has a spliced variant 13b, containing an additional 41 amino acid sequence in the N-terminal domain, which has been seen on carrier vesicles involved in transcytosis and on the apical membrane of Madin-Darby canine kidney cells (Fiedler et al., 1995). Interactions with membranes are by myristoylation thus not regulated by $\text{Ca}^{2+}$ (Wice and Gordon, 1992). Annexins 31 and A10 are the most recently discovered vertebrate annexins with extremely rare expression (Morgan et al., 1999b; Morgan and Fernandez, 1998). Although there is little information on these annexins, their lack of functional $\text{Ca}^{2+}$-binding sites makes them very interesting.

The problem with most localisation studies lies in the possibility of artefacts generated due to methods of cell fixation. Further insight may be gained through the use of GFP chimeras in living cells. Nevertheless, fixed cell studies do provide some useful information regarding annexin behaviour. From the information presented here it appears that most annexins are able to associate with membranes, but it should be noted that this is not a permanent association and the distribution may not always be even. Annexins bind membranes via a $\text{Ca}^{2+}$-bridging mechanism, in which they bind negatively charged phospholipids (Swairjo and Seaton, 1994) leading to an increased affinity for $\text{Ca}^{2+}$. Most require $\text{Ca}^{2+}$ for this activity but there are some exceptions, such as annexins 31 and A10. Annexins 1, 2, 4, 5 and 6 all demonstrate at least some $\text{Ca}^{2+}$-independent membrane association (Tagoe et al., 1994; Blanchard et al., 1996; Harder et al., 1997; Spreca et al., 1992; Kojima et al., 1994). Annexins 13a and b achieve this through myristoylation (Wice and Gordon, 1992; Fiedler et al., 1995). Annexin 2 can bind $\text{Ca}^{2+}$-independently to early endosomes and plasma membranes (Bunting et al., 1981; Jost et al., 1997), while annexin 1 (dephosphorylated) binds $\text{Ca}^{2+}$-independently to multivesicular bodies. Interestingly, phosphorylation by the EGFr kinase changes annexin 1 to a $\text{Ca}^{2+}$-dependent form (Futter et al., 1993).
1.6 Proposed functions of annexins

Many functions have been proposed for the annexins, but there is no general consensus of opinion of function for any member of the family. They often display many different behavioural activities depending on cell type and conditions. Functions such as ion channel activity, vesicle aggregation and inhibition of cPLA$_2$ appear to be common to many of the family but await validation in living cells. This section briefly reviews some of the possible roles that annexins may have in cells.

1.6.1 Exocytosis

The abundance of annexins in many secretory tissues, and their ability to bind the cytoskeleton as well as vesicle and plasma membranes, has led to the idea that annexins may have roles in vesicle trafficking and in fusion events in endocytosis and exocytosis. Many members of the family have been reported to aggregate both biological and synthetic vesicles in the presence of Ca$^{2+}$ in vitro but this can vary depending on the annexin, membrane composition and [Ca$^{2+}$].

Studies of vesicle aggregation by annexins usually require very high, often unphysiological [Ca$^{2+}$], but advocates of this approach point to the small "pockets" of high Ca$^{2+}$ that occur adjacent to plasma membranes and internal stores on Ca$^{2+}$ influx/release (Berridge et al., 1999). The exact values for Ca$^{2+}$ in these areas in vivo are not known but have been suggested to be well in excess of 10$\mu$M (Augustine and Neher, 1992). Vesicle aggregation in high [Ca$^{2+}$] is a property common to most of the family, except for annexins 2 and 7 which can both perform this function at physiological levels of Ca$^{2+}$ (i.e. submicromolar) (Liu et al., 1995; Meers et al., 1988; Drust and Creutz, 1988).

Studies of this type led to the idea that annexin 2 has a pro-secretory role in exocytosis in chromaffin cells, where it becomes phosphorylated during secretion (Waismann, 1995). The heterotetramer was proposed to aggregate secretory vesicles, and upon binding (Nakata et al., 1990) and subsequent phosphorylation, to undergo a conformational change causing destabilisation of the membrane leading to fusion. In support of these ideas, PKC-mediated pre-phosphorylated annexin 2, was shown to induce fusion of aggregated chromaffin granules (Regnouf et al., 1995). The phosphorylation region has been shown to be important by use of a synthetic peptide of this area, not including the p11 binding site, that is reported to block translocation of annexin 2 to the cell boundary on nicotine stimulation in ECs (Chasserot-Golaz et al., 1996; Konig et al., 1998). Translocation is more efficient with the heterotetramer than the annexin 2 monomer.
Annexin 1 has been reported to possess fusogenic activity in neutrophils (Francis et al., 1992) and is also suggested to aggregate vesicles. A recent study of the individual domains of annexin 1, through mutation of the Ca\(^{2+}\)-binding sites, has provided insight into the roles of these regions. Structural domains III and IV exhibited a role in vesicle aggregation, with domain II found not to be directly involved in aggregation but important for vesicle binding (Bitto and Cho, 1998). Another study has shown that Lys-26 and Lys-29 which are components of the N-terminus are important for annexin 1-induced vesicle aggregation (Bitto and Cho, 1999).

GTP promotes Ca\(^{2+}\)-dependent exocytosis in many different cell types. This type of exocytosis has been suggested to be driven by a mechanism in which annexin 7 acts as a Ca\(^{2+}\)-dependent GTP binding protein (Caohuy et al., 1996). Annexin 13b is thought to be involved with vesicle transport as it is found enriched on apical transport vesicles in Madin-Darby canine kidney cells. Antibodies against this protein interfere with transport of vesicles to the apical membrane but not to the basolateral membrane (Fiedler et al., 1995). Other experiments in permeabilised Madin-Darby canine kidney cells support this work and demonstrate that annexin 13b may act in an exocytotic pathway that does not involve the NSF, SNAPs and SNAREs dependent docking fusion mechanism (Ikonen et al., 1995). There is also some evidence from localisation studies for an involvement of annexin-like proteins in exocytosis of lower organisms. Antibodies raised against mammalian annexin 2 and a sequence common to some of the annexins (excluding annexin 2) were found to bind to proteins associated with secretory granules in paramecium cells (Knochel et al., 1996).

### 1.6.2 Endocytosis

In addition to the proposed roles in exocytosis, annexins are implicated in transport and sorting events involved in endocytosis. Most work on annexins in this area has involved the clathrin-dependent and the phagocytic pathways, both of which require fusion events between subcellular compartments. Annexin 1, which is located on early endosomes (Seemann et al., 1996b) and is a major substrate of the EGFr kinase, is phosphorylated in multivesicular bodies (MVB) in NIH 3T3 cells that over-express the kinase. This process has been suggested to be important in the sorting of the EGFr and possibly the inward vesiculation process (Futter et al., 1993). It is normally localised to the plasma membrane and early endosomes but not late endosomes (Seemann et al., 1996b), so the results based on findings in MVBs may be an effect of EGFr overexpression that causes a shift from early endosomes to MVBs. From truncation studies of the N-terminus, residues 13-26 (contains sites of PKC and EGFr kinase phosphorylation) are required for association of annexin 1 with early
endosomes, as this truncation causes a shift to late endosomes and MVBs (Seemann et al., 1996b).

Approximately half the cellular pool of annexin 2 in Baby Hampster Kidney cells has been found to associate with cholesterol-rich membrane domains, typically rich in caveolar proteins. Cholesterol sequestering agents such as digitonin and filipin are reported to release membrane-associated annexin 2 (Harder et al., 1997). Annexin 2 is a major constituent of fusogenic endosomal vesicles and is one of only a few proteins that show efficient transfer between endosomal membranes (Emans et al., 1993). Various lines of evidence support the idea that annexin 2 has a functional role in endocytosis. Antibodies raised against this protein prevent Ca^{2+}-dependent fusion of endosomes (Mayorga et al., 1994), and a mutant with inactive type II Ca^{2+}-binding sites is able to co-fractionate with early endosomes. Removal of the p11 binding region of the N-terminal (residues 1-14) domain did not affect endosomal binding, but deletion of residues 14-26 led to loss of binding (Jost et al., 1997). As a heterotetramer, annexin 2 may be involved in the localisation of early endosomes in polarised cells. Transient transfection experiments using a chimaeric protein comprising the N-terminus of annexin 2 fused with a whole p11 molecule led to all of the endogenous annexin 2 and p11 forming annexin 2/p11 aggregates. This in turn led to translocation of transferrin receptor-containing endosomes from the cell periphery, where the annexin 2 heterotetramer would normally be present, to the region where the aggregates had formed (Harder and Gerke, 1993).

Annexin 2 is not the only member of the family proposed to function in endocytosis. Annexin 6 co-fractionates with early endosomes in rat liver cells (Jackle et al., 1994). It was reported that the ATP and Ca^{2+}-dependent budding of clathrin-coated vesicles was inhibited by use of an annexin 6 depleted cytosol (Lin et al., 1992). The results of this *in vitro* study are challenged by the finding that A431 squamous epithelial cells do not express annexin 6, yet are still able to endocytose normally and that the rate is not enhanced by ectopic expression of annexin 6 (Smythe et al., 1994).

There are many examples of annexin involvement in phagocytosis (which has features in common with endocytosis). Annexin 1 is proposed to have a role in the fusion of phagosomes with lysosomes/endosomes (Emans et al., 1993), and is suggested to be involved in targeting of lysosomes (Futter et al., 1993). Upon ingestion of latex beads by J774 macrophages, annexins 1, 2, 3 and 5 are detected on early endosomes (Diakonova et al., 1997), and annexins 3 and 11 have been found to translocate to the periphagosomal region in neutrophils upon phagocytosis (Ernst, 1991; Sjolin et al., 1997).
Many annexins show differential compartmentalisation between cytosolic and membrane-bound forms, with variability perhaps due to both cell type and the individual annexin. Many annexins are therefore well placed to play a part in connecting secretory vesicles to one another or to the plasma membrane (Nakata et al., 1990), whilst others may link these vesicles to the cytoskeleton. In this context, not only binding of Ca\(^{2+}\) but also that of nucleotides could influence annexin-membrane interactions (Szewczyk and Pikula, 1998).

### 1.6.3 Ca\(^{2+}\) signalling and homeostasis

Annexins have frequently been linked to Ca\(^{2+}\) signalling pathways by virtue of the ability of most members of the family to bind Ca\(^{2+}\), but there are also many links to Ca\(^{2+}\) homeostasis. In addition, many annexins have been proposed to function as ion channels or to be regulators of ion channels. The first suggestion of an annexin acting in this manner was by Rojas and Pollard (1987) for annexin 7. These authors were able to show that annexin 7 formed capacitative gating currents \textit{in vitro}. They also demonstrated that annexin 7 could form a Ca\(^{2+}\)-selective channel, with preference over Ba\(^{2+}\) or Mg\(^{2+}\), in an artificial membrane (Pollard and Rojas, 1988). Purified and recombinant annexin 5 was shown to have an equivalent activity (Rojas et al., 1990). The pharmacology of these annexin channels was shown to be distinct from that of voltage-gated Ca\(^{2+}\) channels, in that channel activity was not blocked by nifedipine (unless used at extremely high levels) or Cd\(^{2+}\) (Pollard and Rojas, 1988).

The elucidation of the crystal structure of annexin 5 by Huber et al. (1990a), added weight to these initial findings by revealing the presence of a central pore, proposed to be the ion conductance pathway. The selectivity for cations was consistent with the presence of acidic amino acid side chains lining the pore. From this crystal structure it was revealed that annexin 5 binds to membranes via the convex face of the molecule, where the Ca\(^{2+}\)-binding sites are located. The calcium ions form bridges with negatively charged phospholipid headgroups, an orientation that configures the pore perpendicular to the membrane (Huber et al., 1992). The publication of other crystal structures for annexins 1, 2, 3, 6 and 12 (Benz et al., 1996; Burger et al., 1996; Favier-Perron et al., 1996; Luecke et al., 1995; Weng et al., 1993) confirmed a high degree of structural conservation for the four repeat annexins, most of which were also shown to have ion channel activity, albeit under rather non-physiological conditions (Pollard et al., 1992; Benz et al., 1996). Despite the overall similarity in structure there are some differences in orientation between domains II and III, and I and IV, which could explain the subtle differences in channel conductances (Liemann and Huber, 1997).
Although these data support the suggestion that the annexin pore acts as an ion channel, there are problems with this idea. In the case of annexin 5, the notion of an ion channel that does not span the membrane presents a major conceptual problem, although this has been countered with a proposed theoretical mechanism of microscopic electroporation. This model postulates that the protein distorts the membrane to create a localised electrostatic field that would allow ions to flow (Huber et al., 1992). However there is no experimental evidence to support this idea. Binding of annexin 5 to synthetic vesicles has been shown to change the fluidity of the bilayer, but it is not known if this is important in the Ca$^{2+}$ channel activity of this protein (Megli et al., 1998).

An alternative mechanism to explain ion conductance by annexins has emerged through observations of annexin 12. Annexin 12 was shown to form a hexameric structure in the crystal form (Luecke et al., 1995). The monomeric form was then shown to insert into membranes at acidic pH. A continuous membrane spanning $\alpha$-helix is formed from a helix-loop-helix motif in the solution structure (see fig. 5). This is proposed to come together with other helices to form an aqueous pore (Langen et al., 1998). Can the findings with annexin 12 be extrapolated to other annexins? Annexin 5 trimers have been reported but not an annexin 5 hexamer (Concha et al., 1992). The formation of an annexin 5 hexamer, modelled on the annexin 12 structure, would lead to a larger pore that would be more consistent with the reported Ca$^{2+}$ conductance data for annexin 5. However, the observation that a protein can display ion channel activity in vitro should be treated with caution, given that under appropriate conditions even a 21 amino acid synthetic peptide of alternating serine and lysine residues can form a cation permeable pore in a lipid bilayer (Hall, 1992). The models proposed are shown in figure 5, with a refinement of figure 5 C shown in figure 4.
Figure 5. Models for ion channels / regulation by annexins. Parts (A) and (B) are adapted from (Gerke and Moss, 1997). The model (A) demonstrates binding of an annexin causing sufficient disruption of the bilayer to allow ion flow across the membrane (Huber et al., 1992). Model (B) is a hexameric formation of annexins held together by Ca2+ forming a large central pore. This model is based on the crystal trimer of annexin 12 (Luecke et al., 1995). The latest model is (C) where the annexin inserts itself into the membrane, suggested for annexins 5 and 12 (Langen et al., 1998; Isas et al., 2000). At present there is no evidence to indicate how many transmembrane domains are formed or if insertion is as a monomer.
Nevertheless, extensive structure-function analyses of annexin 5 strongly argue for a \( \text{Ca}^{2+} \) channel role for annexin 5. Berendes et al. (1993b) demonstrated a change in ion selectivity for annexin 5 through point mutation of a glutamic acid residue in the central pore. Other mutations have led to changes in voltage sensing and ion permeability. The activity of the channel depends not only on the pore but also on the N-terminal domain. Removal of this domain from annexin 5 (or annexin 2) causes channel activity to almost vanish (Burger et al., 1996; Berendes et al., 1993a). A drug identified as an inhibitor of \( \text{Ca}^{2+} \) overload in the heart has been found to bind annexin 5 (Kaneko et al., 1997a) on the concave side of the pore, with \textit{in vitro} binding preventing annexin 5 mediated actin-binding and \( \text{Ca}^{2+} \) influx into vesicles (Kaneko et al., 1997b).

Recently, more direct evidence has emerged to support an ion channel function for annexin 5. In mineralising chondrocytes annexin 5 expression is up-regulated and is located within mineralising matrix vesicles (Kirsch and Wuthier, 1994). These vesicles have a proteinaceous core that binds \( \text{Ca}^{2+} \), forming crystals that ultimately disrupt the vesicles and become embedded in the collagen matrix as part of bone mineralisation. Fusion of matrix vesicles to membranes causes \( \text{Ca}^{2+} \) channel activity that can be mimicked using artificial vesicles containing purified annexin 5 (Arispe et al., 1996). Work from our laboratory showed that targeted disruption of the annexin 5 gene in B-cells led to loss of a peroxide sensitive \( \text{Ca}^{2+} \) influx, and that annexin 5 inserted into the cell membrane in the presence of peroxide, suggesting a role for annexin 5 as either a \( \text{Ca}^{2+} \) channel or as a mediator in the peroxide induced \( \text{Ca}^{2+} \) influx pathway (Kubista et al., 1999).

In non-excitable cells such as B-cells, the mechanism of \( \text{Ca}^{2+} \) signalling is poorly understood, but \( \text{Ca}^{2+} \) influx (see section 1.4) is frequently associated with membrane hyperpolarisation and is insensitive to nifedipine. Annexins are typically very abundant in non-excitable cells and it is here they could act as \( \text{Ca}^{2+} \) channels, especially as they are activated by both depolarisation and hyperpolarisation (Huber et al., 1992), and their conductance values match those recorded electrophysiologically in non-excitable cells (Clapham, 1995).

In addition to functioning as \( \text{Ca}^{2+} \) channels, annexins have also been proposed to influence \( \text{Ca}^{2+} \) homeostasis by acting as \( \text{Ca}^{2+} \) channel regulators. For example, purified annexin 6 was observed to increase the mean opening time and opening probability of ryanodine-sensitive \( \text{Ca}^{2+} \) channels in isolated sarcoplasmic reticulum membrane preparations (Diaz-Munoz et al., 1990), but without effecting their conductance. No effect was observed on \( \text{K}^{+} \) or \( \text{Cl}^{-} \) currents from the SR. The problem
with these results is that the effect required annexin 6 to be on the lumenal side, and
annexin 6 is perceived to have a cytosolic location. The crystal structure showed this
annexin to be similar to annexins 2 and 5, and to have Ca\(^{2+}\) channel activity (Benz et
al., 1996), suggesting that the increased Ca\(^{2+}\) channel activity could have been due to
annexin 6 itself. An investigation of this effect came from a study looking at over-
expression of annexin 6 in mice, where cardiac myocytes had lower resting Ca\(^{2+}\)
levels, and smaller Ca\(^{2+}\) spikes in response to electrical stimulation. This result
suggests that annexin 6 suppresses Ca\(^{2+}\) mobilisation, though it could have been due
to a toxic affect produced by too high a level of the protein, as high levels of
expression in the heart are lethal just after birth (Gunteski-Hamblin et al., 1996).
Further evidence that supports a role for annexin 6 in suppressing Ca\(^{2+}\) mobilisation,
comes from the observation that purified annexin 6 prevents annexin 2-dependent
chromaffin granule aggregation (Pollard and Scott, 1982; Creutz et al., 1992), though
these effects could be due to buffering of Ca\(^{2+}\) by annexin 6. Experiments on dorsal
root ganglia and spinal cord neurones revealed that preventing annexin 6 binding
phospholipids produced an increase in Ca\(^{2+}\) currents (Naciff et al., 1996). In A431
cells, a cell line that does not express annexin 6, introduction of this protein led to
inhibition of Ca\(^{2+}\) influx across the plasma membrane on EGF (epidermal growth
factor) stimulation (Fleet et al., 1999). In general, the findings described here suggest
that annexin 6 may be able to modulate Ca\(^{2+}\) movement, possibly by acting as a Ca\(^{2+}\)
channel regulator.

Annexins have also been shown to modulate chloride channels. Introduction of
purified annexin 4 into a colonic cell line was shown to block Ca\(^{2+}\)-dependent chloride
currents (Kaetzel et al., 1994). In an extension of this work it was shown that
annexin 4 elicited this effect through inhibition of phosphorylation of the channel by
calmodulin-dependent protein kinase II, possibly preventing the interaction between
calmodulin-dependent protein kinase II and the ion channel, as in vitro there is no
direct interaction between the kinase and annexin 4 (Chan et al., 1994). The
heterotetrameric form of annexin 2 has also been suggested to regulate a volume-
activated Cl\(^{-}\) current in ECs (Nilius et al., 1996). Addition of a N-terminal peptide which
included the binding site for p11, that interferes with annexin 2-p11 complex
formation, was found to decrease the volume-activated Cl\(^{-}\) current.

1.6.4 Annexins in disease

Abnormalities in expression or behaviour of some annexins are associated with
clinical disease states. Such diseases have been collectively termed
"annexinopathies" (Rand, 1999). Many of the diseases examined are linked to the
heart and cardiovascular system, but also include cancer, multiple sclerosis, cystic
fibrosis and autoimmune diseases such as inflammatory bowel disease, rheumatoid arthritis and systemic lupus erythematosus.

In healthy individuals, fibrin deposition causes a release of tPA from ECs, which combines with plasminogen at receptors on the endothelial surface to produce plasmin. Fibrin deposition in the presence of damaged ECs is thought to be part of the cause of atherosclerosis. Mammals possess a fibrinolytic surveillance system which when malfunctioning results in cardiovascular disease. Annexin 2, a peripheral EC protein (Hajjar et al., 1996), can act as a receptor for tPA and plasminogen (Figure 6) (Siever and Erickson, 1997; Kassam et al., 1998a). It has been calculated that there is a 60-fold increase in tPA mediated activation of both Lys-plasminogen and Glu-plasminogen when annexin 2 is bound (Cesarman et al., 1994). The annexin 2 heterotetramer is thought to have a greater catalytic effect on tPA-dependent plasmin generation than the monomeric form and has been demonstrated to produce a 90-fold increase in plasminogen activity for Glu-plasminogen (Kassam et al., 1998a). It is interesting to note that binding of plasmin to the annexin 2 heterotetramer inhibits the activity of the enzyme. Incubation of these two proteins led to a 95% decrease in plasmin activity (Fitzpatrick et al., 2000). The heterotetramer appears to exert tight control over plasmin/plasminogen activity on the cell surface, but there has been a report of a second tPA receptor of about 20 kDa. The receptor does not bind anti-annexin 2 antibodies but does show catalytic enhancement of plasminogen activity (Fukao et al., 1997).

![Figure 6. Model for binding of plasminogen and tPA to annexin 2 on the endothelial cell surface.](image-url)

Annexin 2 bound to the endothelial cell surface in a Ca^{2+}-dependent manner binds tPA at its N-terminal region. Plasminogen also binds after cleavage of annexin 2 by a serine protease creating a carboxyl terminal lysine, allowing it to become activated to form plasmin.
Plasminogen binds to a carboxy terminal lysine on annexin 2 (Cesarman et al., 1994). One report suggested that plasminogen binds the C-terminal lysine residues of the p11 subunit in the annexin 2 tetramer (Kassam et al., 1998b). Alternatively tPA requires a series of amino acids within the tail domain of annexin 2 for binding. Mutants of annexin 2 lacking the tail domain or with a substitution of amino acids corresponding to residues 2-13 or 8-19, all block binding to tPA, suggesting residues 8-13 to be of primary importance. This domain contains a cysteine residue, which is interesting because in the condition of homocysteinaemia, this residue can become replaced by a homocysteine residue (Hajjar et al., 1998).

Homocysteinemia is a condition which is associated with cardiovascular disease (see section 1.2.2) and which has been shown to change the binding of tPA but not plasminogen to annexin 2, where homocysteine has been incorporated. Binding was decreased by 65% and plasminogen-activating ability was decreased by 60% (Hajjar, 1993). Homocysteine is thought to incorporate into annexin 2 in the tail domain at the tPA binding site (Hajjar et al., 1998). Collectively these data suggest a role for annexin 2 in the regulation of blood clotting.

Acute promyelocytic leukaemia is associated with a haemorrhagic condition. Annexin 2 has been linked to the tendency to spontaneously haemorrhage, as it is expressed at high levels on leukaemic cells in patients with this condition compared with promyelocytes from normal individuals. Over-expression of annexin 2 is suggested to lead to increased plasmin production, which might explain the haemorrhagic complications observed in this disease. In support of this idea, anti-annexin 2 antibodies decreased fibrinolytic activity whereas over-expression of annexin 2 was associated with an increase in this activity (Rand, 1999). Annexin 8 is also reported to be over-expressed in acute promyelocytic leukaemia, with patients in remission showing a decrease in the levels of annexin 8 (Chang et al., 1992; Sarkar et al., 1994).

Several annexins were first discovered as anticoagulant proteins and were found to be very abundant in the placenta. During pregnancy, patients with antiphospholipid antibody syndrome have a deficiency of annexin 5 in the placenta (Rand et al., 1994). This can be mimicked experimentally in cultured ECs and trophoblasts exposed to antiphospholipid antibodies (Rand et al., 1997). This condition is serious because it is closely linked with foetal loss and thrombosis (Lockshin, 1996; Shapiro, 1996). IgG fractions taken from patients and then exposed to cultured ECs and trophoblasts lead to a reduction of annexin 5 on these cells (Rand et al., 1997). Annexin 5 under normal conditions is proposed to shield procoagulant anionic phospholipids, a notion
supported by the observation that recombinant annexin 5 prevents blood coagulation in venous blood flow conditions (van Heerde et al., 1994b). This effect is thought to be mediated by the ability of annexin 5 to bind phospholipids (Reutelingsperger et al., 1985; Maurer-Fogy et al., 1988), which are important at varying stages of blood coagulation (Davie et al., 1991). In addition, exposure of phosphatidylserine is necessary for the anticoagulant protein C pathway (Solymoss et al., 1988). There is a problem with this model for annexin 5 activity as the protein normally binds negatively charged phospholipids with highest affinity, and these are preferentially located on the inside of the plasma membrane. However, it has been suggested that annexin 5 can bind to as few as two negatively charged phospholipids on the region of the membrane to which it binds, or to other phospholipids at a high Ca^{2+} concentration (van Heerde et al., 1994a).

Binding of annexin 5 to ECs stimulated with phorbol-12-myristate-13-acetate or tumour necrosis factor causes an inhibition of thrombin formation through interference with factors VII/X/tissue factor complex (van Heerde et al., 1994a). In an experiment using phosphatidylcholine/phosphatidylserine vesicles with reconstituted thrombomodulin (inhibitor of thrombin pro-coagulant activity) added to them, annexin 5 caused a 70% decrease in thrombomodulin activity, although on ECs this inhibition was significantly smaller, and both systems showed an inhibition of tissue factor (Ravanat et al., 1992). These results suggest annexin 5 interferes with both procoagulant and anticoagulant systems. Annexin 6 is also thought to inhibit procoagulant activity, as seen in cultured ECs, but this is probably due to the simple Ca^{2+}-dependent sequestration of negatively-charged phospholipids (Yoshizaki et al., 1992).

Annexins are also linked with inflammatory diseases, such as rheumatoid arthritis. The inflammatory symptoms associated with the disease are due at least in part to the activity of cPLA_2, which generates arachidonic acid from membrane phospholipids. This cytosolic protein becomes partially catalytically active in the presence of Ca^{2+}, and requires phosphorylation by MAPK for maximal activity (Lin et al., 1993). Cells stimulated with fibroblast growth factor have been shown to activate cPLA_2 via p42 MAPK activation (Sa et al., 1995). Arachidonic acid is a precursor for platelet activating factor, PGI_2, thromboxanes and leukotrienes amongst other potent proinflammatory mediators (Needleman et al., 1986). Although cPLA_2 is described as being cytosolic, it has been reported to reside in the nuclei of subconfluent ECs whilst being cytosolic in confluent ECs. This change in localisation was found to be independent of the cell cycle as nuclear localisation was observed in both dividing and growth arrested cells (Sierra-Honigmann et al., 1996). These observations have
been suggested to be linked with altered arachidonic acid release in damaged vessels.

Annexins have been suggested to mediate their anti-inflammatory activities by acting as inhibitors of both cPLA₂ and extracellular type II PLA₂ (Capasso et al., 1997). Most attention has focused on annexin 1, but many members of the family are thought to be able to perform this function, though the idea remains controversial. The mechanism of inhibition has been suggested to be Ca^{2+}-dependent substrate sequestration by the annexins, rather than via a direct interaction with the cPLA₂ protein (Davidson et al., 1987). However, a peptide fragment from the N-terminal region of annexin 1 (that does not contain the Ca^{2+} or phospholipid binding sites) was found to suppress arachidonic acid release stimulated by EGF (Croxtall et al., 1995). These data contradict the substrate sequestration idea, though there was no explanation for the mode of action of the peptide. It has also been reported that phosphorylation of annexin 1 reverses the inhibitory effect it has over cPLA₂, leading to suggestions that it may regulate production of PGJ₂ in stimulus-response coupling (Skouteris and Schroder, 1996). Another study using a peptide from the N-terminal domain showed residues 18-25 were capable of blocking arachidonic acid release in A549 cells but that this was inhibited by methoxylation of Tyr^{21} on this fragment (Croxtall et al., 1998). Recent results have shown that peptides derived from the annexin 1 N-terminus can act as ligands for the formyl peptide receptor on neutrophils, preventing transendothelial migration by neutrophils (Walther et al., 2000) and thus explaining part of the annexin 1 anti-inflammatory effects. Cystic fibrosis sufferers who have inflammation of the lungs, have been reported to have annexin 1 degradation in the fluid of the epithelial lining of the lungs. In this case cleavage of a portion of the N-terminal is speculated to result in decreased activity of the protein (Tsao et al., 1998).

Another area in which annexins are associated with disease is during end stage heart failure, where there is a down-regulation of annexin 6 accompanied by up-regulation of annexins 2 and 5. It is thought that these changes may be a compensatory mechanism to help regulate [Ca^{2+}] in the cardiomyopathic heart (Song et al., 1998). To study further the role of annexin 6 in cardiovascular function annexin 6 knockout mice have been generated in our laboratory, but when compared with wild type mice, the null mutant animals were found to show no abnormalities in heart rate or blood pressure (Hawkins et al., 1999), and with no other striking phenotype. Annexin 6 is known to have an effect on Ca^{2+} cycling and contractility of cardiomyocytes, whilst annexin 5, present in most cell types of the heart, may show channel activity (see section 1.6.2). During myocardial infarction the plasma level of annexin 5 in patients increased compared with healthy subjects (Kaneko et al., 1996).
Linked to the proposed annexin 5 Ca$^{2+}$ channel activity discussed in section 1.6.2, research in human failing hearts has found that antibodies to annexin 5 inhibit Ca$^{2+}$-dependent ATPase activity of the SR (Moraru et al., 1998).

Changes in annexin expression are also associated with other important diseases, though any causal link with the cellular physiology is unclear. Annexins 1, 2, 4 and 5 are all expressed at higher levels in the white matter of patients with multiple sclerosis (Elderfield et al., 1992), and are also expressed at high levels in experimental autoimmune encephalomyelitis (Gold et al., 1999). Annexin 11 has been examined in various autoimmune diseases, with anti-annexin 11 antibodies being identified in sera from patients with this type of disease, but not in normal subjects or those with infectious disease (Misaki et al., 1995). Neurones involved in Parkinson's disease show decreased levels of annexin 5, it is suggested that this could occur as a result of annexin 5 adhering to dying cells undergoing apoptosis (Vermes et al., 1999).

The postulated involvement of annexins in tumour progression could be due to either suppressive or progressive activities. In malignant breast cancer annexin 1 expression is induced, whereas it would not normally be present in healthy tissue. In this context annexin 1 could be involved in proliferation and differentiation (Schlaepfer and Haigler, 1990), and is suggested to be most likely involved in the early stages of the development of breast cancer (Ahn et al., 1997). This increase in expression may be linked with reports that a proportion of breast cancer carcinomas show increased EGFr expression (Walker and Dearing, 1999). The EGFr phosphorylates annexin 1 in multivesicular bodies and is suggested to be involved in EGFr sorting in endocytosis (see 1.6.2 Endocytosis). Annexin 1 expression is also increased in tumours of the nervous system (Johnson et al., 1989), hepatocellular carcinoma (Masaki et al., 1996) and in squamous skin carcinoma (Bastian et al., 1993). These increases in annexin 1 may be due to increased growth rate in tumours. Annexin 2 expression has also been observed to be elevated in several cancers (Kumble et al., 1992a; Roseman et al., 1994; Cole et al., 1992) and this too may be attributed to increased growth rate as annexin 2 is normally found at its highest level in HeLa and CHO cells during S-phase (Chiang et al., 1993).

Expression levels of annexin 5 also fluctuate with tumour progression, but in a less defined way, as they are increased in an osteosarcoma cell line (Mohiti et al., 1995) but decreased in uterine cervix and endometrial carcinomas (Karube et al., 1995). Melanoma progression is linked with a decrease in annexin 6 levels, this occurring as the disease progresses from benign to a more aggressive malignant metastatic phenotype (Francia et al., 1996). Here, annexin 6 appears to have a suppressor role.
Consistent with this, transfection of this protein into A431 cells that do not normally express annexin 6, leads to increased serum demand and induction of contact inhibition (Theobald et al., 1994). Annexin 6 A431 cells also produce smaller and slower growing tumours in mice, than do non-expressing clones (Theobald et al., 1995).

In this introduction I have outlined the implications of EC dysfunction in the vasculature. Oxidative stress is one of a group of known causes of cardiovascular disease which is often initiated by EC dysfunction, and can be used as a physiological tool for investigating this subject. Within the endothelium annexins have been proposed to have varying functions that are the focus of much research. Changes in Ca\(^{2+}\) and other signalling events during oxidative stress make this family of proteins worthy of investigation as most annexins are Ca\(^{2+}\) and phospholipid binding proteins. Changes in annexin responses or expression during oxidative stress may therefore be linked with eventual clinical complications that arise from EC dysfunction. The aim of the work in this thesis is therefore to investigate annexin behaviour within these cells under conditions of oxidative stress.
CHAPTER 2: MATERIALS AND METHODS
2.1 Cell culture

HUVEC: Human umbilical vein endothelial cells
A431 cells: Human squamous epidermal carcinoma cells

Obtaining a primary culture

HUVECs were extracted from human umbilical cords obtained from the Department of Obstetrics at University College Hospital. Cords were collected and placed in sterile ice-cold Hank’s balanced salt solution (Sigma) without Mg\(^{2+}\) or Ca\(^{2+}\). Any damaged regions or areas that had come into contact with the clamps during birth were removed. The vein was then cannulated at both ends with 1.1mm blunt needles and held in place with surgical arterial clamps. The vein was perfused with ice-cold Hank’s balanced salt solution to remove the blood and any clots and was then flushed with air. The vein was filled with 0.1% (w/v) collagenase (Lourne) at 37°C, one of the needles was closed off with a stopper and then the vein was filled to distension and the other end closed off. The umbilical cord was then placed in a humidified incubator at 37°C for 15 minutes. The umbilical cord was then massaged manually before recovering the collagenase perfusate. It was then flushed with Hank’s balanced salt solution, and pooled with the collagenase recovered. The suspension was centrifuged at 400xg for 5 minutes, the supernatant discarded and the cells resuspended in 37°C Dulbecco’s modified Eagle’s medium (Gibco BRL). This method was adapted from Jaffe et al., 1973.

2.1.1 Cell culture under normal conditions

Endothelial cells were grown on dishes (Nunc) coated with 2% (w/v) gelatin (Sigma), in Dulbecco’s modified Eagle’s medium (Gibco) containing foetal calf serum (10% v/v) (Gibco) and endothelial cell growth factor at the concentration recommended by the manufacturer (Sigma). Tissue culture medium was supplemented with 100 U/ml penicillin, 100 \(\mu\)g/ml streptomycin and 1.7mM glutamine. Cells were cultured at 37°C in a humidified atmosphere of 21% O\(_2\), 5% CO\(_2\) and balanced N\(_2\). Medium was replaced every other day unless cells were retained at confluency when they received fresh medium every day. For passaging, cells were washed with phosphate buffered saline (PBS) and harvested using Trypsin-EDTA (Gibco), and split in a 1:2 or a 1:3 ratio. For microscopy cells were placed onto freshly flamed coverslips coated with gelatin. HUVECs were not used past passage 10 and were always used at confluency in the experiments for both control and oxidatively stressed cells.
After 2-3 passages, aliquots of $3 \times 10^6$ cells were frozen for future use. Cells were harvested as mentioned, washed with PBS, resuspended in freezing medium (90% FBS, 10% DMSO) and transferred to a room temperature Stratacooler (Stratagene). The Stratacooler was first placed at $-40^\circ$C overnight then transferred to a $-80^\circ$C freezer for 24 hours prior to the cells being placed in liquid nitrogen for storage.

A431 cells were cultured on dishes (Nunc) in DMEM containing foetal calf serum (10% v/v) (Gibco) and supplemented with 100 U/ml penicillin, 100 $\mu$g/ml streptomycin and 1.7mM glutamine. Cells were cultured at 37$^\circ$C in a humidified atmosphere of 20% O$_2$, 5% CO$_2$ and balanced N$_2$. Medium was replaced every other day. For passaging, cells were washed with phosphate buffered saline (PBS) and harvested using Trypsin-EDTA (Gibco), and split in a 1:20 ratio.

2.1.2 Cell culture under hyperoxic conditions

Hyperoxia can be used to oxidatively stress cells. Other groups have demonstrated this at varying levels of O$_2$, 40% O$_2$ for one week (D’Amore and Sweet, 1987), and 65% and 90% O$_2$ for a period of days (Bjerkvig et al., 1992). To simulate conditions of chronic oxidative stress, cells were grown in a specially designed incubator which was humidified at 37$^\circ$C with 40% O$_2$, 5% CO$_2$ and balance N$_2$ (BOC Gases). The incubator was flushed with fresh gas once a day for a few minutes. Cells were placed in this incubator at maximum confluence and were maintained for up to 12 days.

![Hyperoxic culture chamber](image)

**Figure 7. Hyperoxic culture chamber**

The chamber was constructed from Perspex. Gas was passed into and out of the chamber via unidirectional pressure release valves (1atm), one in the base the other in the lid. The temperature of the chamber was maintained by an external thermostat linked to heating pads around the wall of the chamber and a temperature sensor. In the middle of the floor of the chamber was a cell stand, which allowed gas to enter under the cells and permeate out around them. The lid was constructed with a sloping inner surface to prevent air pockets forming.
days under these conditions. During this time, cells were treated in the same way as cells cultured in a normal incubator (see section 2.1) except medium was replaced every day.

2.2 Cell stimulation

To examine the consequences of oxidative stress on stimulus response coupling, cells were exposed to various growth factors, agonists and pharmacological mediators before other experimental procedures were performed. Cells were given fresh medium at least 24 hours before stimulation.

<table>
<thead>
<tr>
<th>Stimulant</th>
<th>Concentration</th>
<th>Supplier</th>
</tr>
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<tbody>
<tr>
<td>Acetylcholine</td>
<td>1µM</td>
<td>Sigma</td>
</tr>
<tr>
<td>ATP</td>
<td>50µM</td>
<td>Sigma</td>
</tr>
<tr>
<td>Bradykinin</td>
<td>1µM</td>
<td>Sigma</td>
</tr>
<tr>
<td>EGF</td>
<td>100ng/ml</td>
<td>Sigma</td>
</tr>
<tr>
<td>Foetal bovine serum</td>
<td>1.5% (v/v)</td>
<td>Gibco</td>
</tr>
<tr>
<td>ionomycin</td>
<td>1µM</td>
<td>Calbiochem</td>
</tr>
<tr>
<td>Pervanadate</td>
<td>0.1µM Na₃VO₄, 0.1µM H₂O₂</td>
<td>Calbiochem</td>
</tr>
<tr>
<td>Thapsigargin</td>
<td>600nM</td>
<td>Calbiochem</td>
</tr>
</tbody>
</table>

Table 3. Concentrations of agonists, growth factors and pharmacological mediators used to activate HUVECs.

Genistein inhibition

In some experiments, cells were incubated with genistein (5µM or 100µM) for 30 minutes before stimulation with EGF or ATP. Cells were then washed, fixed, permeabilised and incubated with annexin 1 antibody / second antibody and examined by confocal microscopy (see section 2.8 for details). Genistein inhibits phosphorylation by PKC and PKA (IC₅₀>350µM) and prevents autophosphorylation of the EGFr (IC₅₀=2.6µM).

2.3 Extraction of cellular protein

Cells were washed in cold PBS. Dishes (90mm) of cells were lysed in situ in 0.5ml of ice-cold lysis buffer (0.5% Triton X-100, 1mM EDTA, 50mM NaCl, 15mM HEPES, 0.5mM phenylmethylsulphonylfluoride (PMSF) and 1µg/ml of leupeptin, pepstatin A and chymostatin). Lysates were centrifuged at 10,000xg for 2 minutes to remove any non-soluble components of the cells. Supernatants were transferred to fresh tubes.
and stored at -20°C until use. Protein concentrations were determined using the Bio-
Rad detergent-compatible protein assay kit. Samples were analysed on a Pharmacia
Ultrospec II spectrophotometer. Standard protein samples were prepared from bovine
serum albumin (Sigma).

Cells used for antiphosphotyrosine western blots were washed first in PBS then
lysed directly into SDS-PAGE sample buffer (0.125M Tris pH 6.8, 0.04g/ml DTT, 4%
(w/v) SDS, 0.2% (w/v) bromophenol blue, 10% (v/v) glycerol). Samples were boiled
for 5 minutes before loading onto a polyacrylamide gel.

2.4 Polyacrylamide gel electrophoresis

Protein extracts not lysed directly into SDS-PAGE sample buffer were added to an
equal volume of 2x SDS-PAGE sample buffer and boiled for 5 minutes, before loading
onto a discontinuous sodium dodecyl sulphate polyacrylamide gel (Hames and
Rickwood, 1983). The gel was of an acrylamide:bisacrylamide ratio of 30:0.8% (w/v)
(Protogel, National Diagnostics). The concentration of acrylamide was 10% in the
resolving gel and 4% in the stacking gel. Prestained molecular weight markers were
from Gibco and New England Biolabs. Gels were run at room temperature at 45 volts,
using apparatus model 400 by Hoefer Scientific Instruments. For examination of total
protein of cell lysates, gels were stained with Coomassie blue.

2.5 Western blotting

Proteins were transferred from polyacrylamide gels onto polyvinyl difluoride
membranes (Immobilon P, Millipore) using BioRad Electroblot Transfer Apparatus
according to the manufacturers' instructions. Transfers were performed at 4°C in
39mM glycine, 48mM Tris, 20% methanol at 0.6 Amps for 4 hours. Membranes were
then blocked in either 0.2% (v/v) Tween-20 in PBS, or 5% (w/v) defatted skimmed milk
(Tesco) in PBS containing 0.05% (v/v) Tween-20 (PBS-T) (Harlow and Lane, 1988).

2.6 Immunoblotting

Membranes were incubated with primary antibodies in PBS-T at room temperature for
90 minutes. Unbound antibody was removed by three ten-minute washes in PBS-T
before addition of the second antibody in the same manner as the primary antibody.
For the majority of antibodies/antisera used no other bands than the ones shown in
the results were detected. In experiments where other bands were seen they were
of a greatly different size to the protein of interest. The following antibodies raised
against human antigens were used for blotting:
Primary antibodies

Annexin 1: Rabbit anti-p35 polyclonal antiserum, raised against human placental denatured annexin 1 (Futter et al., 1993). Used at a dilution of 1:1000.

Annexin 2: Mouse monoclonal antibody, HH7, raised against a synthetic peptide of residues 1 to 18 of human annexin 2 (kindly donated by Prof. V. Gerke, Institute for Medical Biochemistry, ZMBE, University of Muenster, Germany) (Harder et al., 1997). Used at a dilution of 1:2500.

Annexin 4: Goat polyclonal antibody (Santa Cruz Biotechnology), raised against the amino terminus of human annexin 4. Specific for annexin 4. Used at a dilution of 1:1000.

Annexin 5: Anti-p32, immunoglobulin fraction of rabbit polyclonal antiserum, raised against human placental annexin 5 (Shen et al., 1994). Used at a dilution of 1:1000.

Annexin 6: Anti-p68, immunoglobulin fraction of rabbit polyclonal antiserum, raised against human placental annexin 6 (smythe et al., 1994). Used at a dilution of 1:1000.

Annexin 7: Goat polyclonal antibody (Santa Cruz Biotechnology), raised against the C-terminus of human annexin 7. Specific for annexin 7. Used at a dilution of 1:1000.

cPLA$_2$: Mouse monoclonal IgG$_{2b}$ antibody (Santa Cruz Biotechnology), raised against the C-terminus of human cPLA$_2$. Specific for cPLA$_2$. Used at a dilution of 1:1000.

EGFr: Goat polyclonal IgG antibody (Santa Cruz Biotechnology), raised against the C-terminus of human EGFr. Used at a dilution of 1:1000.

p-Erk: Mouse monoclonal IgG$_{2a}$ antibody (Santa Cruz Biotechnology), raised against a short amino acid sequence containing tyrosine 204 of human Erk1 (identical to corresponding Erk2 sequence). Reactive with Tyr-204 phosphorylated ERK 1 and ERK 2; non cross-reactive with JNK1, JNK2, JNK3 or p38. Used at a dilution of 1:1000.

p-38: Rabbit polyclonal IgG MAPK antibody (NewEngland Biolabs), raised against a synthetic peptide corresponding to residues 341 to 360. Detects total levels of p38 MAPK (phosphorylation-state independent). Used at a dilution of 1:1000.
Erk-2: Rabbit polyclonal IgG antibody (Santa Cruz Biotechnology), raised against the c-terminus of rat Erk2-encoded MAPK p42. Reactive to ERK 2 p42 and to a lesser extent ERK 1 p44. Used at a dilution of 1:1000.

phospho-p38: Rabbit polyclonal IgG MAPK (Thr 180/ Tyr 182) antibody (New England Biolabs), raised against a synthetic doubly phosphorylated peptide using the human p38 MAPK sequence. Reactive with thr 180, Tyr-182, does not appreciably react with corresponding forms of p42/44 MAPK or SAPK/JNK. Used at a dilution of 1:1000.

Bax: Mouse monoclonal IgG antibody (Santa Cruz Biotechnology), raised against an epitope corresponding to amino acids 1-171 representing all but the C-terminal 21 amino acids of mouse Bax. Specific for Bax. Used at a dilution of 1:1000.

c-fos: Rabbit polyclonal IgG antibody (Santa Cruz Biotechnology), raised against an epitope corresponding to amino acids 210 – 335 mapping at the c-terminus of human C-fos. Specific for c fos, non cross-reactive with fos B, fra-1 or fra-2. Used at a dilution of 1:1000.

PY20: Mouse monoclonal IgG (Transduction Laboratories), raised against phosphotyrosine. Used at a dilution of 1:10000.

Bcl-2: Mouse monoclonal IgG (Santa Cruz Biotechnology), raised against an epitope corresponding to amino acids 1-205 of human Bcl-2. Specific for Bcl-2, non cross reactive with other apoptosis associated proteins. Used at a dilution of 1:1000.

Bad: Goat polyclonal IgG (Santa Cruz Biotechnology), raised against a peptide mapping near the C-terminus of human Bad. Specific for Bad. Used at a dilution of 1:1000.

Bag-1: Rabbit polyclonal IgG (Santa Cruz Biotechnology), raised against C- terminus of mouse Bag-1 p32 (differs from corresponding human sequence by a single amino acid). Reactive with Bag-1 p32, p36, p50. Used at a dilution of 1:1000.

**Secondary antibodies**

All of the following antibodies were used at a dilution of 1:10000 in western blotting.

Goat anti-mouse IgG-Horseradish peroxidase (HRP) conjugate (Santa Cruz Biotechnology).
Goat anti-mouse IgG (H&L) alkaline phosphatase (AP) conjugate (Promega).

Goat anti-rabbit IgG (Fc) AP conjugate (Promega).

Goat anti-rabbit IgG - HRP conjugate (Santa Cruz Biotechnology).

Goat anti-goat IgG HRP conjugate (Santa Cruz Biotechnology).

Donkey anti-Goat IgG AP (Promega).

Membranes were washed x3 in PBS-T to remove any unbound secondary antibody. For AP-conjugated secondary antibodies, membranes were developed with Western Blue substrate (5-bromo-4-chloro-3-indolyl-1-phosphate and nitro blue tetrazolium) (Promega). Excess substrate was removed by multiple washes with water. For HRP-conjugated secondary antibodies, protein bands were visualised by Enhanced ChemiLuminescence (ECL). The luminol development reagent was prepared from the following two stock solutions:

**Solution 1** : 22.5 mg Luminol (Sigma) was dissolved in 0.5 ml DMSO and added to 50 ml of 0.1 M Tris-HCl pH 8.5. 220 μl of a solution of 37 mg p-Coumaric acid (Sigma) in 2.5 ml DMSO was also added.

**Solution 2** : 178 μl of a 6% stock solution of Hydrogen Peroxide (Sigma) was added to 50 ml of 0.1 M Tris-HCl pH 8.5.

The membrane was washed in a mixture of equal amounts (5 ml) of solutions 1 and 2 for 1 minute, and then excess was dripped off and the membrane was sealed in clear plastic and exposed to hyperfilm (Amersham), or digitally analysed via a LAS1000 chemiluminescent detector (Fuji).

### 2.7 Immunoprecipitation

Cellular protein was extracted according to section 2.3, then incubated with the appropriate antibody for 1 hour at 4°C with mixing. Protein A beads were then added to the protein extracts at 4°C with further mixing for 1 hour. Samples were centrifuged at 10000xg for 20 seconds and the supernatants discarded. The beads were washed multiple times with PBS. Beads were then added to SDS-PAGE sample buffer and boiled for 5 minutes prior to analysis by SDS-PAGE / western blotting.
2.8 Immunofluorescence

Cells grown on coverslips were washed with PBS, then fixed in 3.7% formaldehyde made fresh from paraformaldehyde in PBS (pH 7) at room temperature for 20 minutes, and permeabilised using 80μg/ml LPC (Sigma) at room temperature for 20 minutes. LPC has been found to show better preservation of the integrity of the cytoskeleton compared with acetone and triton permeabilisation (Norman et al., 1994). It is not known if this protocol has been tested for nuclear antigens. This method was chosen as cells were originally fixed and permeabilised with methanol which was observed to lead to disintegration of the actin cytoskeleton.

Cells were exposed to primary antibodies in PBS for 1 hour at 37°C, then washed three times with PBS before incubation for a further hour with a secondary conjugated antibody. Coverslips were then washed again three times with PBS before being mounted onto slides using 1% propylgallate and 20% glycerol in PBS. Controls were performed by staining with only the secondary antibody, and were all found to be clear. The slides were then examined by either epifluorescence or confocal microscopy with oil emersion lenses.

Primary antibodies

Antisera to annexins 1, 2, 4 and 7 were the same as those used in blotting (section 2.6) at the following concentrations. Annexin 1, 1:1000; annexin 2, 1:40; annexin 4, 1:50; annexin 7, 1:50.

Annexin 5: Mouse monoclonal antibody Wac 2A (kindly donated by Prof. C. Reutelingsperger, Department of Biochemistry, University of Limburg, Maastricht, The Netherlands). Used at a dilution of 1:5.

cPLA$_2$: Mouse monoclonal antibody 1.1.1 (Kindly donated by Dr. C. Leslie) (Sierra-Honnigmann et al., 1996). Used at a dilution of 1:500.

Annexin 6: Sheep anti-annexin 6 (a gift from Prof. John Dedman, Department of Physiology and Biophysics, University of Cincinnati, Ohio, USA) (Smith and Dedman, 1986). Used at a dilution of 1:20.


Secondary Antibodies

All antibodies from Sigma
### Table 4. Secondary antibodies used for immunofluorescence.

<table>
<thead>
<tr>
<th>Raised against</th>
<th>Conjugate</th>
<th>Dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mouse</td>
<td>FITC</td>
<td>1:250 / 1:500</td>
</tr>
<tr>
<td>Rabbit</td>
<td>FITC</td>
<td>1:400</td>
</tr>
<tr>
<td>Goat</td>
<td>FITC</td>
<td>1:400</td>
</tr>
<tr>
<td>Sheep</td>
<td>FITC</td>
<td>1:100</td>
</tr>
<tr>
<td>Mouse</td>
<td>TRITC</td>
<td>1:400</td>
</tr>
</tbody>
</table>

#### 2.9 Fluorimetric analysis of calcium mobilisation

Cells \((10^5)\) were loaded for 30 min at 37°C with 1.25μM fura-2/AM (Sigma) and 0.0025% Pluronic F-127 (Calbiochem) in DMSO. Cells were trypsinised and washed with ice-cold PBS and resuspended in supplemented ice-cold HBSS (1.26mM CaCl₂, 0.81mM MgSO₄, 5.4mM KCl, 0.44mM KH₂PO₄, 136.9mM NaCl, 0.34mM Na₂HPO₄, 4.2mM NaHCO₃, 5.5mM D-glucose and 10mM HEPES; CaCl₂ was omitted from nominally Ca²⁺ free HBSS). Cells were washed three times at 4°C and resuspended in ice-cold HBSS and kept on ice in 2ml aliquots until required. Cells were then pre-warmed to 37°C for 5 minutes in a water bath. Fluorescence of the stirred cell suspension was measured ratiometrically by emission at 510nm and excitation at 340nm and 380nm respectively using an LS 50 B fluorimeter with filter wheel attachment (Perkin Elmer).

Results are displayed with arbitrary Y-axis ratio units corresponding to intracellular free calcium levels. Conversion to estimated free calcium levels is inaccurate and not informative since in a cell population where only some cells respond, calcium rises are inevitably underestimated. Comparisons between responses and cell types can however be usefully drawn based on ratio data.

#### 2.10 PCR based analysis of annexin gene expression

Preparations of endothelial cell RNA samples and first strand cDNA synthesis were performed using the Cells-to-cDNA kit (Ambion) according to the manufacturers’ instructions.

PCR reactions were performed using the Long Expand™ PCR kit (Boehringer Mannheim), with concentrations of template, primer (Gibco and Genset) and nucleotides (Invitrogen) as recommended by the manufacturer. Reactions were performed in an Eppendorf Mastercycler gradient thermal cycler. PCR products were
resolved on 2\% agarose gels in the presence of 10\(\mu\)g/ml ethidium bromide and visualised under ultraviolet illumination.

**PCR parameters**

**Stage 1**
- denature template: 2 min at 94°C

**Stage 2**
- denaturing: 10 sec at 94°C
- annealing: 30 sec at 50°C-60°C
- elongation: 1 min at 68°C

10 cycles

**Stage 3**
- denaturation: 10 sec at 94°C
- annealing: 30 sec at 50°C-60°C
- elongation: 1 min at 68°C

20 cycles, each cycle extending the elongation time by 20 seconds.

**Stage 4**
- prolonged elongation: 7 min at 68°C

Annealing temperatures were determined empirically with an initial estimate of 60°C, or 5°C lower than the lowest predicted melting temperature of the oligonucleotides.

**2.11 Oligonucleotide sequences**

The annexin primer pairs (Gibco and Genset) were designed using OLIGO software. For each annexin the primers are shown as forward followed by reverse primer, together with the predicted product sizes.

- **Annexin 1**
  - 360 base pair fragment
  - 5'-ATGGCAATGGTATCAGAA-3'
  - 5'-AGCATCAAATTGCGCTGG-3'

- **Annexin 2**
  - 322 base pair fragment
  - 5'-CTCAGCTTGGAGGGTGAT-3'
  - 5'-TGGAAGCTTTAGCTCAG-3'

- **Annexin 3**
  - 285 base pair fragment
  - 5'-ATGGCATCTATCTGGGTT-3'
  - 5'-ATCAAAGACTGCTGGTG-3'
Annexin 4  258 base pair fragment
5'-ATGGCCATGGCAACCAAA-3'
5'-CATCATCCCCACAATCAC-3'

Annexin 5  228 base pair fragment
5'-GACTTCCCTGGATTTGAT-3'
5'-TTTCATCAGAGCCACAAT-3'

Annexin 6  206 base pair fragment
5'-CACAGGGTGCCAAAGTACC-3'
5'-TCAGCAATGAGGTCCTTG-3'

Annexin 7  180 base pair fragment
5'-TGGGCTGTGACGCTGCTG-3'
5'-TTGGGCACCTTGTTGGGTAGG-3'

Annexin 8  163 base pair fragment
5'-ATGGCCTGGTGAAATCC-3'
5'-GCGTGTTGCTTCTCTTGG-3'

Annexin 11  146 base pair fragment
5'-CTACCACCAGCTGCACC-3'
5'-ATGTTGGGCCATCCC-3'

Annexin 13a  111 base pair fragment
5'-ACCATTCCTAGAAAAAGGA-3'
5'-CCATTTCTTTGCAGGCTT-3'

GAPDH  80 base pair fragment
5'-CCGCGGGGGCTCTCCAGAACATCA-3'
5'-CGTTCACTCACGGATGACCTTG-3'
CHAPTER 3: ENDOTHELIAL MORPHOLOGY AND ANNEXIN EXPRESSION
3.1 Validation of the experimental model

Oxidative stress can be produced by varying methods. Some groups have chosen to use UV-B whilst others prefer $\text{H}_2\text{O}_2$ or t-BOOH which produce rapid, short lived effects in a matter of hours. Another method that has become favoured over the last few years is hyperoxia. Most models that employ hyperoxia to induce oxidative stress use 95% $\text{O}_2$ for 24 hours, which produces a relatively fast onset of oxidative stress in cells. Some studies have used lower $\text{O}_2$ tensions that equate to around 65% $\text{O}_2$, which is shown to produce a slower onset (2-3 days) of oxidative damage.

Here, oxidative stress was generated using 40% $\text{O}_2$ over 12 days to produce a more chronic stress. Clinical conditions such as atherosclerosis are generally the result of decades of chronic oxidative stress. Therefore although the conditions used in this thesis are still not physiological, the longer period of oxidative stress is aimed at producing a more physiological model. For example, a slower induction of endothelial dysfunction is likely to be more effective in eliciting subtle changes in patterns of gene expression.

In this chapter a number of cellular parameters have been investigated. The cell morphology and cell viability were investigated as a function of time of oxidative stress. As this study focused on annexins in HUVECs under oxidative stress the next analysis was to examine changes in gene expression, as these may have a bearing on the localisation studies in Chapter 4.

During extraction of HUVECs from umbilical cords there was the possibility of contamination of smooth muscle cells and/or fibroblasts in the resulting culture. There are three ways of distinguishing EC from other cell types that may contaminate the culture. Identification of Weibel-Palade bodies is one method (Weibel and Palade, 1964), as these are not found in either smooth muscle cells or fibroblasts. Weibel-Palade bodies are endothelial cell-specific organelles that store von willebrand factor (vWF) multimers. Alternatively, the cells can be stained for vWF, which is only expressed by ECs, megakaryocytes and platelets, or by the presence of ABO blood group antigens, as they are also not present on smooth muscle cells or fibroblasts (Jaffe et al., 1973). To confirm that the cells harvested from umbilical cords were indeed ECs, cells were fixed and stained for vWF. vWF is a procoagulant involved in platelet adhesion and aggregation, and acts as the carrier for factor VIII in plasma (Hamer et al., 1986).
Expression of von Willebrand factor was found to be sustained during HUVEC culture in both normal and hyperoxic conditions (figure 8) both in cells freshly harvested from umbilical cords and in cells recovered from storage in liquid nitrogen after two or three passages. VWF expression was seen in cells up to and including passage 10.

3.2 Endothelial cell morphology

Endothelial cells have a distinctive morphology, which when cells are confluent is often described as resembling the pattern of cobblestones. Oxidative stress has previously been reported to generate changes in EC morphology. Hydrogen peroxide has been reported to increase the permeability of EC monolayers, this being associated with formation of paracellular holes (Carbajal and Schaeffer, 1998), while exposure of HUVECs to hyperoxia of 65% or 95% O₂ has also been reported to lead to changes in cell morphology (Bjerkvig et al., 1992).
During oxidative stress induced by culture in 40% O\textsubscript{2}, the distinctive EC morphology along with cell density began to change over a time course of days. Phase contrast photos were taken of cells at 3-day intervals during culture in hyperoxic conditions.

![Phase contrast photos of cells](image)

**Figure 9.** Changes in endothelial cell morphology generated by hyperoxia. HUVECs were grown in 40% O\textsubscript{2} for 12 days and removed for photography on a phase contrast microscope at 3 day intervals. (A) Control cells cultured in 20% O\textsubscript{2}. Cells cultured in 40% O\textsubscript{2} for (B) 3 days, (C) 6 days, (D) 9 days and (E) 12 days (magnification x100), n>10.

The cell density of the HUVECs decreases after 6 days in culture in 40% O\textsubscript{2}. As the cells become more spread out their morphology changes in two ways. A few cells appeared to enlarge as they increased their surface area in contact with the culture dish, but the majority of cells became very thin and elongated, particularly after 9 days in 40% O\textsubscript{2}.

### 3.2.1 Actin cytoskeleton

Filamentous actin has an important structural role in HUVECs in maintaining the permeability barrier between the tissues and the blood stream. As changes in cell morphology were identified in ECs exposed to hyperoxia, the arrangement of actin filaments was investigated in cells fixed then stained with phalloidin and visualised by epifluorescence. HUVECs have been observed to maintain their actin cytoskeleton during several rounds of passage (Galustian et al., 1995), and acute oxidative stress generated by addition of 250 \mu M H\textsubscript{2}O\textsubscript{2} to ECs has been shown to stimulate a rearrangement of actin filaments suggested to involve the p38 MAPK pathway (Huot et al., 1997). In another report low concentrations of H\textsubscript{2}O\textsubscript{2} (10\mu M) induced the
formation of stress fibres, whereas higher concentrations (100μM) led to the disruption of peripheral actin bands (Liu and Sundqvist, 1995).

During chronic oxidative stress caused by culture in 40% O₂ for 12 days, there did not appear to be any significant change in the arrangement of actin filaments when compared with control cells (figure 10). However, oxidatively stressed cells did exhibit a slightly stronger staining for actin around the cell membrane region than in control cells and appeared to contain more stress fibres. The actin peripheral banding is still clearly visible in oxidatively stressed cells (figure 10 B).

3.3 Oxidative damage

Previous studies of HUVECs exposed to 65% and 95% O₂, showed that cells initially entered S-Phase, followed by an almost entire cessation of cell proliferation with a 50% reduction in cell density being observed after 2-3 days (Bjerkvig et al., 1992). In EC, dysfunction generated by oxidative stress is reported to be relieved by addition of antioxidants (Hennig et al., 1994) or by removal of the oxidative source. It has also been reported that lowering cholesterol in atherosclerosis caused by hypercholesterolaemia (a cause of oxidative stress) leads to a regression of
atherosclerotic lesions suggesting an improvement in the endothelium (Benzuly et al., 1994).

To investigate the reversibility of endothelial dysfunction in cells grown under hyperoxic conditions (40% O$_2$), cells were removed from the hyperoxic incubator at 3 day intervals and returned to a normal 20% O$_2$ incubator. Early during the growth of cells in the hyperoxic chamber, a loss of the ability to proliferate was observed, at around two to three days. To assess recovery, cells were observed to see if the dishes of oxidatively stressed cells would become confluent again. Cells did not regain the ability to proliferate after 6 days in culture in 40% O$_2$ after a week spent in a normal incubator.

Following cell cycle arrest the end point of hyperoxic culture is cell death. Within the literature there is a difference of opinion as to whether cell death caused by hyperoxia occurs through a necrotic or apoptotic pathway (see section 1.3.6). To investigate whether culture in 40% O$_2$ leads to changes in the expression of apoptotic genes, members of the Bcl$_2$ family of proteins that interact to regulate programmed cell death or apoptosis were examined by western blotting. Bax and Bad are pro-apoptotic members of the family and were investigated along with Bcl-2 and Bag-1, which are both apoptotic inhibitors.

![Figure 11. Expression of apoptotic proteins.](image)

Cells grown in 40% O$_2$ were washed with PBS and harvested at 3 day time points, lysed, quantified and examined by gel electrophoresis and western blotting. Membranes were probed for Bag-1 and Bax. The molecular masses for each protein are indicated. The blots shown are representative of three separate experiments.

These results indicate that HUVECs may develop an increased tendency to undergo apoptosis during culture in hyperoxic conditions, as Bax was found to increase over the time course of oxidative stress, while Bag-1 expression was decreased in HUVECs that were exposed to hyperoxic conditions. Bad and Bcl-2 were not detected in control cells or during oxidative stress.
3.4 Changes in gene expression

One way in which the effects of oxidative stress may be manifested is through changes in levels of mRNA or protein expression. There is evidence for this in the up-regulation of certain proteins. ECs grown under hyperoxic conditions show increased glutathione mRNA levels, while other proteins may become down-regulated. In endothelial cells there have also been reports of hyperoxia induced up-regulation of glutathione mRNA levels (Jornot and Junod, 1995), and proteins such as VCAM-1 and ICAM-1 (Wiliam et al., 1999).

To examine whether oxidative stress induced any major changes in protein patterns in this experimental model, whole cell lysates were prepared from cells at 3-day intervals (figure 12) and analysed by gel electrophoresis and Coomassie blue

![Figure 12. Coomassie stained gel of whole cell lysates from oxidatively stressed cells.](image)

Cells grown in 40% O$_2$ were washed with ice cold PBS and harvested and lysed in ice cold lysis buffer at 3 day time points (approximately $10^6$ cells). Samples were then quantified for protein and resolved by gel electrophoresis. Protein bands were visualised by Coomassie blue staining. The blot shown is representative of three separate experiments.
staining. The aim of this experiment was to identify any global changes in protein expression, rather than the high resolution 'proteomics' approach using 2D gel electrophoresis. The result shows that the major protein bands remain respectively constant during the experiment, although close inspection reveals a number of minor changes. For example, a band of about 75 kDa appears transiently elevated at 9 days of oxidative stress, whereas bands of about 85, 47 and 50 kDa are reduced from 12 days.

3.4.1 Annexin protein expression

To assess whether or not annexin expression was altered by hyperoxia, protein was extracted from cells at 3 day intervals during culture in 40% O₂, and western blotted for annexins 1, 2, 4, 5, 6 and 7.

![Figure 13. Expression of annexins during incubation in hyperoxic conditions.](image)

Cells grown in 40% O₂ were washed with ice cold PBS and harvested and lysed in ice cold lysis buffer at 3 day time points. Lysates were centrifuged at 10,000xg for 2 minutes to remove any non-soluble components of the cells. Samples were then quantified for protein and resolved by gel electrophoresis. Membranes were probed for annexins 1, 2, 3, 4, 5, 6 and 7, with 40 µg of protein loaded in each track for all of the blots except for annexin 1 where 20 µg of protein was loaded. Bands were visualised by alkaline phosphatase. The blots shown are representative of three separate experiments.

The results show that oxidative stress does not lead to any marked changes in the expression of these annexins (figure 13). Although there were no noticeable
changes in annexin expression, other proteins from HUVECs were found to change their expression levels during culture in hyperoxic conditions (see also figures 11,12 and 36). This suggests that, at least at the level of protein expression, annexin gene expression appears to be relatively unaffected by oxidative stress. Each experiment was performed at least four times.

3.4.2 Annexin mRNA expression

As there did not appear to be any significant changes in protein expression levels of the annexins over a 12 day period of oxidative stress, levels of annexin mRNA expression were examined by RT-PCR. This was to address the possibility that there were changes in annexin gene expression even though no changes in annexin protein expression were observed. Changes in RNA expression occur in the absence of any corresponding changes in protein expression. For example, mRNA levels of glutathione were increased by hyperoxia yet the transcription rate (Jornot and Junod, 1995) was decreased, indicating that the relationship between gene expression and protein expression is not always straightforward. RT-PCR was performed for annexins 1, 2, 3, 4, 5, 6, 7, 8, 11 and 13a.

To determine whether any changes were specific to HUVEC annexins, A431 squamous epithelial carcinoma cells were also examined. RNA was collected from A431 cells after only 5 days in 40% O₂, as longer periods of incubation caused the cells to lose the ability to adhere to the cell culture dish. Annexins 1, 3 and 7 were examined.
Figure 14. RT-PCR analysis of annexin gene expression in control and oxidatively stressed HUVECs and A431 cells.

RNA was extracted and RT-PCR performed for annexins 1, 2, 3, 4, 5, 6, 7, 8, 11 and 13a. PCR products were resolved on 2% agarose gels. Mobility of DNA markers are indicated in bp. (1) RT-PCR from HUVECs RNA samples. (2) RT-PCR from A431 RNA samples. Lanes marked A indicate control cell samples and lanes marked B indicate either HUVECs grown in 40% O\textsubscript{2} for 12 days or A431s grown in 40% O\textsubscript{2} for 5 days. The bands shown are representative of three separate experiments.

All of the annexins examined were found expressed in HUVECs with the exception of annexins 8 and 13. Annexin 8 has previously been reported as not being present in HUVECs (Reutelingsperger, 1994). Although PCR is not a quantitative method for evaluating changes in mRNA expression there do appear to be some changes between control and oxidatively stressed cells for HUVEC annexins 1, 2, 4 and 7 (figure 14 (1)). Annexin 1 appears to increase due to hyperoxia whilst annexins 2, 4 and 7 mRNA become decreased in oxidatively stressed cells. A431 cells showed a change in annexin 3 mRNA expression with a decrease in oxidatively stressed cells (figure 14 (2)).

In the case of HUVEC annexins 1 and 2, where there is a striking change in the level of gene expression, it is interesting to note that no changes in protein expression were seen for these annexins (figure 13). Although a comparative result was not obtained for annexin 5, gene expression of this annexin was confirmed by RT-PCR and western blotting in control HUVECs.
CHAPTER 4: ANNEXIN LOCALISATION
Annexins translocate within cells in response to certain types of stimulation. In many cases, this is likely to be the result of a rise in \([\text{Ca}^{2+}]\), as most of the annexin family are \(\text{Ca}^{2+}\) binding proteins. All of the annexins investigated in this thesis are able to bind both phospholipids and \(\text{Ca}^{2+}\). However since the recent discovery of two annexins that do not bind \(\text{Ca}^{2+}\), other forms of stimulation downstream of the initial \(\text{Ca}^{2+}\) influx may be given more attention. The most frequently identified locations of these proteins within cells are the plasma membrane, nuclear membrane, cytosol or inside the nucleus. Annexins 1 and 5 have been reported to have both a nuclear and cytosolic localisation in ECs (Sun et al., 1992; Raynal et al., 1992). Annexin 2 is partly located on the cell surface (Wright et al., 1994; Hajjar et al., 1996) of HUVECs where it is proposed to act as a receptor for plasminogen and tPA (see section 1.6.4). Annexin 6 has been associated with endocytosis (Lin et al., 1992) and regulation of \(\text{Ca}^{2+}\) channel activity (Fleet et al., 1999; Munoz et al., 1990), and may act as an inhibitor of procoagulant activity on the endothelial cell surface. The ability of annexin 6 to bind negatively charged phospholipids (Yoshizaki et al., 1991) has been suggested to cause inhibition of thrombin generation by factor Xa on the endothelial cell surface. It was reported to have no effect on anticoagulant protein C activation through the thrombin-thrombomodulin system (Yoshizaki et al., 1992).

There are many reports that conditions of oxidative stress lead to changes in \(\text{Ca}^{2+}\) signalling, that these in turn may affect the behaviour of downstream signalling events and the activities of \(\text{Ca}^{2+}\)-binding proteins. To investigate whether annexins might be involved in the cell pathophysiology induced by oxidative stress, cells grown under normal conditions (20% \(\text{O}_2\)) were compared with cells cultured in 40% \(\text{O}_2\) for 12 days (hyperoxic conditions). Cells cultured under normal and hyperoxic conditions were exposed to various stimulants designed to activate the cells in differing ways.

### 4.1 Cell stimulation

To obtain more detailed information about the relationship between oxidative stress and cell signalling, four methods of cell stimulation were used to examine the locational responses of four human annexins; annexins 1, 2, 5 and 6. As the annexins under investigation all have \(\text{Ca}^{2+}\)-binding sites, the first stimulus investigated was thapsigargin. Thapsigargin leads to increased \([\text{Ca}^{2+}]\), as it causes an inhibition of the \(\text{Ca}^{2+}\)-ATPase on the ER, preventing uptake of \(\text{Ca}^{2+}\) by intracellular stores. To investigate the cellular response to higher concentrations of \(\text{Ca}^{2+}\), ionomycin was used. Addition of ionomycin to cells allows \(\text{Ca}^{2+}\) to flow into the cells from the external media, along with \(\text{Ca}^{2+}\) release from intracellular stores and mitochondria via membrane permeabilisation.
To stimulate cellular responses involving more than just a rise in Ca\(^{2+}\), ATP and EGF were used. ATP stimulates P\(_{2Y}\) purinergic receptors in ECs that can lead to release of NO and synthesis of PGI\(_2\) (Boeynaems et al., 1988). The receptors are coupled to PLC via a pertussis toxin-sensitive G-protein, and their activation therefore leads to hydrolysis of phosphatidylinositol-bisphosphate and release of Ca\(^{2+}\) from IP\(_3\)-sensitive internal stores (Dubyak and el-Moatassim, 1993). In ECs ATP mediated release of Ca\(^{2+}\) from IP\(_3\)-sensitive stores in the ER (Missiaen et al., 1996) leads to an endothelial-mediated vasodilation of the vasculature (Furchgott and Vanhoutte, 1989).

Stimulation of HUVECs with EGF, leads to autophosphorylation and activation of the EGFr, with further activation of downstream signalling molecules. Activation of PLC-\(\gamma\) leads to hydrolysis of PIP\(_2\) to yield IP\(_3\), that then causes a release of Ca\(^{2+}\) from internal stores. The downstream effects can also include mitogenesis, enhanced cell motility and differentiation (Wells, 1999).

### 4.2 Method for assessing cell populations with annexins either in the cytosol or the nucleus

Cells were fixed and stained on 13 mm coverslips, then examined by microscopy to determine the percentage of the cell population in which the annexin in question had translocated from the cytosol to the nucleus. Under resting conditions and for all of the annexins examined, the majority if not all of the cells expressed each member of the family in the cytosol. When cells were stimulated, certain conditions led to translocation of annexins to the nucleus. This did not occur in a uniform way across the coverslip, instead large patches of cells with annexins in their nuclei were observed, in which there was often no gradation between such patches and neighbouring cells. A schematic representation of a typical distribution of such cells on a coverslip is shown in figure 15. This observation made quantitative analysis difficult, as random selection of a section of the coverslip could clearly bias the result. Due to this problem, conventional methods for quantitative comparisons of cell populations were not applicable.
Furthermore, it was not possible to measure the size of the patches and then extrapolate that value to estimate the percentage of cells with the annexin in their nuclei, as the patches were irregular in size and shape. The method chosen was to estimate the total percentage of cells with annexin in their nuclei for the entire coverslip for each stimulation time point and to repeat this (n=3). This involved observing thousands of cells per coverslip.

The emergence of these patterns may be explained by cells communicating with each other via gap junctions and thus propagating the signal to many surrounding cells.

### 4.3 Annexin 1 and cPLA₂

Annexin 1 has been reported in both the cytosol and the nucleus of HUVECs (Raynal et al., 1992), where it has been controversially suggested to play a role in the inhibition of cPLA₂ (Solito et al., 1998; Croxtall et al., 1995; Croxtall et al., 1998). Similar roles have been proposed for other family members, but the majority of research has focused on annexin 1 (see section 1.6.4). Cytosolic PLA₂ hydrolyses phospholipids to release arachidonic acid, and was for many years thought to act at the plasma membrane, but a study by Peters-Golden et al. (1996) has revealed that the primary site of cPLA₂ activation occurs when the protein translocates to the nuclear envelope. Thus it was decided to co-stain annexin 1 and cPLA₂ to identify any co-localisation under the conditions being investigated. Cytosolic PLA₂ has also been reported to exhibit differing localisations within HUVECs depending on their confluency. Subconfluent cells have cPLA₂ in their nuclei, while in confluent cells it is predominantly in the cytosol (Sierra-Honigmann et al., 1996). Activation of this protein is reported to require both Ca²⁺ and phosphorylation by a p42 member of the MAPK family (Sa et al., 1995). There are reports of cPLA₂ becoming activated in varying cell types following treatment of cells with ATP (Chen and Chen, 1998), EGF (Sato et al., 1997) thapsigargin (Zhang et al., 1999) and Ca²⁺-ionophores (Chen and Chen, 1998).
Figure 16. Oxidant stress increases translocation of annexin 1 to the plasma membrane region.

Cells were fixed with 3.7% formaldehyde in PBS for 20 min, permeabilised with 80 μg/ml LPC, co-stained for annexin 1 and cPLA₂, then mounted for epifluorescence microscopy. (A) Cells grown in 20% O₂ and stained for annexin 1, (B) a merge of images A and C, (C) cells grown in 20% O₂ and stained for cPLA₂, (D) cells grown in 40% O₂ for 12 days and stained for annexin 1. The arrows show a concentration of annexin 1 in membrane ruffles. (E) is a merge of images D and F, (F) cells grown in 40% O₂ for 12 days and stained for cPLA₂. (G) A 4 micron line scan was taken across the plasma membrane of a cell shown in D, from the cell exterior (Ext) into the cytoplasm (Cyt) as indicated by the line drawn in D. The resulting data were standardised to the peak and background levels, quantified using NIH image. The images shown (magnification x400) are representative of three separate experiments.
Resting cells grown in 20% oxygen (figure 16 A, C), showed a perinuclear distribution within the cytosol for both cPLA\(_2\) and annexin 1, with annexin 1 also showing faint staining around the membrane region. Cells grown under hyperoxic conditions of 40% oxygen for 12 days (figure 16 D, F), manifest an increase in membrane-associated staining of annexin 1 but no change in the distribution of cPLA\(_2\). Increased annexin 1 at the membrane region and in membrane ruffles can also be demonstrated as a line scan taken across the membrane (figure 16 G). Figures 16 B and E, show merged images of annexin 1 and cPLA\(_2\) staining, revealing that under both sets of culture conditions there may be some co-localisation in the perinuclear area of the cytoplasm. Annexin 1 may therefore become membrane bound on oxidative stress.

4.3.1 Annexin 1 and cPLA\(_2\) in cells stimulated with thapsigargin and ionomycin

HUVECs grown under both normal and hyperoxic conditions and then stimulated with thapsigargin (figure 17) showed increased staining for annexin 1 in the membrane region (figure 17 A, D). The perinuclear cytoplasmic staining for cPLA\(_2\) was not influenced by oxidative stress (figure 17 C, F). The merged images (figure 17 B, E), reveal that there may be some co-localisation of the two proteins in the cytoplasm but that following exposure to thapsigargin cPLA\(_2\) does not co-localise with annexin 1 near the plasma membrane.

The stronger annexin 1 membrane staining in figure 17 A may be due to the cells being more densely packed than the cells that have been oxidatively stressed. These results suggest that annexin 1 translocates to the plasma membrane in response to a rise in Ca\(^{2+}\) levels in cultured cells generated by thapsigargin, and that this still occurs after oxidative stress. Oxidative stress and thapsigargin treatment had no effect on cPLA\(_2\) location in HUVECs.
Figure 17. Translocation of annexin 1 to the plasma membrane region upon treatment with thapsigargin is more prominent in control cells than in cells grown in hyperoxic conditions. Cells were stimulated for 5 minutes with 600 nM thapsigargin, fixed with 3.7% formaldehyde in PBS for 20 min, permeabilised with 80μg/ml LPC, stained for annexin 1 and cPLA₂, and mounted for epifluorescence microscopy. (A) Cells grown in 20% O₂ and stained for annexin 1, (B) a merge of images A and C, (C) cells grown in 20% O₂ and stained for cPLA₂, (D) cells grown in 40% O₂ for 12 days and stained for annexin 1, (E) a merge of images D and F, (F) cells grown in 40% O₂ for 12 days and stained for cPLA₂. The images shown (magnification x400) are representative of three separate experiments.

To assess the effects of a more sustained elevation of [Ca^{2+}]_i, control and oxidatively stressed HUVECs were exposed to the Ca^{2+} ionophore, ionomycin.

The results of this experiment (figure 18) were very similar to those obtained with thapsigargin, showing membrane region staining of annexin 1 for both sets of cells (figure 18 A, D). However there was a striking difference in the response to ionomycin of cPLA₂ in cells that had been exposed to oxidative stress, whereby the protein appeared to translocate from the cytosol (figure 18 C) to a more nuclear location (figure 18 F).
Figure 18. Ionomycin causes translocation of annexin 1 to the plasma membrane region and a redistribution of cPLA$_2$ to the nuclei of oxidatively stressed HUVECs.

Cells were treated with 1 µM ionomycin for 5 minutes, fixed with 3.7% formaldehyde in PBS for 20 min, permeabilised with 80 µg/ml LPC, stained for annexin 1 and cPLA$_2$, and mounted for epifluorescence microscopy. (A) Cells grown in 20% O$_2$ and stained for annexin 1, (B) a merge of images A and C, (C) cells grown in 20% O$_2$ and stained for cPLA$_2$, (D) cells grown in 40% O$_2$ for 12 days and stained for annexin 1, (E) a merge of images D and F, (F) cells grown in 40% O$_2$ for 12 days and stained for cPLA$_2$. The images shown (magnification x400) are representative of three separate experiments.

The merged images of the two proteins reveal some co-localisation in the perinuclear cytosolic region in cells grown in 20% O$_2$ (figure 18 B), but no co-localisation in cells grown in 40% O$_2$ for 12 days (figure 18 E), as annexin 1 tends to become plasma membrane associated whilst cPLA$_2$ is predominantly in the nucleus. From these results it can be observed that upon increased [Ca$^{2+}$], annexin 1 translocates to the plasma membrane, but cPLA$_2$ only translocates in response to treatment with ionomycin when the cells are oxidatively stressed.
4.3.2 Annexin 1 and cPLA\textsubscript{2} response to ATP and EGF

Following stimulation of HUVECs with 50 \textmu M ATP for 10 minutes, a partial translocation of annexin 1 and cPLA\textsubscript{2} to the nuclei of some cells was observed (figure 19 A, C, D, F). The intensity of fluorescence of cPLA\textsubscript{2} in the nuclei of these cells was more intense in cells that had been oxidatively stressed (figure 19 F).

A merge of the staining patterns of the two proteins in control cells (figure 19 B), showed co-localisation in cells where annexin 1 had moved into the nucleus. Oxidative stress was associated with more cells expressing both annexin 1 and cPLA\textsubscript{2} in their nuclei after 10 minutes ATP stimulation (figure 19 E). The graph (figure 19 G) indicates that stimulation with ATP leads to more annexin 1 entering the nuclei of a larger percentage of oxidatively stressed HUVECs, the most noticeable difference being after a 20 minute incubation with ATP.

These results show that simple elevation of [Ca\textsuperscript{2+}] using thapsigargin and ionomycin had little effect on the intracellular localisation of annexin 1, irrespective of whether or not the cells were oxidatively stressed. In contrast, exposure to a physiological agonist, namely ATP, which both raises [Ca\textsuperscript{2+}], and activates intracellular signal transduction pathways, leads to a significant accumulation of annexin 1 in the nuclei of oxidatively stressed HUVECs.
Figure 19. The effect of ATP on annexin 1 localisation is enhanced in HUVECs grown under hyperoxic conditions.
Cells were stimulated for the times indicated with 50 μM ATP, fixed with 3.7% formaldehyde in PBS for 20 min, permeabilised with 80μg/ml LPC, stained for annexin 1 and cPLA₂, then mounted for epifluorescence microscopy. (A) Cells grown in 20% O₂ and stained for annexin 1, (B) a merge of images A and C, (C) cells grown in 20% O₂ and stained for cPLA₂, (D) cells grown in 40% O₂ for 12 days and stained for annexin 1, (E) a merge of images D and F, (F) cells grown in 40% O₂ for 12 days and stained for cPLA₂. (G) Cell populations on 13 mm coverslips were estimated and averaged (n=3) for the percentage of cells containing annexin 1 in their nuclei, at each of the time points shown on the graph. The images shown (magnification x400) are representative of three separate experiments.
Because annexin 1 is a major cellular substrate for phosphorylation by the EGFr, and given that cPLA$_2$ is activated by EGFr stimulation, the effects of oxidative stress on EGF signalling in HUVECs were examined. The results show that EGF stimulation elicits different responses in oxidatively stressed and control cells. Control cells stimulated for 5 minutes with EGF showed some translocation of annexin 1 to the membrane region (figure 20 A), whilst cPLA$_2$ maintained a perinuclear distribution (figure 20 C), similar to the ionomycin response. When these two images are merged (figure 20 B), it is seen that both proteins share a similar perinuclear distribution within the cell but are not entirely co-localised. The movement of annexin 1 to the plasma membrane was not shared by cPLA$_2$. A different response of annexin 1 and cPLA$_2$ emerges for cells first grown in 40% O$_2$ for 12 days and then stimulated with EGF. Under these conditions cells stimulated for 5 min (figure 20 D, F) show partial translocation of annexin 1 into the nucleus whereas cPLA$_2$ maintains a predominantly perinuclear distribution with a slight migration of this protein into the nucleus of some cells. A merge of the staining patterns (figure 20 E) shows some areas where the proteins share a similar location but no strong co-localisation. CPLA2 has been reported to show nuclear envelope staining on stimulation of ECs (Sierra-Honnigman et al., 1996), this pattern of staining is not seen in this study but this may only be apparent when using confocal microscopy, where as here the images are obtained on an epifluorescence microscope.

The proportions of cells containing annexin 1 in their nuclei are shown in (figure 20 G), for differing time points during EGF stimulation. A distinct behavioural difference exists between control cells and those that have been oxidatively stressed. Oxidative stress appears to modify the cells in a manner that correlates with annexin 1 translocation into the nucleus upon EGF stimulation. The graphs (figure 20 H, I) show the different fluorescence patterns across the nucleus of a cell grown in control conditions and a cell grown in hyperoxic conditions. The fluorescence decreases in the nuclear region of the line scan in the cell from figure 20 A, whereas in the cell from fig 20 D it increases in this region.

Both ATP and EGF are therefore able to elicit the movement of annexin 1 into the cell nucleus of oxidatively stressed HUVECs, but only ATP is able to do this in control cells. The translocation of annexin 1 to the plasma membrane is similar to that seen after treatment with thapsigargin or ionomycin. In oxidatively stressed cells cPLA$_2$ shows some translocation to the nucleus after both ATP and EGF stimulation but as with the translocation of annexin 1 only ATP elicits this response in control cells.
Figure 20. Annexin 1 translocates to the nuclei of HUVECs upon EGF stimulation in cells grown in hyperoxic conditions.

Cells were stimulated with 100ng/ml EGF for 5 mins or for the times indicated in G, then fixed with 3.7% formaldehyde in PBS for 20 min, permeabilised with 80μg/ml LPC, stained for annexin 1 and cPLA₂, and mounted for epifluorescence microscopy. (A) Cells grown in 20% O₂ and stained for annexin 1, (B) a merge of images A and C, (C) cells grown in 20% O₂ and stained for cPLA₂, (D) cells grown in 40% O₂ for 12 days and stained for annexin 1, (E) a merge of images D and F, (F) cells grown in 40% O₂ for 12 days and stained for cPLA₂. (G) Cell populations on 13 mm coverslips were estimated and averaged (n=3) for the percentage of cells containing annexin 1 in their nuclei for each time point. Two line scans were taken of the nuclear regions of two cells from A and D. The resulting data were standardised to the peak and background levels. Each line scan was taken from top to bottom of the lines draw. (H)The line scan for A and (I) is the line scan for D. The images shown (magnification x400) are representative of three separate experiments.
4.3.3 Inhibition of tyrosine phosphorylation

The preceding results show that nuclear translocation of annexin 1 requires more than a simple rise in [Ca^{2+}]. As annexin 1 is known to be a substrate for phosphorylation by both tyrosine and serine/threonine kinases, this raised the possibility that the results obtained with ATP and EGF in oxidatively stressed cells could be linked to tyrosine phosphorylation of annexin 1.

The protein tyrosine kinase inhibitor genistein was chosen as at high concentrations it partially inhibits PKC and PKA (IC_{50}>350 \ \mu M) and at lower levels prevents autophosphorylation and therefore activation of the EGFr (IC_{50} =2.6 \ \mu M). Annexin 1 contains phosphorylation sites for all of these kinases. Cells were thus preincubated with 5\mu M or 100\mu M genistein and then stimulated with ATP and EGF, fixed and stained, then examined by confocal microscopy.

![Figure 21. Addition of genistein to HUVECs.](image)

Cells cultured in 20% O_{2} were incubated with 5 \ \mu M genistein for 30 minutes, fixed with 3.7% formaldehyde in PBS for 20 min, permeabilised with 80\mu g/ml LPC, stained for annexin 1 and mounted for confocal microscopy. The image shown a single image taken through the middle of the cells, and is representative of three separate experiments.

Addition of genistein to HUVECs at both 5\mu M or 100\mu M was observed to cause annexin 1 to partially translocate into the nucleus (figure 21). The observed translocation was similar to that seen in oxidatively stressed cells stimulated with ATP (figure 19) or EGF (figure 20) except in this case almost the entire population of cells exhibited annexin 1 in the nucleus. Addition of ATP or EGF to cells after preincubation with genistein did not show any further changes in annexin 1 translocation (not shown). Due to this result it was not possible to examine in this way the effect of inhibiting tyrosine phosphorylation on the translocation of annexin 1 seen in oxidatively stressed cells.
4.4 Annexin 2 localisation

Annexin 2 is of particular interest in ECs since the discovery that it acts as a cell surface receptor for tPA and plasminogen, catalysing the conversion of plasminogen to plasmin (Hajjar et al., 1994) (see section 1.6.4). Along with this observation in endothelial cells annexin 2 has also been reported to bind alternatively spliced tenasin C, an extracellular matrix glycoprotein (Chung and Erickson, 1994) and directly interact with the cytomegalovirus glycoprotein B (Pietropaolo and Compton, 1997).

Cells grown under normal and hyperoxic conditions were used to examine the behaviour of annexin 2. Under both culture conditions cells were fixed and stained for annexin 2 after treatment with the following agonists and pharmacological reagents; thapsigargin, ionomycin, ATP and EGF. In both control and oxidatively stressed cells annexin 2 exhibited a perinuclear distribution, with a representative image shown in figure 22 (A). Following treatment of the cells, regardless of the agent used, annexin 2 maintained its perinuclear location with some translocation to the plasma membrane region (figure 22 B), although this was less noticeable in cells stimulated with EGF. A line scan (figure 22 C) across the plasma membrane where two cells stimulated with ATP for 20 minutes meet (figure 22 B), shows increased immunoreactivity for annexin 2 at the cell-cell interface.
Figure 22. Annexin 2 translocates to the plasma membrane after ATP stimulation.
Cells grown in 20% O₂ were either washed in PBS or stimulated with 50 μM ATP, then fixed with 3.7% formaldehyde in PBS for 20 min, permeabilised with 80 μg/ml LPC, stained for annexin 2 and mounted on coverslips for examination by epifluorescence microscopy. (A) resting cells, (B) Cells stimulated with 50 μM ATP for 20 min. (C) Line scan of membrane region where two cells meet. An 8 micron line scan was taken from the cytoplasm (Cyt) of one cell to the cytoplasm of the other as indicated by the line drawn (from left to right) in C. The resulting data were standardised to the peak and background levels, quantified using NIH image. The images shown (magnification x400) are representative of three separate experiments.

4.5 Cellular distribution of annexin 4 after oxidative stress
The literature for annexin 4 is not as extensive as that for some of the other annexins investigated in this chapter. The crystal structure of this protein reveals that it forms a trimeric arrangement similar to that seen for annexin 5 (Zanotti et al., 1998). Localisation studies have shown annexin 4 to have an unstructured cytoplasmic staining in human fibroblasts, with a higher concentration present in the nucleus...
(Barwise and Walker, 1996), a similar pattern has been described in bovine mammary gland (Katoh et al., 1995). Increasing $[Ca^{2+}]$ in these cells using an ionophore promotes translocation of annexin 4 to the nuclear and plasma membranes (Barwise and Walker, 1996). However the translocation of annexin 4 did not occur when using an ionophore and a $Ca^{2+}$ free external solution, implying a requirement for $Ca^{2+}$ influx (Raynal et al., 1996).

Annexin 4 has been reported to inhibit $Ca^{2+}$/calmodulin-dependent anion current activation, it does not interact with calmodulin-dependent protein kinase II but is thought to prevent it from interacting with the ion channel (Chan et al., 1994). Annexin 4 in ECs is of interest as patients with antiphospholipid syndrome and systemic lupus erythematosus both show levels of anti-annexin 4 in their serum (Satoh et al., 1999). In addition, annexin 4 is expressed in human cardiac tissue but shows up-regulation in failing hearts (Matteo and Moravec, 2000).

Annexin 4 was observed to have a punctate cytosolic distribution in resting HUVECs (figure 23 A). After culture in hyperoxic conditions, annexin 4 partially translocated to the nuclei of HUVECs while still exhibiting some cytoplasmic staining (figure 23 B). However, the staining pattern changed after exposure to hyperoxia, to become less punctate and more diffuse. The resting pattern of annexin 4 differs from that reported in fibroblasts but this may just be attributed to the different cell types or the substrate that the cells are plated on.
4.6 Translocation of annexin 5 under oxidative stress

Within the cardiovascular system annexin 5 is widely expressed, being abundant in HUVECs and one of the major cardiac annexins along with annexin 6 (Luckcuck et al., 1997; Doubell et al., 1993). Patients with anti-phospholipid syndrome and systemic lupus erythematosus have anti-annexin 5 antibodies in their sera along with an increased occurrence of thrombosis (Satoh et al., 1999). As with annexin 6, annexin 5 has been reported to have an anticoagulant effect on the EC surface, by inhibiting the effect of prothrombin. Annexin 5 has been suggested to do this by forming lipid-bound clusters that exist as a two-dimensional lattice across the membrane preventing lateral movement of prothrombin (Andree et al., 1992). Experiments performed with blood flow across tumour necrosis factor-stimulated ECs showed that preincubation of the cells with recombinant annexin 5 led to less deposition of fibrin, supporting the idea that annexin 5 can prevent blood coagulation (van Heerde et al., 1994b).

Annexin 5 was present throughout the cytoplasm of HUVECs and had a punctate pattern, but was excluded from the nuclei of these cells (figure 24 A). After 12 days of culture in 40% O₂ the staining pattern changed. Annexin 5 redistributed into the nuclei, but was absent from the nucleoli (figure 24 B, C). In the nucleus annexin 5 retained a punctate appearance similar to the pattern in the cytosol. In cells that had been oxidatively stressed some showed staining in both the cytosol and nucleus, whereas others showed a translocation of almost all the cellular annexin 5 to the nucleus. The change in fluorescence across the nuclear region under both conditions is shown by two line scans (figure 24 D and E). These results show that oxidative stress causes a striking change in localisation of annexin 5 in HUVECs from the cytosol to the nucleus.
Figure 24. Annexin 5 in control and oxidatively stressed cells.
Cells were fixed with 3.7% formaldehyde in PBS for 20 min, permeabilised with 80 µg/ml lysophosphatidyl choline, stained for annexin 5 and mounted on coverslips for examination by confocal microscopy. (A) resting cells grown in 20% O₂. (B) cells grown in 40% O₂ for 12 days. (C) enlargement of nuclear region, where annexin 5 appears to be excluded from the nucleolus. Two line scans were taken of the nuclear regions of two cells from images A and B. The resulting data were standardised to the peak and background levels, quantified using NIH image. Each line scan was taken from top to bottom of the lines drawn. (D) The line scan for image A and (B) is the line scan for image B. The image shown a single image taken through the middle of the cells, and is representative of three separate experiments.

4.6.1 HUVEC annexin 5 does not respond to thapsigargin but does translocate in response to ionomycin

Stimulation with thapsigargin had no effect on the localisation of annexin 5. Figure 25 A shows cells grown under normal conditions where the staining pattern for annexin 5 remains the same as is seen in resting cells (figure 25 A). After oxidative stress, cells treated with thapsigargin showed no change in annexin 5 location, it remains predominantly in the nuclei of the cells (figure 25 B). Figure 25 (C) shows how the percentage of cells with annexin 5 in their nuclei does not change under either culture condition during a time course of thapsigargin treatment. Oxidatively stressed cells
may show no response to thapsigargin as annexin 5 is already in the nuclei of unstimulated cells grown under these conditions.

![Image](image-url)

Figure 25. Annexin 5 in control cells and oxidatively stressed cells does not change distribution upon treatment with thapsigargin.

Cells were stimulated for the times indicated with 600 nM thapsigargin, fixed with 3.7% formaldehyde in PBS for 20 min, permeabilised with 80 µg/ml LPC, stained for annexin 5 and mounted on coverslips for examination by epifluorescence microscopy. (A) cells grown in 20% O₂ and stimulated with thapsigargin for 10 min. (B) cells grown in 40% O₂ for 12 days and stimulated with thapsigargin for 10 min. (C) Cell populations on 13 mm coverslips were estimated and averaged (n=3) for the percentage of cells containing annexin 5 in their nuclei, for each of the time points. The images shown (magnification x400) are representative of three separate experiments.

Ionomycin produces a translocation of annexin 5 from the cytosol to nuclei after 20 minutes in cells grown under normal conditions (figure 26 A). This produced a cell population, of which approximately half presented annexin 5 in their nuclei (figure 26 C). Cells exposed to hyperoxic conditions (in which annexin 5 was already nuclear) showed no change in the localisation of annexin 5 during ionomycin stimulation (figure 26 B).
Figure 26. Ionomycin causes increased translocation of annexin 5 to the nuclei of HUVECs in control cells but has no effect on oxidatively stressed cells.

Cells were treated with 1 μM ionomycin for the time indicated, fixed with 3.7% formaldehyde in PBS for 20 min, permeabilised with 80 μg/ml LPC, stained for annexin 5 and mounted on coverslips for examination by epifluorescence microscopy. (A) shows cells grown in 20% O₂ and stimulated with ionomycin. (B) cells grown in 40% O₂ for 12 days and stimulated with ionomycin. (C) Cell populations on 13 mm coverslips were estimated and averaged (n=3) for the percentage of cells containing annexin 5 in their nuclei for all of the time points. The images shown (magnification x400) are representative of three separate experiments.

These studies show that control cells stimulated with thapsigargin, which elicits a smaller and more transient rise in [Ca²⁺], did not display any translocation of annexin 5, but stimulation with ionomycin which produces a larger rise in [Ca²⁺] (see Chapter 5.1, figure 35), did lead to translocation of annexin 5 to the nuclei of the cells.
4.6.2 The response of HUVEC annexin 5 to ATP and EGF

With regard to annexin 5, stimulation of HUVECs with ATP yielded similar results to those obtained with thapsigargin. Thus ATP had no effect on annexin 5 location within cells grown under either culture condition (figure 27 A and B). The staining patterns were the same as those shown in figure 24 A and B, in resting cells grown under both conditions. The graph in fig 27 (C) shows a quantitative summary of these results, demonstrating that ATP has no effect on annexin 5 location in HUVECs before or after exposure to oxidative stress during a time course.

![Image](https://via.placeholder.com/150)

**Figure 27.** ATP has no effect on annexin 5 localisation of both control and oxidatively stressed HUVECs.

Cells were stimulated with 50 μM ATP for the times indicated, fixed with 3.7% formaldehyde in PBS for 20 min, permeabilised with 80 μg/ml LPC, stained for annexin 5 and mounted on coverslips for examination by confocal microscopy. (A) cells grown in 20% O₂ and stimulated with ATP. (B) cells grown in 40% O₂ for 12 days and stimulated with ATP. (C) Cell populations on 13 mm coverslips were estimated and averaged (n=3) for the percentage of cells containing annexin 5 in their nuclei. The images shown a single image taken through the middle of the cells, and is representative of three separate experiments.
In contrast to the results obtained with ATP, stimulation of control HUVECs with EGF caused the translocation of annexin 5 from the cytosol to the nuclei (figure 28 A). Cells that had been oxidatively stressed prior to stimulation with EGF, showed no change in annexin 5 distribution (figure 28 B). These results can be seen more clearly in the graph (figure 28 C) which shows the percentage of cells with annexin 5 in their nuclei during an EGF time course.

Figure 28. EGF stimulation changes the distribution of annexin 5 in control but not oxidatively stressed cells.
Cells were stimulated with 100 ng/ml EGF for the times indicated, fixed with 3.7% formaldehyde in PBS for 20 min, permeabilised with 80 μg/ml LPC, stained for annexin 5 and mounted on coverslips for examination by epifluorescence microscopy. (A) cells grown in 20% O$_2$ and stimulated with EGF. (B) cells grown in 40% O$_2$ for 12 days and stimulated with EGF. (C) Cell populations on 13 mm coverslips were estimated and averaged (n=3) for the percentage of cells containing annexin 5 in their nuclei for each time point. The images shown (magnification x400) are representative of three separate experiments.

The response of HUVEC annexin 5 to EGF is not clear. Unlike annexin 1, annexin 5 is not a substrate for EGFr phosphorylation, nor is it phosphorylated by any of the
known downstream kinases such as the Erks. However, EGFr activation does cause a rise in [Ca^{2+}], and experiments using ionomycin suggest that Ca^{2+} is likely to at least play a part in the nuclear translocation of annexin 5.

4.7 Response of annexin 6 to oxidative stress

Within the cardiovascular system annexin 6 is a major cardiac annexin. When over-expressed in the heart, it causes altered cardiomyocyte behaviour (Guntleski-Hamblin et al., 1996), but in annexin 6 knockout mice there were no detrimental effects on cardiovascular function (Hawkins et al., 1999) although the cardiac myocytes do have defective Ca^{2+} handling and contractile properties (Moss, S.E. and Song, G., personal communication). In human end-stage heart failure annexin 6 becomes down-regulated whilst annexins 2 and 5 become up-regulated (Song et al., 1998). It has also been suggested to have an antithrombotic property when bound to the surface of cultured ECs, but this may simply be attributed to its ability to bind phospholipids (Yoshizaki et al., 1992). Treatment of cells with H_2O_2 is reported to cause annexin 6 to translocate from the plasma membrane to the cytosol. This change in location correlates with an increase in [Ca^{2+}], observed during treatment with H_2O_2, and annexin 6 was suggested to be the source of Ca^{2+} that generated this elevation in Ca^{2+}-levels (Hoyal et al., 1996).

Other studies have reported annexin 6 to be capable of regulating Ca^{2+} channel activity from the SR (Munoz et al., 1990; Diaz-Munoz et al., 1990). In A431 epithelial carcinoma cells, ectopic expression of annexin 6 leads to an inability to sustain a prolonged Ca^{2+} increase following EGF stimulation (Fleet et al., 1999). These results suggest a role for annexin 6 in Ca^{2+} channel regulation.
Figure 29. Annexin 6 localisation in resting control and oxidatively stressed HUVECs.
Cells were fixed with 3.7% formaldehyde in PBS for 20 min, permeabilised with 80 μg/ml LPC, stained for annexin 6 and mounted on coverslips for examination by confocal microscopy. (A) and (C) annexin 6 observed in resting cells grown in 20% O₂ and 40% O₂ for 12 days. (B) and (D) show enlargements of the nuclear regions. Two line scans were taken of the nuclear regions of two cells, one from A and the other from C. The resulting data were standardised to the peak and background levels, quantified using NIH image. Each line scan was taken from top to bottom of the lines drawn. (E) is a line scan from A and (F) is a line scan from C. The images shown a single image taken through the middle of the cells, and is representative of three separate experiments.

In unstimulated control cells annexin 6 exhibited a cytoplasmic distribution with some punctate staining, but was excluded from the nucleus (figure 29 A, B). The exclusion from the nucleus of one of the cells (figure 29 A), is clearly illustrated in figure 29 E, a line scan taken across the nuclear region. However the location of annexin 6 in oxidatively stressed cells can be both cytosolic and nuclear (figure 29 C, D). A line scan (figure 29 F) of the nuclear region of a cell from figure 29 (C) shows a higher fluorescence in the nucleus than in the adjacent cytoplasm. The pattern of annexin 6 cytoplasmic staining (figure 29 A) in HUVECs shows a strong similarity with annexin 5.
(figure 24 A). However, upon translocation into the nuclei the straining pattern is no longer comparable between annexins 5 and 6.

4.7.1 Effect of thapsigargin and ionomycin on annexin 6 localisation

Annexin 6 failed to respond to addition of thapsigargin to HUVECs under normal conditions. The majority of cells continued to display annexin 6 in the cytoplasm (figure 30 A). In cells exposed to hyperoxia before treatment with thapsigargin, translocation of annexin 6 from the cytosol to the nuclei of these cells was observed. Two distinct patches of cells are shown 5 minutes after thapsigargin addition (figure 30 B, C). During the time course there was a steady increase in the percentage of oxidatively stressed cells with annexin 6 in their nuclei, illustrated by the graph (figure 30 D). This clearly shows that oxidative stress causes changes that allow HUVECs to respond to addition of thapsigargin by translocation of annexin 6 to the nuclei.

![Figure 30](image-url)

Figure 30. Thapsigargin causes translocation of annexin 6 to nuclei of HUVECs grown in hyperoxic conditions.

Cells were stimulated with 600 nM thapsigargin, then fixed with 3.7% formaldehyde in PBS for 20 min, permeabilised with 80µg/ml LPC, stained for annexin 6 and examined by confocal microscopy. (A) Cells grown in 20% O₂ treated with thapsigargin. (B) cells grown in 40% O₂ for 12 days and treated with thapsigargin. (D) Cell populations on 13 mm coverslips were estimated and averaged (n=3) for the percentage of cells containing annexin 6 in their nuclei for each of the time points shown. The images shown a single image taken through the middle of the cells, and is representative of three separate experiments.
Figure 31. Ionomycin has a similar effect on annexin 6 in control and oxidatively stressed cells.
Cells were stimulated with 1μM ionomycin for the times indicated, then fixed with 3.7% formaldehyde in PBS for 20 min, permeabilised with 80μg/ml LPC, stained for annexin 6 and examined by confocal microscopy. (A) and (B) Cells grown in 20% O2 and treated with ionomycin. (C) and (D) cells grown in 40% O2 for 12 days and treated with ionomycin. (E) Cell populations on 13 mm coverslips were estimated and averaged (n=3) for the percentage of cells containing annexin 6 in their nuclei for each time point. The images shown a single image taken through the middle of the cells, and is representative of three separate experiments.

With a larger rise in [Ca^{2+}], generated by ionomycin, annexin 6 in cells grown under both control and hyperoxic conditions responded by translocating to the nucleus
Both groups of cells showed a similar time-dependent increase in the percentage of cells with annexin 6 in their nuclei (figure 31 E). Figure 31 A and B show cells stained for annexin 6, grown under normal conditions and stimulated with ionomycin for 3 minutes. Figures 31 C and D show oxidatively stressed cells treated with ionomycin for 3 minutes. In HUVECs annexin 6 translocates to the nuclei of cells on treatment with ionomycin but not with thapsigargin. However, after culture in hyperoxic condition annexin 6 translocates to the nucleus upon treatment with both pharmacological agents.

4.7.2 Response of annexin 6 to ATP and EGF stimulation

Addition of ATP to HUVECs did not have any effect on the localisation of annexin 6 in control or oxidatively stressed cells, as can be seen in the graph in Fig 32 (D). Figure 32 (A) shows control cells after a 10 minute incubation with ATP. Figures 32 B and C, 40% O<sub>2</sub>, ATP 10 min

Cells with ANX 6 in their nuclei (%)

□ 20% O<sub>2</sub> ■ 40% O<sub>2</sub>

HUVECs stimulated with 50uM ATP

Figure 32. ATP has no effect on annexin 6 in control or oxidatively stressed HUVECs.

Cells were stimulated with 50μM ATP for the times indicated, then fixed with 3.7% formaldehyde in PBS for 20 min, permeabilised with 80μg/ml LPC, stained for annexin 6 and examined by confocal microscopy. (A) Cells grown in 20% O<sub>2</sub> and stimulated with ATP. Images (B) and (C) of cells grown in 40% O<sub>2</sub> for 12 days and stimulated with ATP show two different types of cell clusters, (B) those with annexin 6 in their nuclei and (C) those without annexin 6 in their nuclei. (D) Cell populations on a 13 mm coverslip were estimated and averaged (n=3) for the percentage of cells containing annexin 6 in their nuclei for each of the time points. The images shown a single image taken through the middle of the cells, and is representative of three separate experiments.
show the different cell patches seen in the oxidatively stressed cells after incubation with ATP for 10 minutes.

In contrast to the results obtained using ATP, stimulation of HUVECs with EGF caused translocation of annexin 6 to the nuclei of cells cultured under both normal and hyperoxic conditions. Both groups of cells responded with similar kinetics during an EGF time course (figure 33 D). Figure 33 A shows a group of cells stimulated with EGF for 5 minutes, by which time a few cells displayed annexin 6 in the nucleus. For oxidatively stressed cells, two separate patches of cells are shown (figure 33 B, C), one in which translocation has not occurred and the other in which annexin 6 is present in the nuclei of all cells in the field of view. Therefore oxidative stress did not appear to have any effect on EGF-stimulated translocation of annexin 6 in HUVECs.

![Figure 33. Effect of EGF on annexin 6 in cells culture in 20% and 40% O₂.](image)

Cells were stimulated with 100 ng/ml EGF for the times indicated, then fixed with 3.7% formaldehyde in PBS for 20 min, permeabilised with 80μg/ml LPC, stained for annexin 6 and examined by confocal microscopy. (A) Cells grown in 20% O₂ and stimulated with EGF. (B) and (C) cells grown in 40% O₂ for 12 days and stimulated with EGF, shown are two different cell clusters, (B) those with annexin 6 in their nuclei and (C) those without annexin 6 in their nuclei. (D) Cell populations on a 13mm coverslip were estimated and averaged (n=3) for the percentage of cells containing annexin 6 in their nuclei for each of the time points. The images shown a single image taken through the middle of the cells, and is representative of three separate experiments.
4.8 Localisation of annexin 7 in HUVECs

Annexin 7 was originally discovered as a protein (synexin) able to aggregate isolated chromaffin secretory granules (Creutz et al., 1978), where it was suggested to aid membrane fusion (Kuijpers et al., 1992). A further investigation of annexin 7 function has been performed by generation of knock-out mice. Although these proved to be embryonic lethal, the heterozygous mice expressing low levels of annexin 7, exhibited defective insulin secretion thought to be caused by a change in the ability of IP$_3$ agonists to release intracellular Ca$^{2+}$ (Srivastava et al., 1999). Like annexin 5, annexin 7 has been suggested to have the ability to function as a Ca$^{2+}$ channel (Pollard et al., 1992; Burns et al., 1989). Alternatively, it has also been suggested to function as a tumour suppressor (Kataoka et al., 2000) (see 1.6.3 Annexins in disease). There is little information on annexin 7 in the cardiovascular system.

**Figure 34. Culture in hyperoxic conditions does not affect localisation of annexin 7 in HUVECs.**

Cells were fixed with 3.7% formaldehyde in PBS for 20 min, permeabilised with 80 µg/ml LPC, stained for annexin 7 and mounted on coverslips for examination by confocal microscopy. (A) cells grown in 20% O$_2$, (B) enlargement of part of a cell from A, (C) cells grown in 40% O$_2$ for 12 days. The images shown a single image taken through the middle of the cells, and is representative of three separate experiments.

Annexin 7 showed a punctate staining pattern in HUVECs (figure 34 A, B), similar to annexins 4 (figure 23) and 5 (figure 24). Cells that had been grown for 12 days in...
hyperoxic conditions showed no change in the distribution of annexin 7 (figure 34 C). These results suggest that oxidative stress generated by 40% $O_2$ over 12 days does not modulate annexin 7 localisation in HUVECs.

The results in this chapter show that oxidative stress influences both the localisation of certain annexins in HUVECs, and differentially modulates the responses of these annexins to a range of pharmacological agents. Although no clear pattern emerges from these studies, it is clear that nuclear translocation of annexins can be influenced by oxidative stress in combination with cell activation, and that in most cases this cannot be explained by a simple rise in [Ca$^{2+}$]].
CHAPTER 5: CELL SIGNALLING
Cell signalling is mediated by many factors, and although calcium often plays a pivotal role, serine/threonine or tyrosine phosphorylation and consequent activation of various signalling molecules is frequently a major component of signalling pathways. Oxidative stress is known to affect several signal transduction cascades. To examine potential changes in cellular signalling caused by hyperoxia in HUVECs, Ca\(^{2+}\) signalling, some signalling proteins and tyrosine phosphorylation were investigated.

5.1 Calcium signalling

Calcium signalling in HUVECs is of importance in cell homeostasis and controlled release of factors required for the maintenance of a healthy vasculature (see sections 1.1 and 1.4). Changes in Ca\(^{2+}\) signalling may also be significant in the context of the annexin family as all of the annexins investigated in this thesis are Ca\(^{2+}\)-binding proteins. For example, membrane-bound annexin 6 has been suggested to release Ca\(^{2+}\) into the cytosol during oxidant stress generated by H\(_2\)O\(_2\) (Hoyal et al., 1996) and annexin 5 has been seen to be required for a normal Ca\(^{2+}\) response to H\(_2\)O\(_2\) in B-cells (Kubista et al., 1999).

There are numerous reports of oxidative stress leading to changes in Ca\(^{2+}\) signalling in HUVECs (see section 1.4.2). The model for much of this work is based on oxidative stress being induced by H\(_2\)O\(_2\) or t-BOOH. Oxidant stress produced by addition of H\(_2\)O\(_2\) to ECs has been found to produce defective Ca\(^{2+}\) signalling in response to addition of bradykinin. With increased time under oxidative stress cells showed less response to bradykinin, measured by changes in [Ca\(^{2+}\)]\(_i\) (Schilling and Elliott, 1996). Addition of H\(_2\)O\(_2\) to ECs has been observed to produce Ca\(^{2+}\) oscillations generated by release of Ca\(^{2+}\) from internal stores (Hu et al., 1998).

Changes in Ca\(^{2+}\) seen during oxidative stress have been linked to annexins. Addition of t-BOOH to alveolar macrophages has been observed to produce a rise in [Ca\(^{2+}\)]\(_i\) in conjunction with a release of annexin 6 from the plasma membrane. The rise in Ca\(^{2+}\) is not from influx or release from intracellular stores but is suggested to be released from annexin 6 when the protein dissociates from the plasma membrane (Hoyal et al., 1996).

To investigate Ca\(^{2+}\) signalling in cells oxidatively stressed by culture in 40 % O\(_2\) for 12 days, cells were stimulated with a range of pharmacological reagents. Cells were harvested and examined whilst in suspension, in order to obtain average data from large numbers of cells.
Figure 35. Calcium signalling in control and oxidatively stressed HUVECs. Ratiometric analysis of cytosolic intracellular free calcium was determined fluorometrically in suspensions of Fura-2-AM loaded cells as described in section 2.9. In (A) cells were treated with 500μM Mn^{2+}, (B) with 600 nM thapsigargin, (C) with 1μM ionomycin, (D) with pervanadate (0.1μM Na<sub>2</sub>VO<sub>4</sub>, 0.1μM H<sub>2</sub>O<sub>2</sub>), (E) with 0.5% (v/v) foetal calf serum at 180 seconds and again at 360 seconds, and in (F) cells were treated with 50μM ATP. For each of the figures shown above n=3.

The first consideration was to establish resting Ca<sup>2+</sup>, to do this Mn<sup>2+</sup> was used, as when cells are in a cuvette as used here, fura 2-AM can leak into the buffer in which the cells are suspended, and this can anomalously elevate the fluoresence measured.
Fura 2-AM has a higher affinity for Mn$^{2+}$ than for Ca$^{2+}$, and when added to the buffer will quench any fura 2-AM that has leaked from the cells. Fura-2AM when bound to Mn$^{2+}$ does not fluoresce, thus allowing ratiometric analysis of the basal calcium levels of the two sets of samples. The results in figure 35 show that HUVECs that have been oxidatively stressed have a slightly higher basal level of [Ca$^{2+}$], than control cells (figure 35 A), which is indicated by the ratio levels after the Mn$^{2+}$ has been added.

The next area investigated was mobilisation of Ca$^{2+}$ from internal stores by use of thapsigargin (figure 35 B). The response produced is bi-phasic with an initial rapid release from internal stores followed by capacitative calcium entry causing the sustained phase. No differences were found between control and oxidatively stressed cells. As some of the experiments on annexin localisation in chapter 4 had shown differing responses of annexins to thapsigargin and to ionomycin it seemed sensible to examine Ca$^{2+}$ signals produced by ionomycin to compare the amplitude of change in [Ca$^{2+}$], generated by the two types of cell treatments. Mobilisation of Ca$^{2+}$ using ionomycin (figure 35 C) revealed no differences in oxidatively stressed cells compared with control cells, although the rise in [Ca$^{2+}$], produced by ionomycin was greater than that generated by thapsigargin with the concentrations used here.

Stimulation using pervanadate was also explored to examine different forms of cell activation. A similar response was elicited in oxidatively stressed cells compared with control cells, except for a slightly faster initial rise in [Ca$^{2+}$], in the oxidatively stressed cells (figure 35 D). The response also appeared to be bi-phasic under both conditions.

The main differences in Ca$^{2+}$ signalling were observed during ATP and serum stimulation of oxidatively stressed cells. Both these responses comprised a transient response of approximately 100s duration. The peak Ca$^{2+}$ response to serum was decreased in cells that had been grown in 40% O$_2$ (figure 35 E). Upon addition of a second aliquot of serum, control cells showed a further small rise in [Ca$^{2+}$], whereas oxidatively stressed cells showed no further response (figure 35 E). Previous experiments measuring serum responses in buffer containing no Ca$^{2+}$ (not shown) showed that the Ca$^{2+}$ mobilisation profile was reproducible, although the amplitude diminished with repetition. This indicated the Ca$^{2+}$ response to serum to be a release from intracellular stores. The lack of a second response in oxidatively stressed cells may reflect a difference in refilling of intracellular stores after initial release.

Oxidatively stressed cells also demonstrated a smaller rise in [Ca$^{2+}$], compared with control cells following exposure to 50μM ATP. The response under both conditions
was rapid, and under both conditions the ratio remained slightly elevated after the initial transient rise in Ca²⁺ (figure 35 F).

Experiments using acetylcholine and bradykinin were also performed but neither agonist generated a measurable response in either control or oxidatively stressed cells. This may have been caused by the cells having been treated with trypsin prior to addition of these agonists where trypsin may have caused partial hydrolysis of the respective receptors. Responses to these agonists have previously been reported in bovine aortic endothelial cells in experiments to investigate oxidative stress generated by t-BOOH (Schilling and Elliott, 1996). Cells were harvested using 0.25% trypsin-EDTA and then immediately resuspended in medium containing 10% serum, whereas in this project they were harvested using 1% trypsin-EDTA, washed and resuspended in HBSS. ECs are extremely sensitive to trypsinisation and the procedural differences in collecting the cells may explain the lack of response in these studies of HUVECs to acetylcholine and bradykinin.

5.2 Signalling proteins

Oxidative stress has also been reported to have effects on signalling pathways, perhaps through changes in protein expression, and/or phosphorylation (see section 3.3). In ECs hyperoxia has been demonstrated to cause an increase in expression of ICAM-1 and VCAM-1 (Willing et al., 1999), and in rats exposed to hyperoxia an increase in Mn²⁺-SOD was observed (Choi et al., 1995). In blood samples collected from diabetic patients (diabetic patients showing greater oxidative stress than control patients, see section 1.3.4) increased levels of ICAM-1 expression and SOD activity have been reported (Elhadd et al., 1999).

Here, aspects of the EGFr signalling pathway were examined, this being an archetypal example. Activation of the EGFr can stimulate ion influxes, changes in gene expression and reorganisation of the cytoskeleton. In human fibroblasts, EGFr internalisation is inhibited after treatment with H₂O₂ (de Wit et al., 2000), though binding of EGF to the receptor was not affected. As all of the cell activation conditions used in these studies (including addition of EGF) lead to activation of cPLA₂, and given that annexin 1 is proposed to act as an inhibitor of cPLA₂ expression of cPLA₂ was also investigated. Furthermore, oxidative stress induced by UV-B irradiation leads to phosphorylation of cPLA₂ which in turn corresponds to an increase in arachidonic acid release and production of PGE₂ (Chen et al., 1996). Finally the early response gene c-fos was examined, as changes in gene expression are known to occur after exposure of cells to oxidative stress. For example, experiments on rats exposed to
hyperoxia revealed an increase in expression of a variety of stress-response genes which included c-jun and c-fos (Choi et al., 1995). Use of H\textsubscript{2}O\textsubscript{2} is reported to up-regulate expression of c-fos mRNA levels in rabbit lens epithelial cells (Li and Spector, 1997).

![Figure 36. Modulation of EGFr, cPLA\textsubscript{2} and c-fos expression by oxidative stress.](image)

Protein samples were collected in cell lysis buffer at 3 day intervals during culture of HUVECs in hyperoxic conditions. Lysates were then quantified and examined by SDS-PAGE. The top panel shows cell lysates stained for the EGFr. The middle panel was stained for cPLA\textsubscript{2} and the bottom panel stained for c-fos. Each gel was loaded with 40\(\mu\)g of protein per track. Protein bands were visualised by ECL. The blots shown are representative of three separate experiments.

Both cPLA\textsubscript{2} and the EGFr were down-regulated during culture under conditions of hyperoxia (figure 36). The decrease in expression of the EGFr occurred steadily during the period of the experiment, whereas cPLA\textsubscript{2} expression decreased more abruptly at 12 days of oxidative stress. Preliminary results of cell lysates blotted for their cytoplasmic component showed a decrease in EGFr with time spent in hyperoxia (not shown), this agrees with the report that oxidative stress causes an inhibition of EGFr internalisation (de Wt et al., 2000). The most dramatic effect was the up-regulation of c-fos protein expression, which is most striking at 9 and 12 days of culture in hyperoxia.

**MAPK Family**

The MAP kinases are a multigene family of dual specificity protein kinases involved in many signalling pathways. Members of the p38 and Erk subgroups of this family are directly implicated in the activation of other proteins including c-fos and cPLA\textsubscript{2}. Some members of the MAPK family become activated by ROS. For example, addition of H\textsubscript{2}O\textsubscript{2} to endothelial cells has been shown to activate Erk1 and Erk2 (Samanta et al., 1998) which in turn have been suggested to be important for long-term cell survival (Wang
et al., 1998). Interestingly, synthesis of arachidonic acid was also increased though this was not blocked by addition of a MAPK inhibitor (Samanta et al., 1998). In another study, an increase in phosphorylated p38 MAPK was produced on EGF treatment after a preincubation in H$_2$O$_2$, yet there was no increase in EGF-mediated phosphorylation of Erk1/2 after the same preincubation (Zhang and Jope, 1999).

![Western blot images showing phosphorylation of p38 and Erk2](image)

Figure 37. Modulation of Erk and p38 phosphorylation by oxidative stress. HUVECs were grown in 40% O$_2$ for 12 days, protein was extracted from the cells into lysis buffer at 3 day intervals. The top two panels show western blots of phospho-p38 and p38. The bottom two panels show comparable blots of p-Erk and Erk2. Each gel was loaded with 40 µg of protein per track and bands were visualised by alkaline phosphatase with western blue. The blots shown are representative of three separate experiments.

Western blotting results failed to reveal any major changes in expression or phosphorylation of p38 or Erk2 members of the MAP kinase family (figure 37). Although there may appear to be slight fluctuations in some bands the results are not thought to be conclusive, but there may be some upregulation of p38 MAP kinase expression.

### 5.3 Protein tyrosine phosphorylation

Reactive oxygen species are thought to act as second messengers and activate both tyrosine and serine/threonine kinases. There are suggestions that tyrosine phosphorylation is involved in part of the signalling mechanism that leads to translocation of annexins to the nucleus from the cytoplasm (chapter 4). Studies of nuclear translocation of annexin 5 in a human osteosarcoma cell line has been reported to be blocked by the tyrosine kinase inhibitor genistein (Mohiti et al., 1997). As annexins 1, 4, 5 and 6 all move into the nuclei of oxidatively stressed HUVECs, the
tyrosine phosphorylation of proteins in oxidatively stressed cells was investigated (figure 40).

Figure 40. Anti-phosphotyrosine western blot of cell lysates from HUVECs exposed to hyperoxia.
Cells were grown in 40% O₂ for 12 days, protein was extracted from the cells into lysis buffer at 3 day intervals. 50 μg of protein was loaded per track. The positions of molecular mass markers, given in kDa are indicated. Results were visualised by ECL. The blot shown are representative of three separate experiments.

These results show that culture in 40% O₂ produces an increase in intensity of a band at around 120-130 kDa and the emergence of a tyrosine phosphorylated protein from 3 days of culture at around 35-40 kDa. It is interesting to note that extracts of wounded HUVEC monolayers (when compared with intact monolayers), showed by anti-phosphotyrosine western blotting an increase of a band at 120-130 kDa. This was suggested to be pp125FAK as immunoprecipitates of this protein showed increased phosphorylation in wounded cells (Romer et al., 1994).

Annexin 1 has been identified as a substrate for phosphorylation on tyrosine by the EGFr kinase (Fava and Cohen, 1984). Annexin 5 is generally thought not to be tyrosine phosphorylated as it does not contain any tyrosine residues located within the appropriate consensus sequences. However there is one report where annexin 5
has been reported to become phosphorylated by the vascular endothelial growth factor receptor (Wen et al., 1999).

The additional band that appears on the blot at between 35-40 kDa during oxidative stress was shown not to be annexin 1, as immunoprecipitation studies showed that although the protein is tyrosine phosphorylated in control cells, the level of phosphorylation does not change over a time course of hyperoxia (not shown).
CHAPTER 6: DISCUSSION
Vascular endothelial cells play a vital role within the vasculature, and changes in the behaviour of these cells are widely believed to be of fundamental importance in the development of cardiovascular disease. Although there are many primary effectors that lead to endothelial dysfunction, oxidative stress appears to be responsible for the cell pathology in many cases. Of the many consequences of oxidative stress, changes in cell signaling, and in particular in calcium handling and homeostasis, have been extensively reported in the literature. The importance of calcium in vascular endothelial cell function, and the fact that annexins are implicated in cardiovascular disease prompted the studies in this thesis.

Most studies into the effects of oxidative stress on vascular endothelial cells have relied on the short term application of potent oxidants such as t-BOOH or hydrogen peroxide. However, in preliminary studies (not shown) to assess the suitability of this approach, these agonists elicited rapid cell blebbing and then cell death, within hours of being added to the cells. Furthermore, it is unlikely that acute doses of such powerful oxidants would reliably reproduce any of the normal cell pathophysiology that occurs over decades in human disease. For these reasons, an alternative approach was devised, in which HUVECs were cultured in 40% O\textsubscript{2} over several weeks. Clearly, a few weeks of oxidative stress is unlikely to replicate the pleiotropic effects of years of mild oxidative stress, but it was reasoned that this time period should be sufficient to permit more gradual effects, such as changes in gene expression.

### 6.1 Endothelial morphology

HUVECs harvested from freshly obtained umbilical cords were identified by staining for von Willebrand factor. Following two or three rounds of passage, cultures were transferred to a custom-built hyperoxic incubator for a further 12 days. During this period the cells lost their classic 'cobblestone' morphology, gradually becoming more sparse in appearance and also markedly thin and elongated. Morphological changes have been reported elsewhere in HUVECs cultured in 65% and 90% O\textsubscript{2}, where it was shown that hyperoxia initially caused cells to enter S-phase, then G\textsubscript{2}M, and ultimately a total cessation of cell proliferation (Bjerkvig et al., 1992). Interestingly, annexin 1 becomes phosphorylated on tyrosine residues during S-phase in HUVECs (Patte and Blanquet, 1992a), whilst annexin 6 becomes phosphorylated in the late G1 phase of the cell cycle in HUVECs (Patte and Blanquet, 1992b). In another report, annexin 6 was shown to undergo a cell cycle dependent post-translational modification during mitosis in Swiss 3T3 fibroblasts (Moss et al., 1992). There are also reports that annexin 2 expression may be influenced by the cell cycle (Chiang et al., 1993) and
that several annexins exhibit cell density dependent modulation of expression (Schlaepfer and Haigler, 1990).

Elongation of endothelial cells, similar to that seen in these studies, has been reported in response to shear stress (Girard and Nerem, 1995), and a similar morphology was induced by a conditioned medium believed to contain a combination of tumour necrosis factor-α and interleukin-1 (Heffernan et al., 1994). These observations prompted the idea that endothelial cell elongation induced by oxidative or other stresses, may be mediated at least in part by changes in cytokine-mediated cell signalling. In a similar approach to that used in this thesis, increased spreading was reported in microvascular endothelial cells grown in 40% O₂ for one week (D’Amore and Sweet, 1987). In this study cells were shown to be irreversibly growth arrested after 6 days in culture, possible via a mechanism involving protein kinase C. PKC is believed to be activated by oxidative stress, since experiments using specific inhibitors have shown that PKC is required for peroxide mediated activation of Erks (Clerk et al., 1998), and studies of cell cycle in endothelial cells have shown G2 arrest to be a consequence of (indirect) negative regulation by PKC of cdk2 activation (Thomas et al., 1998; Kosaka et al., 1996).

As cell morphology is largely determined and regulated by the actin cytoskeleton, oxidatively stressed HUVECs were examined by immunofluorescent staining of polymerised actin. Moreover, actin has been shown to play an important role in regulating the permeability of endothelial cell monolayers. Treatment of endothelial cells with hydrogen peroxide has been observed to lead to the disappearance of peripheral actin banding (Liu and Sondqvist, 1995). Here, in cells grown in 40% O₂ for 12 days, the banding of F-actin was still visible, and there may even have been a slight increase in the prevalence of actin stress fibres in oxidatively stressed cells - which would be consistent with the cells acquiring a more elongated morphology. A similar increase in the number of actin stress fibres has been reported to be a consequence of the addition of thrombin to endothelial cells, and was accompanied by diminished peripheral actin banding (Ehringer et al., 1999). These authors also reported that histamine had a similar effect on stress fibres but no effect on peripheral actin banding, suggesting that histamine stimulation more closely mirrors the effects of oxidative stress. Whatever mechanism underlies the changes in morphology and actin cytoskeleton it is perhaps not surprising that such changes occur. Oxidative stresses, such as hydrogen peroxide, are known to activate components of the stress response pathway including p38 MAP kinase, which in turn is linked to cdc42 and rac1, known effectors of actin polymerisation (Huot et al., 1997). Nevertheless, experiments in this thesis failed to identify any activation of p38.
MAP kinase, so the observed cytoskeletal and morphological changes may have been
the consequence of other signalling pathways.

The decrease in cell density observed here, could either have been due to cell
shrinkage and elongation, or ultimately cell death and detachment from the culture
dish. Indeed, at time points beyond 12 days cell death was clearly increasingly
responsible for the apparent ‘thinning’ of the cell population (results not shown).
However, adhesion molecules such as VCAM-1 (Marui et al., 1993) have been
shown to exhibit increased expression during oxidative stress, so loss of
adhesiveness is unlikely to be a major cause of cell disappearance. The mechanism
of cell death induced by oxidative stress is not clear. Both apoptosis and necrosis
have been postulated, the different views perhaps being a consequence of
differences between cell types or culture conditions, or alternative methods for
inducing oxidative stress. Experiments with hydrogen peroxide on rat thymocytes
reported an apparent role for annexin 1 in defining the type of cell death. In the
presence of purified annexin 1 cell death occurred by apoptosis, but in the presence
of anti-annexin 1 antibodies, cell death was by necrosis, with the switch being
suggested to be the result of cPLA2 inhibition by annexin 1, rather than through any
effect on lipid peroxidation (Sakamoto et al., 1996). In endothelial dysfunction there is
typically an increased production of prostaglandins suggesting that if anything, cPLA2
is more active. Extrapolating results from rat thymocytes to human endothelial cells is
risky, but if common mechanisms underlie these sets of observations then the cell
death reported in this thesis is more likely to be necrotic than apoptotic, though this
again is countered by western blot results showing increased expression of certain
pro-apoptotic members of the Bcl2 family. Thus, Bax and Bag-1, which are pro- and
anti-apoptotic Bcl2 family members, increased and decreased respectively during
culture of HUVECs in 40% O2.

Surprisingly, Bcl2 itself and Bad were not detected in these cells, presumably they
were below the levels of detection using the available antibodies. Experiments on
PC12 phaeochromocytoma cells in which apoptosis was induced by hydrogen
peroxide, showed increases in both Bax as well as Bad, with Bcl2 being
undetectable (Maroto and Perez-Polo, 1997). Bax expression was also reported to
increase in endothelial cells undergoing apoptosis induced by thrombospondin-1, with
a concomitant reduction in the expression of Bcl2 (Nor et al., 2000). It should be noted
that although Bad was not detected in studies in this thesis, it has been reported in
endothelial cells (Hermann et al., 2000).
Collectively, the results obtained in these studies, using this model of oxidative stress, are in approximate agreement with reports elsewhere. To summarise, culture of HUVECs in 40% O\textsubscript{2} leads to a loss of proliferative capacity within three to six days, during which time the cell morphology remains fairly constant. After six days the cells become increasingly elongated and might have an increased tendency towards apoptosis, and from twelve days onwards cell death occurs.

### 6.2 Annexin expression

Oxidative stress is well known to induce changes in the level of expression of numerous genes in many different cell types. In endothelial cells ICAM-1 and VCAM-1 have been reported to become elevated (William et al., 1999), though western blot analysis of ICAM-1 in this thesis gave inconclusive results (not shown). Other reports have implicated annexins in the cellular response to oxidative stress. A plant annexin (showing particularly high similarity to human annexins 1, 3, 6 and 7) was identified in a screen for proteins conferring resistance to peroxide in E.coli (Gidrol et al., 1996). In support of this, the purified isolated annexin was found to have peroxidase activity, and areas of Arabidopsis thaliana exposed to H\textsubscript{2}O\textsubscript{2} had higher levels of expression of this protein. As yet no mammalian annexins have been shown to possess this kind of activity, or indeed enzyme activity of any sort. Mammalian annexins 1 and 2 have both been identified as being modulated by oxidative stress. Annexin 2 was shown to increase in response to oxidative stress in rat renal carcinoma cells (Tanaka et al., 2000), and annexin 1 was reported to be elevated at both the protein and mRNA levels in A549 and HeLa cells following exposure to H\textsubscript{2}O\textsubscript{2}. Concomitant with this increase, the protein also relocated from being predominantly cytosolic to having a nuclear and perinuclear location (Rhee et al., 2000). These results conflict with other findings in which oxidative stress induced physiologically through application of tumour necrosis factor-\(\alpha\), failed to enhance annexin 1 expression in the murine L929 cell line, though expression of SOD was increased (Polla et al., 1996).

In this study, changes in annexin gene expression during exposure of HUVECs to hyperoxic conditions, were evaluated by western blotting and RT-PCR analysis of mRNA. The results of such analyses failed to identify any significant changes in expression for annexins 1, 2, 3, 4, 5, 6 and 7. From the PCR analysis it was found that annexin 11 is also expressed in HUVECs but not annexins 8 or 13, but it is not known whether the other two human annexins (A10 and annexin 31) are expressed in HUVECs, though the generally restricted pattern of expression of these annexins suggests that this is unlikely. The RT-PCR analysis revealed some differences in yield of PCR products between control and oxidatively stressed cells for some of the
annexins. From this type of experiment it is difficult to say whether this means there are changes in mRNA levels but it suggests that there may be some interesting differences, but a more sensitive quantitative experiment should be used to address this question, such as a ribonuclease protection assay. However, changes in protein levels are not the only mechanism of regulation of protein activity. Thus, protein activity may be regulated either by post-translational modifications such as phosphorylation, or by changes in intracellular localisation. Instances of this type are not detectable by proteomic or DNA chip approaches and are therefore more difficult to identify and assess. To further investigate any possible relationship between oxidative stress and HUVEC annexins, a systematic analysis was undertaken to reveal the effects of oxidative stress on the intracellular localisation of annexins, and the responses of annexins to various physiological and pharmacological agonists.

6.3 Annexin localisation

A defining property of annexins is their ability to bind Ca^{2+}-dependently to negatively charged phospholipids, a feature reflected in vivo by their translocation from a cytosolic to a membrane-bound configuration following a rise in [Ca^{2+}]. In this study, annexins 1 and 2 were observed to partially translocate from the cytosol to the plasma membrane region of HUVECs. For annexin 1 this occurred during oxidative stress, and also in control cells exposed to Ca^{2+}-mobilising agonists such as thapsigargin and ionomycin. These reagents also stimulated the movement of annexin 2 from cytosol to plasma membrane although this was less noticeable with EGF stimulation, but oxidative stress alone had no effect on the intracellular distribution of annexin 2. These findings are consistent with studies in other cell types. For example, in anterior pituitary cells annexins 1 and 2 were reported to associate with the plasma membrane where they were suggested to be involved in exocytosis (Turgeon et al., 1991), and annexin 1 was also found associated with secretory granules. Raynal et al., (1992) also reported annexin 1 to be associated with the plasma membrane of endothelial cells.

Annexin 2 is unusual in being able to bind to membranes in both a Ca^{2+}-dependent and Ca^{2+}-independent manner. Thus, extraction of lung epithelial cell membranes with buffer containing EGTA leaves a substantial amount of annexin 2 still associated with the membrane (Liu et al., 1997). Similar Ca^{2+}-independent binding of annexin 2 was also reported in adrenal chromaffin cells (Creutz et al., 1987). However, most of the cellular annexin 2 exists as the heterotetrameric form which is largely associated with the subplasma membrane cytoskeleton, with any monomeric annexin 2 being predominantly cytosolic (Thiel et al., 1992). The annexin 2_{2-p11_2} heterotetramer has
been shown to require less Ca\textsuperscript{2+} than the monomer for membrane association (Evans and Nelsestuen, 1994). The tetrameric complex has been suggested to be required for regulated exocytosis in endothelial cells (Konig et al., 1998), as microinjection of synthetic peptides corresponding to the region of the N-terminal domain involved in complex formation was shown to alter membrane conductance during Ca\textsuperscript{2+}-dependent exocytosis. An additional role for annexin 2 was suggested in endothelial cells, with the report that the annexin 2 complex was required for the normal activity of volume activated chloride channels (Nilius et al., 1996).

In all experiments in this study in which either or annexins 1 and 2 were shown to translocate to the plasma membrane, the movement could be associated with a rise in [Ca\textsuperscript{2+}], perhaps with the exception of oxidatively stressed cells in which annexin 1 was constitutively more closely associated with the plasma membrane, but in which resting [Ca\textsuperscript{2+}] is in any case somewhat elevated. Whilst it is impossible to say what effects on endothelial cells, if any, might result from an increased presence of annexins 1 and 2, changes in cytoskeletal function, exocytosis or growth factor signalling are all feasible. Indeed, in endothelial dysfunction associated with atherosclerosis, endothelial cells increase rates of synthesis and release of various physiological mediators (see section 1.1), supporting the idea that pro-secretory mechanisms might be enhanced by oxidative stress.

**Nuclear localisation**

Perhaps the most striking effect of oxidative stress on endothelial cell annexins was the increased tendency of annexins 1, 4, 5 and 6 to acquire a nuclear localisation. Annexin 1 moved into the nucleus of HUVECs only in response to stimulation with ATP, an effect that was enhanced in oxidatively stressed cells. There has been a previous report describing annexin 1 in the nuclei of control unstimulated endothelial cells (Raynal et al., 1992), which is slightly at odds with the observation here that significant nuclear staining of annexin 1 was actually seen in only a very small proportion of control HUVECs. This may have been due to the use of HUVECs in this study and bovine aortic ECs by Raynal et al., or it could be a difference produced by the substrate that the cells were grown on, the HUVECs were grown on gelatin where this other group used fibronectin. Different coatings on dishes used to grow ECs are known to lead to some changes in localisation of some proteins. The fact that annexin 1 did not relocate to the nucleus following exposure of HUVECs to a Ca\textsuperscript{2+} ionophore indicates that a simple rise in [Ca\textsuperscript{2+}] is insufficient to elicit this response. In contrast, ATP stimulation of HUVECs, which causes both a transient rise in [Ca\textsuperscript{2+}], in addition to activation of intracellular signalling pathways, was much more effective in driving annexin 1 into the nucleus.
Unlike annexin 1, annexin 5 required only oxidative stress to induce nuclear expression, with the proportion of cells having nuclear annexin 5 increasing with culture time in 40% O$_2$. In control HUVECs, both Ca$^{2+}$ ionophore and stimulation with EGF led to nuclear translocation of annexin 5. Studies elsewhere have reported annexin 5 to be located in both nucleus and cytosol in HUVECs (Sun et al., 1992), with expression in the nucleolus, but this was not observed in oxidatively stressed cells in this study, as judged by confocal microscopy. In human neuroblastoma cells annexin 5 was reported to be in the nucleus, and to translocate to the nuclear membrane on cell stimulation (Blanchard et al., 1996). This study produced different results to those observed in this thesis but here there are differences in culture media and permeabilisation techniques, which may possibly lead to the appearance of such differences.

In oxidatively stressed HUVECs, further stimulation failed to change the location of annexin 5. Interestingly, in control HUVECs ionomycin but not thapsigargin stimulated the translocation of annexin 5 from cytosol to nucleus, indicating that the magnitude and perhaps the kinetics of the intracellular Ca$^{2+}$ rise might be important factors in determining the response of annexin 5. EGF treatment of control HUVECs, which generates a smaller rise in [Ca$^{2+}$], than thapsigargin but which stimulates intracellular protein kinases, also caused the nuclear translocation of annexin 5. Studies elsewhere reported that translocation of annexin 5 to the nucleus of a human osteosarcoma cell line was blocked by inhibition of tyrosine phosphorylation (Mohiti et al., 1997). Collectively, these studies suggest the subcellular localisation of annexin 5 in HUVECs is regulated by multiple factors including protein phosphorylation, oxidative stress and intracellular [Ca$^{2+}$].

Oxidative stress did not have any discernable effect on the localisation of annexin 6 in unstimulated cells, but did change the responses of annexin 6 to certain pharmacological agents. In control HUVECs annexin 6 translocated from the cytosol to the nucleus in response to stimulation with ionomycin and EGF. Following oxidative stress, cells also exhibited annexin 6 nuclear translocation after treatment with thapsigargin. The difference between control and oxidatively stressed cells with regard to the thapsigargin response, may be due to the somewhat elevated resting [Ca$^{2+}$], in the latter, or it could be because the thapsigargin response in oxidatively stressed cells occurs against a background of altered protein phosphorylation and perhaps gene expression. Certainly, this is not the first description of annexin 6 in the nucleus, as both smooth muscle cells and endothelial cells have been reported to express nuclear annexin 6 (Doubell et al., 1993).
How annexins get into the nucleus, and what function they might have there is far from clear. It has been suggested annexins might translocate into the nucleus by active transport through nuclear pores, due to the presence of nuclear localisation signals (Burke, 1990). Annexin 2, although not detected in the nucleus of HUVECs in this study has been identified in the nucleus in HeLa cells where it was proposed to bind to the nuclear matrix (Kumble et al., 1992b). It is possible that annexins bind to cytoskeletal proteins in the nucleus in a similar manner as they do in the cytosol. Within the nucleus, annexin 2 has been suggested to act as a primer recognition protein, acting as a co-factor for DNA polymerase-α, possibly having a role in lagging strand DNA replication (Vishwanatha et al., 1992) though this is highly speculative.

Punctate staining
ANNEXINS 4 (FIGURE 23), 5 (FIGURE 24) AND 7 (FIGURE 34) SHARE A SIMILAR PUNCTATE STAINING PATTERN. THIS PUNCTATE STAINING PATTERN DID NOT CO-LOCALISE WITH VWF, AND THE PATTERN OF STAINING OF ANNEXIN 5 EXTENDED INTO THE NUCLEI OF HUVECs. THIS CURIOUS OBSERVATION SUGGESTS THAT ANNEXIN 5 IS UNLIKELY TO EXHIBIT THIS PATTERN BY ASSOCIATION WITH VESICLES. CLOSER OBSERVATION USING THE HIGHEST MAGNIFICATION ON THE CONFOCAL MICROSCOPE DID NOT REVEAL ANY ADDITIONAL DETAIL ABOUT THIS PUNCTATE PATTERN. ONE POSSIBILITY IS THAT THE PUNCTATE STAINING IS ARTEFACTUAL, BEING CAUSED BY FORMATION OF AGGREGATES OF THE PROTEIN, ALTHOUGH IT COULD BE BOUND TO SMALL INTRACELLULAR STRUCTURES. FURTHER INVESTIGATION IS REQUIRED TO REVEAL THE REASONS FOR THIS STAINING PATTERN.

Localisation conclusions
Several of the localisation results from this project suggest that the nuclear translocation of annexins requires a phosphorylation event as well as a rise in [Ca^{2+}], and that oxidative stress itself may be sufficient to trigger this event. Although there is no set pattern, it does appear that protein phosphorylation plays a part in signalling cascades that lead to some annexins translocating to the nucleus. Other groups have tried to clarify similar observations and have utilised phosphotyrosine inhibitors such as genistein, for example in the case of annexin 5 translocation to the nucleus of an osteoscarcoma cell line (Mohiti et al., 1997). In this study, genistein was used to try to block annexin 1 movement into HUVEC nuclei, but the results were inconclusive as genistein itself was observed to cause translocation of annexin 1 to the nucleus. The time period of incubation in genistein was different to that used in the reported work for annexin 5. In the early stages, genistein addition may cause the cells to become transiently activated in some way, and that further time is necessary to allow cells to return to a resting state before performing the experiment.
6.4 Cell signalling

Changes in transmembrane signalling mechanisms are widely believed to be early events associated with endothelial dysfunction. In this project calcium signalling, the EGF signalling pathway and tyrosine phosphorylation were investigated to try and provide some insight as to events occurring within HUVECs that may indirectly effect annexins.

Calcium signalling

The majority of annexins are Ca⁡2⁺-binding proteins, and as such this makes it important to consider the effects of hyperoxia on Ca⁡2⁺-signalling, but Ca⁡2⁺ signalling may also have downstream effects on annexins. At neutral pH annexin 5 has been reported to insert into vesicle membranes in the presence of H₂O₂. This is of particular interest as some annexins, and in particular annexin 5, have been suggested to have roles as Ca⁡2⁺ channels. This study went on to show that in DT40 cells annexin 5 is required for H₂O₂ induced Ca⁡2⁺ influx (Kubista et al., 1999). This response of annexin 5 is especially interesting as the findings in chapter 5 for Ca⁡2⁺ signalling indicate that oxidatively stressed (but otherwise unstimulated) HUVECs may have a higher basal [Ca⁡2⁺] level. Despite this, stimulation with pharmacological reagents or agonists produced equal or smaller Ca⁡2⁺ responses in oxidatively stressed cells compared with control cells.

Most work to investigate the modulation of EC Ca⁡2⁺ signals by oxidant stress has been done using H₂O₂, xanthine/xanthine oxidase or t-BOOH. After exposure of endothelial cells to 40% O₂, the basal Ca⁡2⁺ level was increased. Similar results have also been reported by other groups, where oxidative stress generated by xanthine/xanthine oxidase in HUVECs caused the resting intracellular levels of Ca⁡2⁺ to rise from 90-100 nM to 500nM (Geeraerts et al., 1991), the increase suggested to be due to a release of Ca⁡2⁺ from internal stores. Other experiments using this system of oxidative stress have shown that the rise in Ca⁡2⁺ can be reversed on addition of catalase (a H₂O₂ scavenger) (Saeki et al., 2000). In contrast, hyperoxic exposure of rat alveolar macrophages was reported not to cause an increase in basal Ca⁡2⁺ levels (Forman et al., 1986).

Changes in [Ca⁡2⁺], induced by thapsigargin were the same in control and oxidatively stressed HUVECs. This indicates that the filling state of intracellular stores in the ER of oxidatively stressed HUVECs is normal. However, ratiometric measurement of intracellular Ca⁡2⁺ levels on addition and re-addition of serum to HUVECs showed that there may be some alteration in the capacity of intracellular stores to refill, as unlike
control cells, oxidatively stressed cells failed to respond to readdition of serum. These results indicate that the stores appear to be able to empty normally but that subsequent replenishment of Ca\(^{2+}\) may be delayed in oxidatively stressed cells. In support of this idea, treatment of rat alveolar macrophages with concavalin A was found to elicit a reproducible rise in [Ca\(^{2+}\)]\(_i\) in control cells upon readdition of agonists, but not in cells that had been exposed to hyperoxia (Forman et al., 1986). Certainly, annexins have been suggested to modulate Ca\(^{2+}\) release from stores. For example, annexin 1 may mediate Ca\(^{2+}\) release from intracellular stores. Cells transfected with sense or antisense annexin 1 showed changes in Ca\(^{2+}\) signalling suggested to be related to IP\(_3\) sensitive intracellular stores, whereas increased levels of annexin 1 attenuated a stimulus-evoked Ca\(^{2+}\) response (Frey et al., 1999). Annexin 6 has been shown to modify gating of Ca\(^{2+}\) release channels of the sarcoplasmic reticulum by increasing their opening probability and opening time (Diaz-Munoz et al., 1990).

Experiments in which ECs were stimulated with bradykinin and ATP, showed that the Ca\(^{2+}\) responses produced by both agonists were attenuated by oxidative stress generated by t-BOOH (Schilling and Elliott, 1996). Consistent with this, stimulation of HUVECs by ATP produced a diminished Ca\(^{2+}\) response after culture in 40% O\(_2\) for 12 days. Stimulation of control HUVECs with pervanadate yielded a biphasic response, whereas HUVECs that had been cultured in hyperoxic conditions showed a quicker onset of the first stage of the signal. Pervanadate is known to cause an increase in [Ca\(^{2+}\)]\(_i\), probably by stimulating protein tyrosine phosphorylation and subsequent formation of IP\(_3\) (Teshima et al., 1994). Experiments designed to explore the roles of protein tyrosine kinases and phosphatases showed that changing the levels of phosphotyrosine had profound effects in Ca\(^{2+}\) signalling on endothelial cells. In ECs tyrosine phosphatase inhibitors were found to produce increased levels of [Ca\(^{2+}\)]\(_i\), which could be attenuated by inhibition of tyrosine kinases (Fleming et al., 1996).

**Protein phosphorylation**

Oxidative stress is known to lead to the initiation of phosphorylation events. Considerable work has been done to investigate the involvement of tyrosine phosphorylation and the activity of tyrosine kinases in cells that have been treated with H\(_2\)O\(_2\). Some studies have shown that peroxide induced tyrosine phosphorylation in ECs, leading to a rise in [Ca\(^{2+}\)]\(_i\), (Flemming et al., 1996), an effect that was attenuated on inhibition of the tyrosine kinases. This work is backed up by the observations of another group showing release of Ca\(^{2+}\) from intracellular stores being reduced by up to 70% by addition of genistein to ECs (Sharma and Davis, 1996). These results suggest that phosphotyrosine levels may influence Ca\(^{2+}\) signalling in ECs. As is suggested by the annexin translocation data (Chapter 4) and the anti-
phosphotyrosine blot (Chapter 5.3), oxidatively stressed cells may already show higher basal levels of phosphotyrosine, and this may therefore contribute to a faster Ca^{2+} response on addition of pervanadate.

H_{2}O_{2} has been reported to stimulate increased tyrosine phosphorylation of many proteins in many different cell types. In lymphocytes, tyrosine phosphorylation of STAT3 is induced on treatment with H_{2}O_{2} (Carballo et al., 1999). Studies of EC monolayers showed increased tyrosine phosphorylation of multiple unidentified proteins along with increased permeability of the endothelial monolayer, both of which were prevented by addition of genistein (Carbajal and Schaeffer, 1998). Another group showed the PDGF receptor and the cytoplasmic tyrosine kinase pp60^{src} to be among proteins phosphorylated on treatment with H_{2}O_{2} in mesangial cells (Gonzalez-Rubio et al., 1996). Phosphorylation of both proteins was prevented by pre-treatment of the cells with catalase (see section 1.3.2).

In experiments conducted in this project, two major proteins of M, 120-130 and 35-40 kDa were seen to have increased tyrosine phosphorylation in HUVECs exposed to hyperoxia. Neither of these proteins were identified, but the larger protein is similar in size to pp125FAK, which becomes phosphorylated in mechanically wounded HUVEC monolayers (Romer et al., 1994). Blockade of Ca^{2+} influx in HUVECs is known to prevent phosphorylation of p125FAK, a focal adhesion protein kinase important in cell motility (Alessandro et al., 1998). If the upper band is pp125FAK, the basal rise in Ca^{2+} in oxidatively stressed cells may be enough to allow for the phosphorylation of this protein. Interestingly, phosphorylation of p125FAK leads to increased actin stress fibre formation (Abedi and Zachary, 1997), which would be consistent with reports elsewhere in oxidatively stressed cells (see section 3.2.1).

Some annexins are well established as substrates for serine/threonine and tyrosine kinases. The results from the translocation experiments (Chapter 4) suggest that phosphorylation events in oxidatively stressed HUVECs may lead to the enhanced translocation of some annexins to the nucleus. Annexin 2, which demonstrated plasma membrane translocation on oxidative stress, is suggested to be involved in the secretory granule to membrane fusion process in exocytosis of chromaffin cells as annexin 2 pre-phosphorylated by PKC and then added to chromaffin cells either with depleted PKC activity or into cells in the presence of a phosphatase inhibitor, led to induction of secretion (Sarafian et al., 1991). Annexin 5 was observed to translocate to platelet plasma membranes in response to inhibition of protein phosphatases, whilst inhibition of protein kinases prevented this redistribution of annexin 5 (Trotter et al., 1997). In other work, genistein prevented the relocation of annexin 5 to nuclei (Mohiti
et al., 1997). In this case, the effect is probably indirect as annexin 5 is not believed to be a protein kinase substrate itself, although there has been one report of annexin 5 becoming phosphorylated on stimulation of ECs by vascular endothelial growth factor (Wen et al., 1999). Annexin 1, which is phosphorylated by the EGFr kinase (Fava and Cohen, 1984), was investigated by immunoprecipitation to examine potential changes in phosphotyrosine content. Annexin 1 did not exhibit any changes in expression level or in phosphorylation status throughout the period in culture in hyperoxic conditions. This result suggests that, at least in the case of nuclear translocation of annexin 1, any tyrosine phosphorylation events that may be involved are likely to affect other proteins, and that these may exert an indirect effect on annexin 1.

**EGFr signalling pathway**

Part of the work in this project focused on aspects of the EGFr signalling pathway in HUVECs that had been oxidatively stressed. The level of expression of the EGFr was found to decrease during culture in 40% O₂, and preliminary results of the cell cytoplasmic fraction showed a more striking decrease in EGFr. These data agree with the previous discovery that receptor internalisation is inhibited in fibroblasts treated with H₂O₂ (de Wit et al., 2000).

Another protein that exhibited decreased expression during oxidative stress was cPLA₂, although here the change was not observed until 12 days of hyperoxia. Oxidant stress generated by UVB was reported to increase cPLA₂ activity, and also to increase its expression in epidermis (Chen et al., 1996). The results here, showing a decrease in cPLA₂ expression in HUVEC during oxidative stress may differ from other reports because of the experimental model of oxidant stress or the cell type investigated. Another report contrasts the UVB study, with the results showing that in cells treated with t-BOOH the level of cPLA₂ activity remain unchanged (Akiba et al., 1997).

Activation of the EGFr leads to signal transduction events that culminate in the activation of the early response gene c-fos. During oxidative stress c-fos demonstrated a large increase in expression. A similar increase has been reported by other groups that have investigated oxidative stress. For example, one group have shown c-fos mRNA expression to increase in a rabbit lens cell line in response to treatment with H₂O₂ (Li and Spector, 1997). Shear stress of HUVECs (known to generate ROS) also produced an increase in c-fos expression that was shown to be decreased by catalase (Hsieh et al., 1998).
Some members of the MAP kinase family are involved in the EGFr signalling cascade, though during investigation of expression and activation of p38 MAP kinase and Erk2, no clear changes in either of these proteins was observed during hyperoxia in HUVECs. Activation of Erk1, Erk2 and p38 has been reported in response to treatment of myocytes with H$_2$O$_2$ (Clerk et al., 1998). Activation of p38 by H$_2$O$_2$ has been associated with mitotic arrest at low levels of oxidative stress, in both cases the effects were reversed by glutathione precursor (Kurata Si, 2000). Interestingly, the activity of cPLA$_2$ is thought to be linked with activation of Erk, as inhibitors of cPLA$_2$ activity are able to prevent activation of Erk by H$_2$O$_2$ (Tournier et al., 1997). Full activation of cPLA$_2$ activity is known to require phosphorylation by p42 MAP kinase (Sa et al., 1995). The apparent absence of any changes in the expression or activation of these proteins may have been due to the more chronic exposure to oxidative stress used here.

6.5 Summary

Oxidative stress generated by hyperoxia has many effects on HUVECs. From this study it has become clear that the main effect on annexins is manifested as changes in localisation, with annexin 5 showing the most dramatic response. It is possible that annexin 5 may have a protective role in HUVECs during oxidative stress, but it is not known how it enters the nucleus or what function it performs there. Many of the responses of annexins produced by culture in hypoxic conditions resemble those seen in control cells activated by pharmacological reagents. Addition of these reagents and agonists to oxidatively stressed cells generally had a more potent effect. As suggested from this aspect of the project, phosphorylation appears to be an important factor in mediating the changes seen during oxidative stress. This point is supported by studies of Ca$^{2+}$ signalling where there have been reports of phosphorylation leading to increased [Ca$^{2+}$], as was seen here in oxidatively stressed HUVECs. This project has also shown increased tyrosine phosphorylation in oxidatively stressed cells but curiously did not show changes in phosphorylation of p38 MAPK and Erk2, although this has been reported during oxidative stress by other groups. ECs are known to adapt to oxidative stress in many different ways via changes in protein expression, signalling events etc. The changes in annexin localisation reported in this project may therefore be an adaptive response of HUVECs to oxidative stress.

Much work remains to be done to clearly explain the findings of this study. The punctate staining patterns for annexins 4, 5 and 7 need to be resolved, as do the mechanisms by which annexins move into the nucleus, why this is more likely for
some annexins in oxidative stress, and in what way the relocation of annexins affects the cells. To help address these questions it would be advantageous to determine the identity of the tyrosine phosphorylated proteins that appear during hyperoxia, as these proteins may have roles in signalling pathways that lead to nuclear translocation of annexins.

Much work has been published on annexin 2 on the cell surface of ECs, and it would also be interesting to see if the levels of cell surface annexin 2 change in oxidative stress and EC dysfunction, as such changes may help explain the appearance of a more thrombogenic surface in cardiovascular disease.

In conclusion, the findings of this project therefore leave many questions unanswered but show that annexins exhibit differential behaviour in HUVECs exposed to hyperoxia. If annexins do have roles to play in endothelial homeostasis, then further research of annexin behaviour in EC dysfunction will be essential to our understanding of mechanisms involved in health and disease of the vascular endothelium.
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