Hemodynamic Regulation of Occludin and ZO-1: Effect on Barrier Function

A dissertation submitted for the degree of Ph.D

By

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Declaration

I hereby certify that this material which I now submit for assessment on the program of study leading to the award of Doctor of Philosophy is entirely my own work and has not been taken from the work of others save and to the extent that such work has been cited and acknowledged within the text of my own work.

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Date: 7/09/06

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Abstract

The vascular endothelium constitutes a highly effective fluid and solute barrier through the regulated apposition of tight junction protein complexes between adjacent endothelial cells. As endothelial cell-mediated functions and pathology are sensitive to hemodynamic forces (cyclic strain and shear stress), we hypothesised a dynamic regulatory link between endothelial tight junction assembly/function and hemodynamic stimuli. We have investigated this hypothesis via examination of the precise effects of cyclic strain on the expression, modification, and function of occludin and ZO-1, pivotal components of the tight junction complex. Moreover, the mechanotransduction pathway by which tight junction regulation occurs was also investigated.

For these studies, cultured bovine aortic endothelial cells (BAECs) were subjected to physiological levels of equibiaxial cyclic strain (0 or 5% strain, 60 cycles/min, 24 h).

In response to strain, protein expression of both occludin and ZO-1 increased. Increased mRNA levels were also observed for occludin, but not ZO-1. These changes were accompanied by tyrosine dephosphorylation of occludin and serine/threonine phosphorylation of ZO-1, modifications that could be completely blocked by pharmacological inhibition with dephostatin (tyrosine phosphatase) and rottlerin (protein kinase C), respectively. The effects of cyclic strain on the association and subcellular localisation of occludin and ZO-1 was also investigated. In response to cyclic strain we observed a significant increase in endothelial occludin/ZO-1 co-association in parallel with increased localisation of both proteins to the cell-cell border. Moreover, these events were completely blocked by dephostatin and rottlerin and were accompanied by a strain–dependent reduction in transendothelial permeability to FITC-dextran, an event that could also be blocked by inhibitor addition indicating a causal relationship between biochemical changes and barrier function.

We observed that these modifications of the tight junction following strain were mediated via Gi-proteins, the small GTPase Rac-1, and the signaling molecule p38.

Overall, these findings indicate that physiological cyclic strain up-regulates vascular endothelial tight junction assembly, with subsequent consequences for barrier integrity, putatively via tyrosine phosphatase- and PKC-dependent modification of occludin and ZO-1. The signal is transduced via G-proteins, Rac-1 and p38.

Abbreviations

BAEC	Bovine Aortic Endothelial Cell
BCA	Bicinchoninic Acid
BSA	Bovine Serum Albumin
CACO-2	Human Colorectal Adenocarcinoma Cell Line
cDNA	Complimentary DNA
CVD	Cardiovascular Disease
DAG	Diacylglycerol
DMSO	Dimethylsulfoxide
DNA	Deoxyribose nucleic acid
EC	Endothelial Cell
ECM	Extra Cellular Matrix
EDTA	Ethylenediamine Tetracetic Acid
EGF	Epidermal Growth Factor
EGFR	Epidermal Growth Factor Receptor
eNOS	Endothelial Nitric Oxide Synthase
ERK	Extra Cellular Regulated Kinase
ET-1	Endothelin-1
FAK	Focal Adhesion Kinase
FBS	Fetal Bovine Serum
FGF	Fibroblast Growth Factor
FGFR	Fibroblast Growth Factor Receptor
FD40	Fluorescein Isothiocyanate Dextran 40kda
GAPDH	Glyceraldehyde Phosphate Dehydrogenase
GEF	Guanine Nucleotide Exchange Factor
GFP	Green Fluorescent Protein
GPCR	G-Protein Coupled Receptor
Grb-2	Growth Factor Binding Protein-2
HBSS	Hanks Buffered Saline Solution
HPF	High Power Field
HUVEC	Human Umbilical Vein Endothelial Cells

IB	Immunoblot
ICAM-1	Intracellular Adhesion Molecule-1
IHF	Irish Heart Foundation
ILGF	Insulin Like Growth Factor
ILGFR	Insulin Like Growth Factor Receptor
IP	Immunoprecipitation
JAM-1	Junctional Adhesion Molecule 1
JNK	C-Jun N-Terminal Kinase
LDL	Low Density Lipoprotein
MAPK	Mitogen Activated Protein Kinase
MAPKK	Mitogen Activated Protein Kinase Kinase
MAPKKK	Mitogen Activated Protein Kinase Kinase Kinase
MCP-1	Monocyte Chemo Attractant Protein-1
MDCK	Madin-Darby Canine Kidney Cells
MEK	MAPK Kinase
MMP	Matrix Metalloproteinase
mRNA	Messenger Ribonucleic Acid
NOS	Nitric Oxide Synthase
PAGE	Polyacrylamide Gel Electrophoresis
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PDGF	Platelet Derived Growth Factor
PDGFR	Platelet Derived Growth Factor Receptor
PI-3 Kinase	Phosphoinositide 3 Kinase
PKA	Protein Kinase A
PKC	Protein Kinase C
PMA	Phorbol 12-Myristate 13-Acetate
PTK	Protein Tyrosine Kinase
PTX	Pertussis Toxin
RNA	Ribonucleic Acid
ROCK	Rho Kinase

ROS	Reactive Oxygen Species
RTK	Receptor Tyrosine Kinase
RT-PCR	Reverse Transcriptase Polymerase Chain Reaction
SDS	Sodium Dodecyl Sulphate
Shc	Src Homology/Collagen
siRNA	Small Interfering RNA
SMC	Smooth Muscle Cell
SNP	Single Nucleotide Polymorphism
SSRE	Shear Stress Response Element
TBS	Tris Buffered Saline
TEE	Transendothelial Exchange
TEER	Transendothelial Electrical Resistance
TJ	Tight Junction
TGF	Transforming Growth Factor
TNFα	Tumour Necrosis Factor-Alpha
VEGF	Vascular Endothelial Growth Factor
ZO-1	Zonula-Occludens 1

Units

bp	Base Pairs
cm	Centimeters
cm^2	Centimeter Squared
°C	Degree Celsius
g	Grams
h	Hours
kDa	Kilo Daltons
L	Liter
M	Molar
mg	Miligrams
min	Minute
ml	Mililiter
mm	Milimeter
mM	Milimolar
ng	Nanogram
OD	Optical Density
pM	Picomolar
rpm	Revolution Per Minute
sec	Seconds
U	Enzyme Units
µg	Microgram
µl	Microliter
µm	Micrometer
µM	Micromolar
V/v	Volume per volume
W/v	Weight per volume
V	Volts
W	Watts
x g	G force

Publications and Poster Presentations

Publications

N.T. Collins, O.C. Colgan, Y.A. Birney, G. Ferguson, R.P. Murphy, P.A. Cahill and P.M. Cummins. Cyclic strain-mediated regulation of vascular endothelial tight junction assembly: putative roles for integrins, G-proteins and Rho-GTPase signaling pathways. *Arteriosclerosis, Thrombosis, & Vascular Biology.* 2006; Submitted.

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Chapter 1

Introduction

1.1 Cardiovascular Disease

Cardiovascular disease (CVD) refers to the class of diseases that involve the heart and blood vessels: arteries and veins. Over 50 million Americans have cardiovascular problems, and most other Western countries face high and increasing rates of cardiovascular disease. It is the number 1 cause of death and disability in the United States and most European countries. More than 8,700 people in Ireland died from heart disease in 2004 and the number of people diagnosed with the disease has increased rapidly in recent years and now stands at 25,000 per annum [David Labanyi, Irish Times health supplement 7/3/06]. Ireland is one of the worst countries in the European Union in terms of cardiac disease prevalence and death rates [Dr. Brian Maurer, IHF]. The cost of cardiovascular disease in Europe is estimated to be at €169 billion per annum or €3,724 per capita [David Labanyi, Irish Times health supplement 7/3/06].

It is clear to see that CVD is extremely prevalent in our society today. However much can be done to prevent or limit the damage caused by this disease. There is increased emphasis on preventing CVD by modifying risk factors, such as healthy eating, exercise and not smoking. In fact women's deaths from heart disease in Europe could be halved if they stopped smoking and improved their diet, a major European conference on women's cardiovascular health was told in March 2006. However some risk factors for developing the disease, such as gender, age, family history and ethnicity, cannot be modified. Hemodynamic forces within the vasculature also influence CVD. These forces associated with the flow of blood through the vasculature affect the initiation and progression of CVDs, including atherosclerosis, hypertension and

pathological vascular remodelling [Lusis *et al.*, 2000; Frangos *et al.*, 2001].

1.2 The Endothelium

The vascular endothelium is a dynamic cellular interface between the vessel wall and bloodstream where it regulates the physiological effects of humoral and biomechanical stimuli on vessel tone and remodelling. The endothelial monolayer forms the lining of the entire inner surface of the vascular system from the heart to the capillaries. It is therefore well located to respond to and monitor changes in blood flow and biochemical composition. The single layer of cells that is the endothelium, is not just an inert container for blood but a vital organ whose health is essential to normal vascular physiology and whose dysfunction can be a critical factor in the pathophysiology of diseases, such as hypertension and atherosclerosis .

Blood vessels are active, integrated organs composed of endothelial cells (EC), smooth muscle cells (SMC) and fibroblasts divided into structural layers (See Fig. 1.1). The innermost layer is composed of a single layer of contact inhibited ECs. The tunica intima is a simple squamous epithelium surrounded by a connective tissue basement membrane with elastic fibres. The tunica media is primarily comprised of SMCs, which play a key role in maintaining vascular tone and function. The outermost layer, which attaches the vessel to the surrounding tissue, is termed the tunica adventitia. This is a layer of connective tissue, with varying amounts of elastic and collagenous fibres.

The inner most layer of cells, the endothelial cells, will form the focus of this

study, and in particular the junctions that form between adjacent cells. Because of their structure, location and intercellular arrangement, ECs are able to respond to a wide variety of stimuli and are employed in a number of unique roles within the vasculature, such as maintenance of a semi-permeable barrier that allows specific substances to move between the blood and the interstitium. ECs are also involved in regulation of haemostasis. This involves maintaining a balance between pro- and anti-coagulant forces in the circulation, insuring fluid movement of blood. Another role of the endothelium is to carry out inflammatory responses that involve recruitment of leukocytes, leading to a potentiation of the healing process. The endothelium also serves to regulate vascular tone. The vessel has the ability to respond to changes in the microenvironment to maintain optimal blood flow.

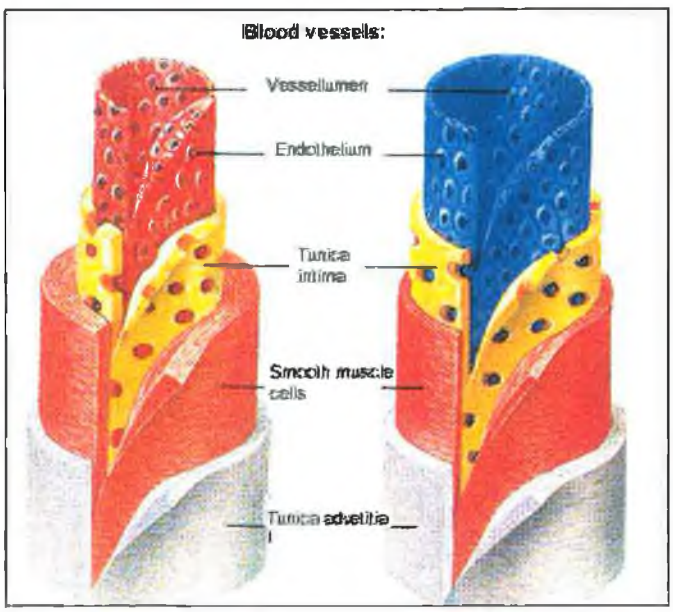

Fig. 1.1. Structure of Blood Vessels. Diagrammatic representation of an artery and vein, showing different layers of the vessel. [http://www.chemie.tu-darmstadt.de/bt/Endothel.html].

1.3 Mechanical Forces and the Endothelium

Among the physiological stimuli that impact on the endothelium, mechanical or hemodynamic forces associated with blood flow are of central importance. These include cyclic circumferential strain, caused by a transmural force acting perpendicularly to the vessel wall, and shear stress, the frictional force of blood dragging against cells (See Fig. 1.2). Both of these forces are essential to maintain a healthy vessel. They also have a profound impact on endothelial cell metabolism and can induce qualitative and quantitative changes in endothelial gene expression leading to changes in cell fate [Patrick *et al.*, 1995; Traub *et al.,* 1998; Chien *et al.,* 1998].

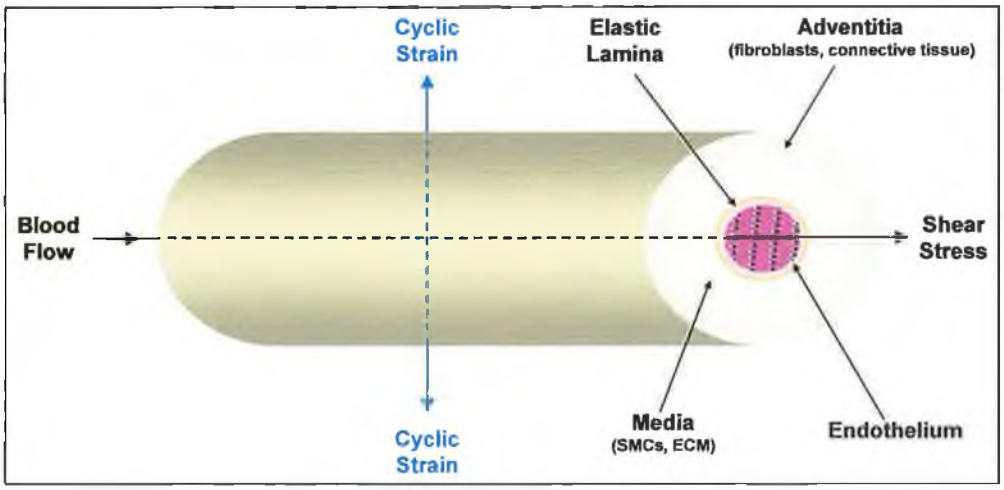

Fig. 1.2. Biomechanical stimulation of a blood vessel. Hemodynamic forces associated with blood flow play a pivotal role in the physiological control of vascular tone, remodelling and the initiation and progression of vascular pathologies. These forces include cyclic circumferential strain acting perpendicularly to the vessel wall causing outward stretching of *both* vascular ECs and SMCs and thus, rhythmic distension of the arterial wall, and fluid shear stress, the frictional force generated as blood drags against vascular endothelial cells.

1.3.1 Cyclic strain

1.3.1.1 Modeling of Cyclic Strain

All cells of the vessel wall can detect and respond to the physical force cyclic strain, which results from the pulsitile flow of blood in the vessel. The effects of circumferential stress on ECs have been investigated by applying cyclic stretch to endothelial cells cultured on an elastic membrane mounted in a stretch device such as that shown in Fig. 1.3. During this procedure, ECs are grown on a flexible membrane, which can be precisely deformed by a microprocessor-controlled vacuum, providing equibiaxial tension. This allows the cells to be subjected to defined levels of cyclic strain, in a variety of wave patterns [Banes *et al.*, 1985]. In this way, we can monitor the effect of various levels of cyclic strain, physiological levels or detrimentally high levels for example, on specific endothelial cell markers. Normal blood pressure is considered to be 120/80 mm Hg, whereas blood pressures of above 140/90 mm Hg and below 90/60 mm Hg are considered high and low respectively. Factors ranging from physical exertion to psychological stress can result in a transient rise in blood pressure, and a consequent transient increase in cyclic stress. Genetic predisposition to hypertension can lead to a chronic increase in cyclic stress, resulting in potentially serious clinical manifestations. Conversely, factors such as electrolyte imbalance, ischemic heart disease and systemic sepsis can result in transient hypotension, and a consequent transient decrease in cyclic stress (American Heart Association).

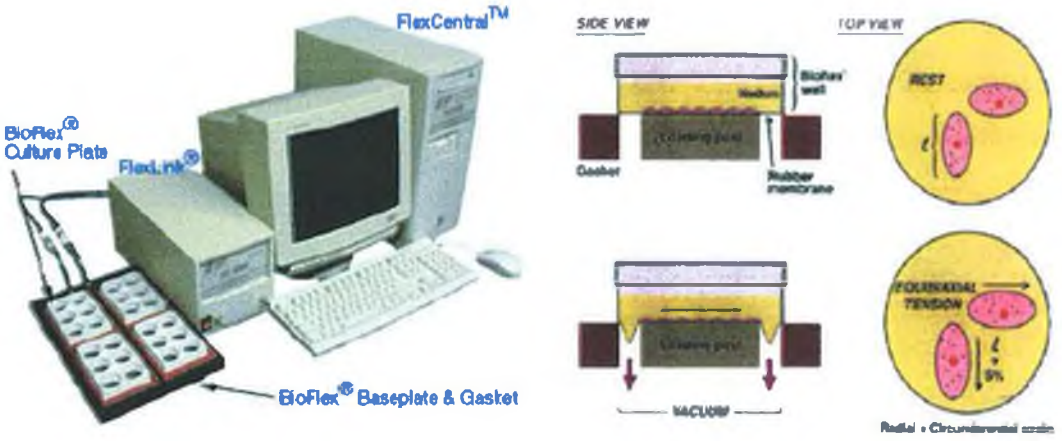

Fig. 1.3. In vitro cyclic strain device; The Flexercell™ Tension Plus FX-4000T™ system (Flexcell International Corp.-Hillsborough NC).

1.3.1.2 Effect of Cyclic Strain on ECs

There have been many studies investigating the effects of cyclic strain on aortic ECs (Lehoux *et al.*, 2005; Sipkema *et al.*, 2003]. Both in vitro and ex vivo models reveal that cyclic strain has a profound impact on endothelial metabolism and can induce qualitative and quantitative changes in gene expression leading to changes in cell fate, with consequences for endothelial phenotype and vessel wall homeostasis [Patrick *et al.*, 1995; Traub *et al.,* 1998; Chien *et al.,* 1998]. In addition to affecting the expression and/or activation of numerous signaling molecules associated with mechanotransduction, cyclic strain has been shown to regulate the expression and/or activation of several classes of effector genes (and gene products) in vascular ECs, including those regulating: (i) **Vessel diameter** - nitric oxide (NO), nitric oxide synthase (NOS), cyclooxygenase-2 (COX-II), endothelin-1 (ET-1) and thimet oligopeptidase

[Coen *et al.*, 2004; Cotter *et al.*, 2004]; (ii) **Proliferation** - platelet-derived growth factor (PDGF) and vascular endothelial growth factor [Sumpio *et al.*, 1998; Zheng *et al.*, 2001]; (iii) **Migration** - urokinase plasminogen activator (uPA), plasminogen activator-inhibitor type-1 (PAI-1), monocyte chemotactic protein type-1 (MCP-1), MMP-2, MMP-9 and MT1-MMP (membrane type-1 matrix metalloproteinase, MMP-14) [Ulfhammer *et al.*, 2005; von Offenberg Sweeney *et al.*, 2004a; von Offenberg Sweeney *et al.*, 2004b; von Offenberg Sweeney *et al.*, 2005; Wung *et al.*, 1997]; (iv) **Cell-cell communication/barrier function** - intracellular adhesion molecule type-1 (ICAM-1), zonula occludens 1 (ZO-1) and occludin [Collins *et al.*, 2006; Pradhan *et al.*, 2004]; and (v) **Angiogenesis** - MMP-2, MMP-9, MT1-MMP, uPA and RGD-dependent integrins [von Offenberg Sweeney *et al.*, 2005; Yamaguchi *et al.*, 2002].

Vascular ECs sense and respond to cyclic strain both morphologically and phenotypically. The influence of cyclic strain on ECs is visually apparent as early as 15 min post-strain with the formation of actin stress fibres and morphological alignment of cells perpendicular to the force vector [Iba *et al.*, 1991]. Alignment is subsequently followed by phenotypic or cell fate changes, such as those mentioned above. Physiological levels of cyclic strain on aortic ECs for example have been shown to increase both migration [von Offenberg Sweeney *et al.*, 2005] and proliferation [Iba *et al.*, 1991; Li *et al.*, 2005], whilst concomitantly reducing apoptosis [Haga *et al.*, 2003; Liu *et al.*, 2003].

1.3.2 Shear Stress

The endothelium is also exposed, and sensitive to, shear stress, which results from blood flow through the vessel. The mean shear stress to which the endothelium is subjected to is approximately 10–70 dynes/cm^2. However, arteries do experience variations in pressure and blood flow, which can be influenced by the architecture of the vasculature. At certain positions, such as bifurcations in the vessel, or points of extreme curvature the vessel may be exposed to turbulent flow, oscillatory shear stress and eddy currents, all of which can abrogate the protective effects of laminar shear.

Laminar blood flow within a vessel can be described by the equation: $\tau = 4\mu Q / \pi r^3$; where τ is shear stress, μ is blood viscosity, Q is flow rate and r is the vessel radius. The term r is raised to the third power thus where Q is constant, therefore a small change in r will result in a large change in τ [Lehoux *et al.*, 2003].

1.3.2.1 Effect of Shear Stress on ECs

In vitro studies in which endothelial monolayers have been subjected to defined levels of shear stress have been essential to our understanding of shear stress-related molecular responses. The complexity of the shear stress response is only now being elucidated and some of the best characterised responses include, reorganization of actin containing stress fibers, alterations in metabolic activities and changes in cell cycle kinetics [Davies *et al.*, 1993; Davies 1995].

Shear stress induces extensive changes in endothelial cell behaviour and has been implicated in vasculogenesis, re-endothelialisation of vascular grafts, atherosclerosis, and angiogenesis [Urbich *et al.*, 2002]. Shear stress stimulates several signaling cascades in endothelial cells including: potassium channel activation (the earliest), elevation of inositol trisphosphate and diacylglycerol, an increase in intracellular calcium levels, G protein activation, MAPK signalling, and activation of transcription factors, such as nuclear factor κB (Davies, 1997; Azuma *et al.*, 2001]. The early signaling events are followed by changes in gene expression and alignment of actin filaments and microtubules within the flow direction, resulting in changes in cell shape and directional migration [Braddock *et al.*, 1998; Hsu *et al.*, 2001].

Recent studies have also shown that laminar shear stress modulates many genes which regulate cell fate (i.e. cell cycle progression and apoptosis). Physiological levels of laminar shear stress prevent apoptosis in ECs in response to a variety of stimuli, including tumour necrosis factor-α, oxidized LDL and angiotensin 11 [Dimmeler *et al.*, 1999]. Shear stress also upregulates gene products involved in actin remodelling (e.g. β-actin and myosin heavy chain). Fluid shear stresses generated by blood flow in the vasculature can also profoundly influence the phenotype of the endothelium by regulating the activity of certain flow-sensitive proteins (for example, eNos) [Topper *et al.*, 1999]. Flow-dependent activation of eNOS has been observed both *in vitro* and *in vivo*, and the resultant release of NO has been related to SMC relaxation as a response to increases in flow. In conjunction with flow-mediated increases in vasodilators such as NO, levels of vasoconstrictors including ET-1 have been found to be decreased. A

number of genes involved in thrombosis, homeostasis, and inflammation such as thrombomodulin, tissue plasminogen activator (t-PA) and vascular cell adhesion molecule-1 (VCAM-1), have all been identified as being shear responsive. Thus, shear stress profoundly affects the health and functions of the endothelium.

1.4 Mechanotransduction

Vascular cells have the ability to respond to the mechanical forces cyclic strain and shear stress. The cells must be able to detect this hemodynamic stimulus in order to respond to it. This is achieved by several mechanically sensitive receptors/detector molecules present in vascular cells. These receptors can then elicit a signaling pathway, which culminates in the recruitment/activation of an effector molecule(s) to mediate the cellular response. This process is referred to as mechanotransduction. Mechanical forces initiate complex signal transduction cascades that lead to functional changes in the cell. The main classes of mechanotransduction receptors present on EC are integrins, G-proteins, protein tyrosine kinases (PTKs) and ion channels, each of which will be discussed in greater detail.

There has been very little investigative work in determining the mechanotransduction pathway by which cyclic strain and shear stress illicit their effects on tight junction permeability (See section 1.5.4 for information on tight junctions). In fact, to the best of our knowledge, this study is the first definitive attempt to determine the pathway from onset of cyclic strain to tightening of the EC barrier.

Essential to the coordination of cellular functions in response to hemodynamic stimuli is the ability of cells to communicate with each other. The process of intercellular signaling is achieved through numerous molecules, which interact specifically with specialized docking sites on the cell surface, called receptor proteins. The source of these molecules may be; i) autocrine secretion of a signaling molecule, that targets the secretory cell itself, ii) paracrine secretion of a signaling molecule that targets a cell close to the signal releasing cell or iii) endocrine secretion of a signaling molecule from a gland, that targets a cell distant from itself. Intracellular signaling, results in the coordination and synchronization of cell function within a given tissue (e.g. the vessel wall). Following the binding of a ligand to a receptor, intracellular effector molecules are activated leading to alterations in cell structure and/or function [Stone 1998]. As the small GTPases RhoA and Rac-1 have been implicated as key permeability regulators, especially with regards to barrier dysfunction, it was decided to look at the effect of these intracellular signaling molecules on the cyclic strain-regulation of tight junctions in ECs. We have also elected to examine the signaling molecules p38 and MEK, as the MAPK pathway can be activated in response to mechanical forces. Furthermore, several studies have linked MAPK pathways to TJ protein regulation (See section 1.4.5.1.1).

1.4.1 Integrins

1.4.1.1 Structure of Integrins

Integrins are integral membrane proteins that are found in the basolateral plasma

membrane of cells. They are obligate heterodimers comprised of α and β subunits (See Fig. 1.4). There are eighteen α and eight β subunits. In addition, variants of some of the subunits are formed by differential splicing. Each possible combination of subunits has its own binding specificity and signaling properties [Giancotti *et al.,* 1999]. These subunits can form twenty-four different integrins, sixteen of which are reportedly involved in the vasculature with seven expressed in endothelial cells [Rupp *et al.,* 2001].

Each subunit consists of a large NH_2-terminal extracellular domain, a single membrane-spanning domain and a short cytoplasmic domain [Shyy *et al.,* 2002].

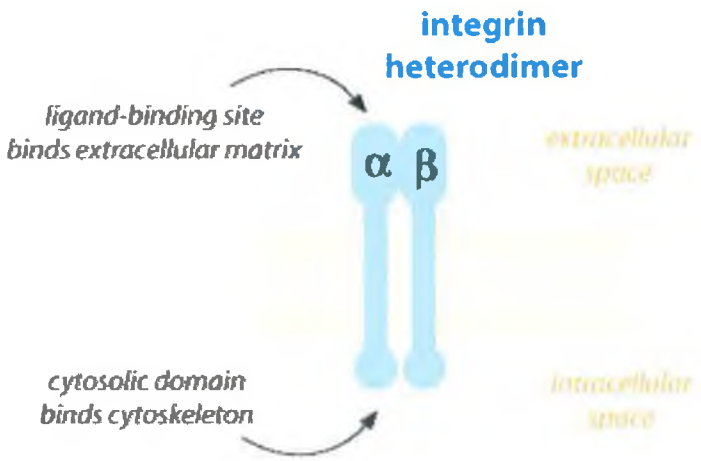

Fig. 1.4. The basic structure of the integrin, a cell surface protein receptor. [http://www.bioteach.ubc.ca].

1.4.1.2 Function of Integrins

The first main function of integrins is attachment of the cell to the extracellular matrix (ECM). The extracellular domain binds directly to ECM proteins such as

fibronectin, vitronectin, collagen, laminin, fibrinogen and osteopontin. The connection between the cell and the ECM anchors the cell and enables it to endure physical forces. The second integrin function is signal transduction from the ECM to the cell. The cytoplasmic tail of integrins are generally devoid of enzymatic activity. As a result of this, integrins transduce signals via adaptor proteins such as Shc, which connect the integrin to the cytoskeleton, cytoplasmic kinases and transmembrane growth factors [Giancotti *et al.,* 1999]. It is the role integrins play in cell signaling, where hemodynamic forces are concerned that we are most interested in.

1.4.1.3 Integrins in Mechanotransduction

Integrin signaling is a crucial component in development, maintenance and function of the vascular system [Ruoslahti *et al.*, 1997]. Integrins have the unique characteristic that they can signal through the cell membrane in either direction, essentially forming a bridge between the ECM and the cytoskeleton. Shear stress and cyclic strain activates integrins by switching them into an active conformation, which increases the extracellular domains affinity and avidity for the ECM proteins. The cytoplasmic domains of both the α and β subunits interact with intracellular signaling molecules and cytoskeletal proteins to regulate cellular events, such as signal transduction, cytoskeletal reorganization and cell motility [Shyy *et al.*, 2002].

Evidence for mechanical activation of integrins is provided by both direct assessment of integrin conformational changes in response to these forces and blockade of the induced responses by antibodies or blocking peptides such as the Arg-Gly-Asp

(RGD) peptide [Lehoux *et al.,* 1998]. In this way it has been shown that shear stress and cyclic strain causes an increase in integrin activity in ECs.

Integrin activation is directly associated with members of the Rho small GTPase family (see section 1.4.5.2.1), including RhoA, Cdc42 and Rac-1 [Shyy *et al.,* 2002]. Other families associated with integrin activation include multiple kinases (FAK, and c-Src), adaptor molecules ($p130^{cas}$ and Shc), and guanine nucleotide exchange factors (C3G and SOS). When these signaling molecules are activated by integrins they can then go on to activate MAPKs such as ERK, which can then illicit cellular responses to shear stress or cyclic strain. See Fig. 1.5 for an example of integrin-mediated mechanotransduction.

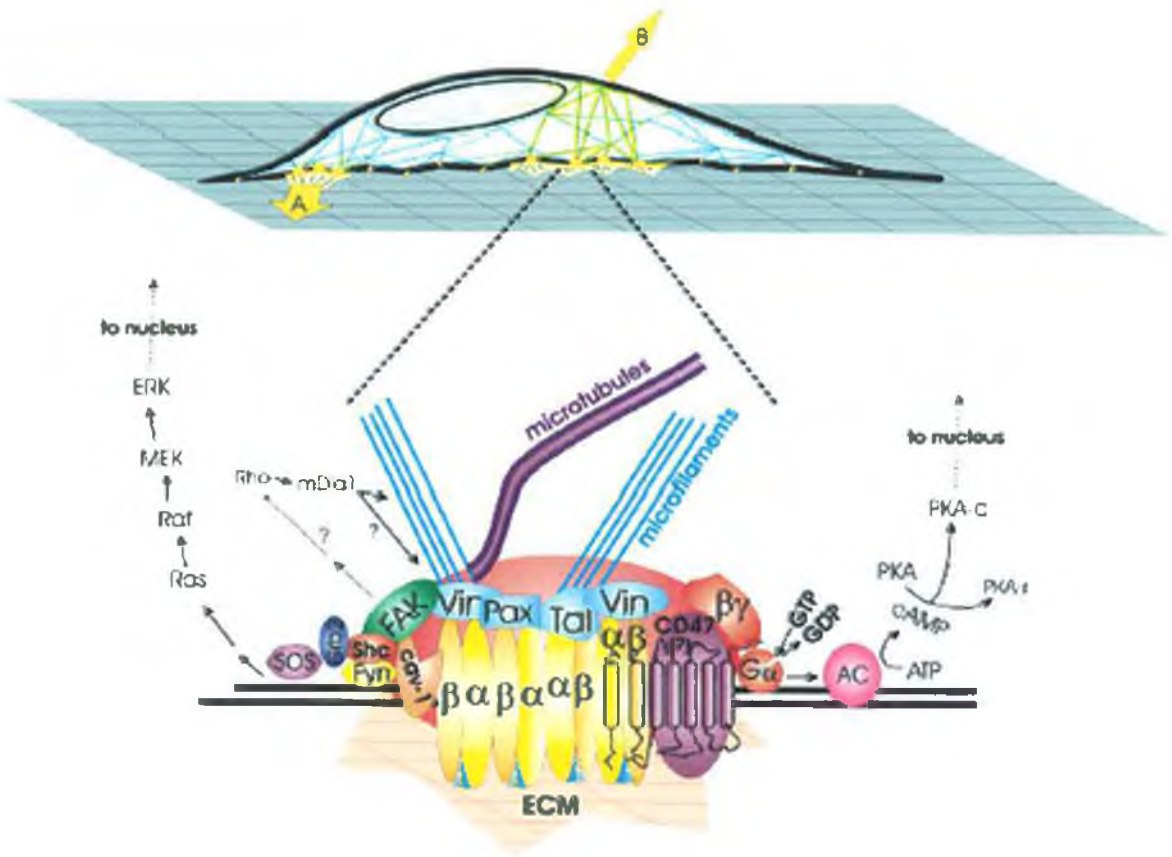

Fig. 1.5. Schematic diagram of how forces applied through the ECM (**A**) or directly to the cell surface (**B**) travel to integrin-anchored focal adhesions through matrix attachments or cytoskeletal filaments, respectively. Internally generated tension and forces transmitted through cell-cell contact similarly reach focal adhesions through the cytoskeleton. Forces concentrated within the focal adhesion (magnified at bottom of figure) can stimulate integrin clustering and induce recruitment of additional cytoskeletal linker proteins (Vin, vinculin; Pax, paxillin; Tal, talin) that connect directly to microfilaments and indirectly to microtubules. Forces applied to this specialised cytoskeletal adhesion complex also activate integrin-associated signal cascades. Focal adhesion kinase (FAK) may be involved in Shc recruitment as well as modulation of Rho activity which, in turn, can regulate the force response through mDia1. Caveolin-1 (cav-1) can also recruit Shc to integrins to activate the ERK cascade. CD47 associates with the integrin heterodimer to form a protein complex with seven transmembrane segments that mimics the action of G protein-coupled receptors. In the case shown, when integrins are mechanically stressed, the complex stimulates Gs-mediated up-regulation of the cAMP cascade through adenylate cyclase (AC), resulting in nuclear translocation of the catalytic subunits of protein kinase A, PKA-c. MEK, mitogen-activated protein kinase or extracellular signal- regulated kinase kinase; ERK, extracellular signal-regulated kinase; GDP, guanosine diphosphate; ATP, adenosine triphosphate [Alenghat *et al.*, 2002].

1.4.1.4 Integrins and Tight Junction Regulation

There has been very little research looking at the direct effect of integrin activation by mechanical forces on tight junction permeability. However, integrins and Rho family GTPases are intimately connected at multiple levels and control many of the same cellular events. Furthermore, it appears that integrins regulate Rho family GTPases and Rho family GTPases regulate integrins [Schwartz *et al.,* 2000]. Integrins and Rho family GTPases function coordinate to mediate adhesion-dependent events in cells. Integrins and GTPases might therefore be organized into complex signaling cascades that regulate cell behavior.

Therefore, integrins regulate multiple signaling pathways. In fact, dynamic coupling of Rho family GTPase activation to the hemodynamically sensitive integrin signaling system is now well documented [Grande-García *et al.*, 2005]. In this study, integrins were found to independently control the translocation of GTP-bound Rac-1 to the plasma membrane in mouse fibroblast cells. This step is essential for Rac-1 binding to effectors and eliciting its effect within the cell such as stabalising the tight junction (See section 1.4.5.2.1). Integrins control Rac-1 signaling by preventing the internalization of its binding sites. Therefore, it is possible that hemodynamic forces elicit changes in endothelial permeability by engaging an integrin/RhoA/Rac-1-GTPase signaling axis, leading to modulation of actin cytoskeletal organisation with direct consequences for tight junction assembly and function. In fact, Rac-1 downstream signaling is strictly dependent on integrins [Grande-García *et al.*, 2005]. Pak, a Rac-1 effector that is activated by serum in attached cells is not activated in suspended cells

after serum stimulation, indicating that adhesion to the ECM couples Rac-1 with its effector Pak.

A direct link between integrins and tight junction function was discussed in a study by Tafazoli *et al.* It was found that *Yersinia* bacteria attach to beta1-integrins at the tight junctions of MDCK cells. Here the bacteria perturb the F-actin structure and distribution of occludin and ZO-1, thereby promoting paracellular translocation of bacteria and soluble compounds [Tafazoli *et al.*, 2000].

1.4.2 Heterotrimeric G-proteins

1.4.2.1 Structure and Function of Heterotrimeric G-proteins

G-proteins discovered by Alfred Gilman and Martin Rodbell (Nobel Prize for Physiology or Medicine, 1994), are pivotal membrane signaling systems. They are a vital intermediary between the activation of receptors on the cell membrane and signal transduction within the cell. Rodbell demonstrated in the 1960s that GTP was involved in cell signaling, whilst Gilman discovered the proteins that interacted with the GTP to initiate cell signaling cascades within the cell.

Receptor activated G-proteins are bound to the inside surface of the cell membrane. They are comprised of a G-protein coupled receptor (GPCR) and the heterotrimeric G-protein complex in addition to the more recently identified regulators of G protein signaling (RGS-proteins) and activators of G-protein signaling (AGS-

proteins) [Offermanns 2003]. All of these receptors have seven membrane spanning elements that use intracellular loops and their C-terminal tails for interaction with heterotrimeric G proteins. They consist of the Gα and the tightly associated Gβγ subunits (See Fig. 1.6).

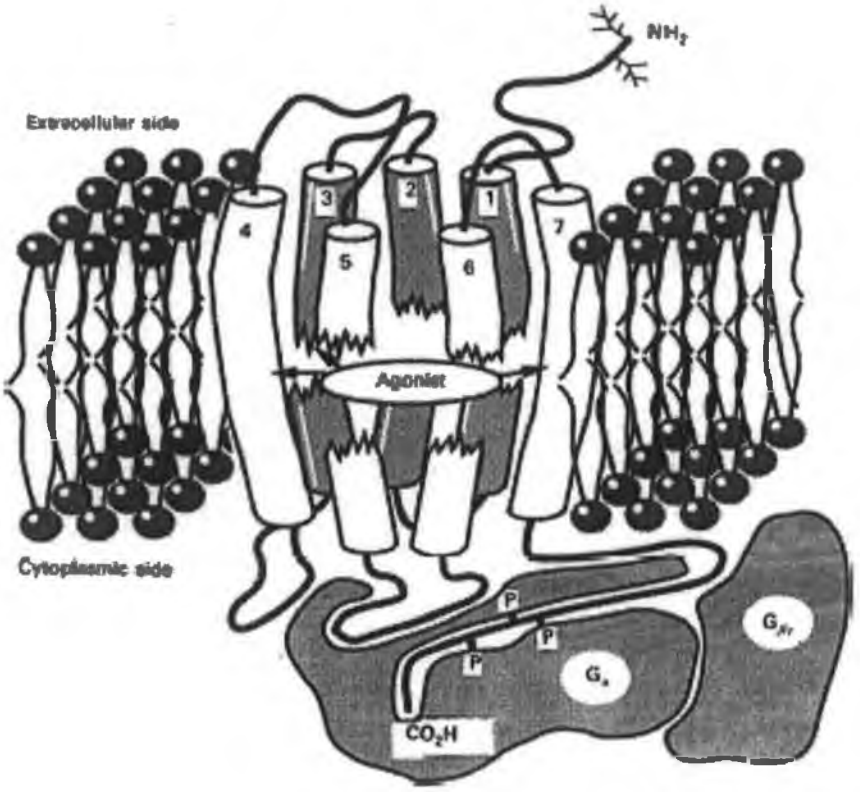

Fig. 1.6. Shared features of receptors coupled to G proteins. The model illustrates some of the features predicted to be shared by all receptors that interact directly with G proteins [Taylor *et al.*, 1990].

When G-proteins are activated, ligand receptors catalyses the GDP/GTP exchange at the α subunit of the coupled G-protein and promotes dissociation of the α and βγ components, which subsequently activate their effectors, for example adenylyl cyclase. The duration of G-protein activation is controlled by the intrinsic GTPase

activity of Gα. Following GTP hydrolysis the Gα subunit returns to the GDP-bound conformation and re-associates with the Gβγ subunit (See Fig. 1.7).

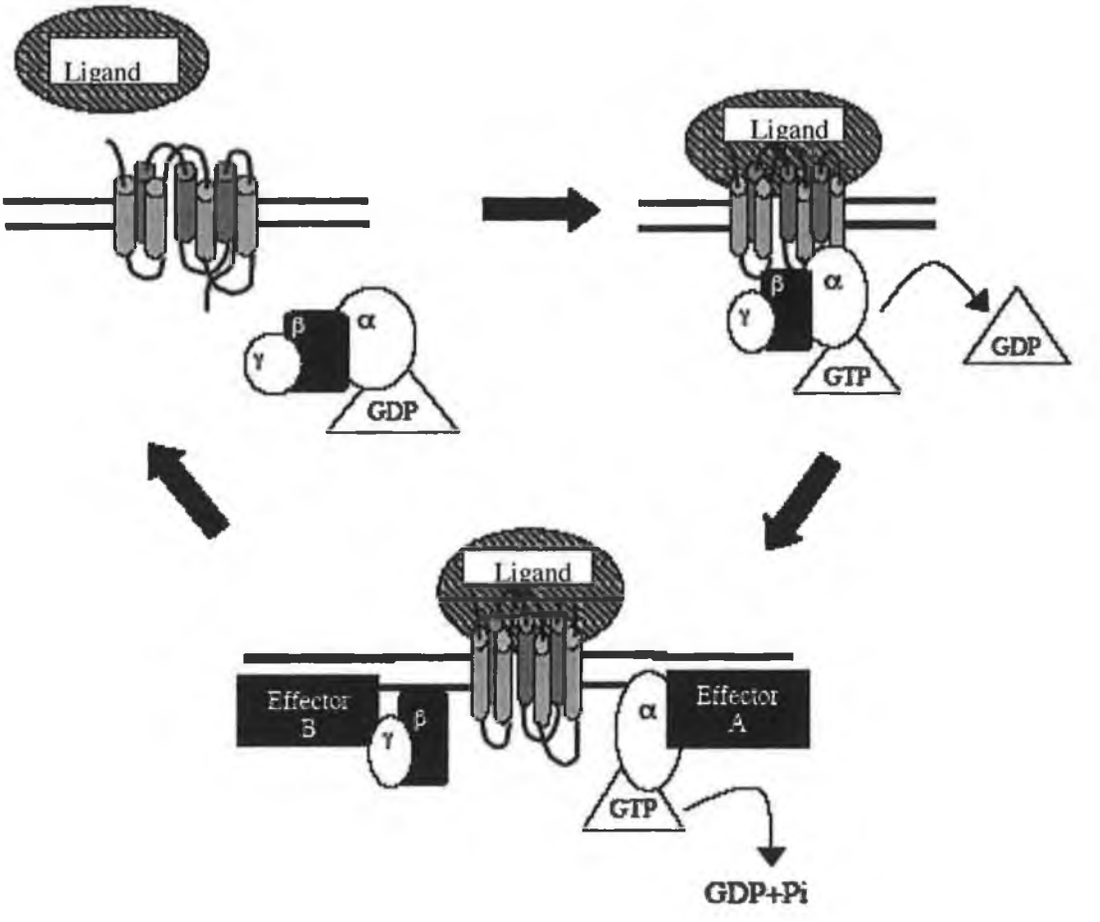

Fig. 1.7. Following binding of a ligand, the activated receptor catalyses GDP/GTP exchange at the α subunit which promotes dissociation of the G-protein complex, the α and βγ subunits, which subsequently activate their effectors. Following hydrolysis of GTP by the α subunit the heterotrimer re-associates.

More than twenty G-protein α-subunits have been described which have been divided into four families based on structural and functional homologies, α_s, $\alpha_{i/o}$, α_q, and α_{12}. The majority of GPCRs are capable of activating more than one G-protein subtype, which leads to initiation of various signaling cascades. There are some characteristic patterns of G-protein activation by specific receptors; the cellular or physiological effect

of a receptor is dependent on which G-protein sub-type it is coupled to.

The α subunits:

• **$G\alpha_s$ - family:** There are two members of this family, α_s and α_{olf}, as well as four known splice variants. Upon activation, this group stimulates adenylyl cyclase to increase levels of intracellular cAMP and to activate calcium channels. α_s is ubiquitously expressed while α_{olf} is restricted to neuronal cells specifically olfactory sensory neurons.

• **$G\alpha_{i/o}$ family:** This family consists of α_{i1}, α_{i2}, α_{i3}, α_{o1}, α_{o2}, $\alpha_{t\text{-}rod}$, $\alpha_{t\text{-}cone}$, α_{gust} and α_z, all of which are highly homologous and have the ability to inhibit adenylyl cyclase, in addition to activation of potassium channels. The high degree of homology between sub-types may suggest partially redundant functions. Expression of the various α_i sub-types is very diverse depending on tissue examine (e.g. α_{12} is predominant in the mammalian heart). A defining characteristic of the $G\alpha_{i/o}$ family is sensitivity to pertussis toxin. Pertussis toxin is produced by *Bordetella pertussis* and catalyzes the adenosine diphosphate (ADP)-ribosylation of α_I and α_o subunits at a cysteine residue near the C-terminus resulting in uncoupling of receptor and G-protein. α_z unlike the other members of this family has been found to be pertussis toxin insensitive and is expressed in various tissues. It can inhibit adenylyl cyclase but its physiological function is somewhat ambiguous, although α_z-deficient mice point to roles in platelet activation.

• **$G\alpha q$ family:** This family stimulates phospholipase C in a pertussis toxin-insensitive manner. α_q and α_{11} are ubiquitously expressed. Moreover receptors activating α_q family members do not discriminate between α_q and α_{11} [Wange *et al.,* 1991]. $\alpha_{15/16}$ are only expressed in hematopoietic cells and α_{14} is restricted to the kidney, lungs and testis.

• **Gα12 family:** α_{12} and α_{13} constitute the members of this family and appear to be widely expressed. The function of these proteins is somewhat unclear. One recently discovered function is the interaction of these proteins with cadherins causing the release of transcriptional activator β-catenin [Meigs *et al.,* 2001].

The Gβγ subunit:

This complex is assembled from a repertoire of five β subunits and twelve γ subunits. The sequence similarity is higher between β subunits (79-90% homology) than γ subunits and it is not yet clear how many combinations will actually form stable dimers [Clapham *et al.,* 1993]. β subunits are believed to posses a propeller structure formed by seven β sheets. The γ subunit is located at one end of the propeller and associates with the β subunit by a coiled structure [Bohm *et al.,* 1997]. βγ-sensitive effectors include adenylyl cyclase, phospholipase C, phospholipase A, potassium channels, calcium pumps, and phosphoinositide 3 kinase (PI-3 kinase) [Exton 1996; Yamada *et al.,* 1989; Lotersztajn *et al.,* 1992]. With a few exceptions there appears to be no major differences between different βγ-subunit combinations in their ability to activate effector enzymes.

1.4.2.2 G-Proteins as Mechanotransducers

Membrane localisation and rapid activation strongly implicate G-proteins as a primary sensor of hemodynamic forces [Gudi *et al.*, 1996]. In fact, G-protein activation by mechanical forces represents one of the earliest mechanotransduction events reported. Both PTX-insensitive (Gαq) and PTX-sensitive (Gαi) subunits are involved in this rapid

response [Gudi *et al.,* 1996; Clark *et al.,* 2002]. G-proteins may detect mechanical forces in one of two ways, either via GPCR or they may be stimulated directly by the deformation of either the actin cytoskeleton or the membrane phospholipid bilayer during exposure to cyclic strain or shear stress.

Shear stress and cyclic strain-induced activation of G-proteins results in several responses which function in the regulation of vascular tone, including release of vasodilators or vasoconstrictors such as ET-1 [Liu *et al.,* 2003; Pirotton *et al.,* 1987]. Changes in G-protein expression have been observed within the physiological range of cyclic strain and shear stress. These changes have been correlated with enhanced NO and PGI2 release as well as increased G-protein functionality [Redmond *et al.,* 1998].

1.4.2.3 G-Proteins and Tight Junction Regulation

Although there has been very little research looking at the role of G-proteins as mechanotransducers in regulation of occludin and ZO-1, there have been several studies looking at the expanding role of G-proteins in tight junction regulation.

Saha *et al.* observed that a fraction of $G\alpha_s$ was co-localised with ZO-1 in the TJ of MDCK cells, supporting the model of multiple $G\alpha$ subunits interacting with TJ proteins to regulate the assembly and maintenance of the TJ [Saha *et al.*, 2001]. Dodane *et al.* speculated that the formation and permeability of tight junctions are actively regulated by second-messenger-generating systems involving G-proteins. They reported

that $G\alpha_{12}$ was present at the cell borders along the areas of lateral cell-cell contact in MDCK and Caco-2 epithelial cells. This isoform formed a pattern of distribution very similar to ZO-1, indicating that it may be part of the zonula occludens complex and may locally regulate formation and permeability of tight junctions [Dodane *et al.* 1996].

Experiments by Balda *et al.* in 1991 observed that pertussis toxin increased TEER in epithelial cells, indicating that junction formation may be controlled by a network of reactions including G-protein activation [Balda *et al.,* 1991]. Although this finding differs from ours, it indicates that G-proteins may be involved in TJ regulation. Studies by Denker *et al.* also alluded to G-protein involvement in TJ biogenesis. They observed that the heterotrimeric G-proteins, $G\alpha_{i\text{-}2}$, and the $G\alpha$ family member, $G\alpha_{12}$ may participate in the maintenance and/or regulation of TJ assembly in MDCK cells [Denker *et al.* 1998].

1.4.3 Protein Tyrosine Kinase (PTK)

Tyrosine phosphorylation of various proteins is a very important step in the regulation of normal cell proliferation, migration, differentiation and survival. PTKs are integral components of signal transduction cascades that involve tyrosine phosphorylation. They can be separated into two distinct categories: receptor and non-receptor tyrosine kinases.

1.4.3.1 Receptor Tyrosine Kinases

Receptor Tyrosine Kinases are enzyme-linked membrane receptors displaying intrinsic tyrosine kinase activity within the receptor itself. Examples of receptor tyrosine kinases include the insulin-like growth factor receptor (ILGFR), the epidermal growth factor receptor (EGFR) and the platelet-derived growth factor receptor (PDGFR). These receptors have an extracellular domain responsible for the binding of a ligand, for example, a circulating hormonal stimuli, a transmembrane domain and an intracellular domain with tyrosine kinase activity.

When an agonist binds to the extracellular domain of a receptor tyrosine kinase, it leads to the dimerisation of the receptor if it is made up of two chains, such as the insulin receptor, or in the case of the EGF receptor, which is a monomer, binding of a ligand to the receptor leads to dimerisation of two receptors. This leads to a conformational change that causes auto-phosphorylation of the cytoplasmic domain of the receptor (See Fig. 1.8). This subsequently causes the activation of the tyrosine kinase activity and can also create a binding site for intracellular adaptor molecules such as Sos and Grb-2. These adaptor molecules bring other signaling molecules such as the small GTPase Ras, into close proximity to the receptors' intracellular domain, thereby eliciting a signaling cascade leading to a functional cellular response [Stone 1998].

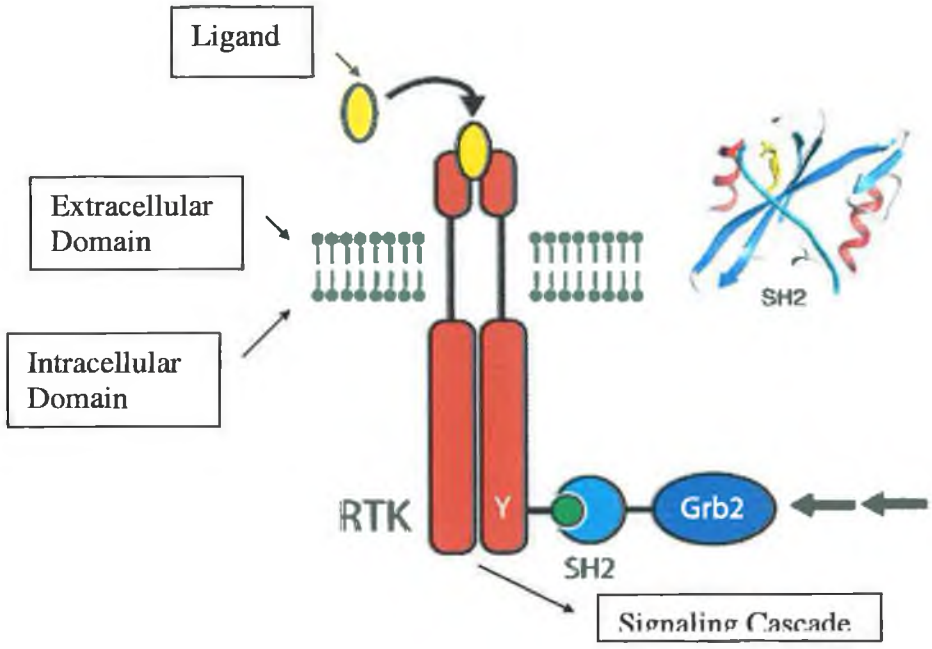

Fig. 1.8. Diagrammatic representation of the initial events in receptor tyrosine kinase (RTK) signaling. Extracellular ligand (yellow) binds resulting in oligomerisation. The intracellular tyrosine kinase domains phosphorylate each other, generating a binding site for SH2-containing adaptor proteins such as Grb2. (Y is phosphorylated tyrosine amino acid) [http://www.nimr.mrc.ac.uk].

1.4.3.2 Non- Receptor Tyrosine Kinases

Non-receptor tyrosine kinases differ from RTKs as they do not have an extracellular domain. They are intracellular enzymes that also possess an intrinsic tyrosine kinase activity. Examples of non-receptor tyrosine kinases molecules include the FAK, c-Src and Jak families. These molecules may phosphorylate receptors, which lack intrinsic tyrosine kinase activity or they may recruit other downstream signaling molecules such as PI-3 kinase [Stone 1998].

1.4.3.3 PTKs in Mechanotransduction

Protein tyrosine kinases play an important role in transducing the mechanical signal. In fact, the activities of PTKs in cardiac myocytes, platelets, and ECs are increased by mechanical stimuli such as cyclic stretch and shear stress [Sadoshima *et al.,* 1993; Ishida *et al.,* 1996]. Focal adhesion-associated tyrosine kinases, e.g., FAK and c-Src, are rapidly activated in ECs by shear stress [Li *et al.,* 1997; Jalali *et al.,* 1998]. PTKs play an important role in activating MAPKs, as genistein, a PTK inhibitor attenuates the shear stress-induced activation by ERK and JNK. PTKs are also crucial in the shear stress regulation of cell shape and stress fibers.

PTK-mediated mechanotransduction often involves the participation of other receptors such as integrins and G-proteins [Soldi *et al.,* 1999; Linseman *et al.,* 1995; Eguchi *et al.,* 1998; Zwick *et al.,* 1997].

1.4.3.4 PTKs and Tight Junction Regulation

There has been quite a lot of research focusing on the role of non-receptor tyrosine kinases in TJ regulation. In fact the activity of Src kinases appears to play a role in both assembly and disassembly of tight junctions. A study by Busaroy *et al.* demonstrated that oxidative stress-induced disruption of tight junctions is mediated by the activation of c-Src in Caco-2 cells [Busaroy *et al.*, 2003]. In another study by Kale *et al.* it was reported that c-Src binds to occludin and phosphorylates it on tyrosine residues leading to disruption of the tight junction in Caco-2 cells [Kale *et al.*, 2003]. Occludin has also

been found to colocalise with the non-receptor tyrosine kinase, c-Yes, at cell junction areas and to form an immunoprecipitable complex with c-Yes *in vivo* [Chen *et al.*, 2002]. This study provided strong evidence that occludin tyrosine phosphorylation is tightly linked to tight junction formation in MDCK cells, and that the non-receptor tyrosine kinase c-Yes is involved in the regulation of this process.

There has been very little research regarding receptor tyrosine kinase-mediated mechanotransduction in TJ regulation. However a study by Buchert *et al.* showed that the junction-associated protein AF-6 interacts and clusters with specific Eph receptors, which are a subfamily of receptor tyrosine kinases, at specialized sites of cell-cell contact in the adult rat brain, indicating that TJ proteins may directly associate with RTKs [Buchert *et al.*, 1999].

1.4.4 Ion Channels

Some ion channels function as mechanotransducers and therefore play a role in cell signaling. Two different mechano-sensitive channels have been identified in vascular cells: shear stress activated potassium channels and stretch activated cationic channels. The exact mechanisms by which mechanical forces regulate ion channel conformation remains indistinct, though deformation of the cytoskeleton is thought to be an important contributor. This premise is supported by a number of studies, including a study that demonstrates cytoskeleton G-protein coupling in shear-induced potassium channel opening and integrin-cytoskeleton activation of ion channels [Lehoux *et al.*, 2003].

There has been very little research investigating the role of ion channels in mechanotransduction in relation to TJ formation. However ion channels have been implicated in TJ function in a study by Broughman and co-workers. In this study it was reported that NC-1059, a synthetic channel-forming peptide, transiently increased trans-epithelial electrical conductance across MDCK cell monolayers in a time- and concentration-dependent manner. However, concomitant alterations in junctional protein localisation (zonula occludens-1, occludin) and cellular morphology were not observed [Broughman *et al.*, 2004]. Calcium channels also play an important role in tight junction function. Indeed, calcium is critical for the maintenance of cell-cell junctions in various cell types. A study by Chen *et al.* showed that when Ca^{2+} was depleted from MDCK cell culture medium, there was a significant reduction in TEER, indicating a global loss of the tight junction barrier function. Reconstitution of Ca^{2+} resulted in an increase in TEER [Chen *et al.,* 2002]. In another study in primary pulmonary endothelial cultures, chelation of extracellular calcium increased albumin transfer by 125%, decreased TEER, and caused retraction of adjacent cells. Restoration of extracellular calcium restored normal barrier function [Shasby *et al.*, 1986].

1.4.5 Intracellular signaling molecules

1.4.5.1 Mitogen-activated Protein Kinases

The mitogen-activated protein kinase (MAPK) signaling cascade is an important

pathway whose activation can lead to, or stimulate, gene transcription and/or protein synthesis. The MAPK super-family is comprised of three main signaling pathways:

- the extracellular signal-regulated protein kinase (ERK),
- the c-jun N-terminal kinases or stress-activated protein kinases (JNK/SAPK),
- the p38 family of kinases.

Each of the MAPK modules operates as a three-tier system. The MAPK module is activated by a MAPK kinase (MAPKK), which is a dual-specific kinase, which phosphorylates ERK, JNK and p38 at both Ser/Thr and Tyr sites. The MAPKK is activated by a MAPKK kinase (MAPKKK), which receives its stimulus from receptors on the cell surface. MAPK have a key role in the regulation of many genes because the end targets of these cascades are often nuclear proteins or transcription factors [Cowan *et al.*, 2003].

Activation of the MAPK pathway in response to mechanical stimuli may occur by various means including G-proteins, integrins, receptor tyrosine kinases and cytoskeleton-associated non-receptor tyrosine kinases. Phosphorylation of the α and $\beta\gamma$ subunit of a G-protein can lead to MAPK activation in kidney fibroblast cells [Crespo *et al.*, 1994]. Shear stress has been found to activate ERK1/2 via a $G_{i}\alpha$/Ras pathway and JNK via a $G\beta\gamma$/Ras tyrosine kinase pathway in endothelial cells [Jo *et al.*, 1997]. Similarly, small GTPases such as Ras or RhoA are stimulated by mechanical strain and may regulate ERK1/2 or JNK activation in endothelial and smooth muscle cells [Wung *et al.*, 1999; Numaguchi *et al.*, 1999]. Similarly integrins have been shown to be

involved in the activation of members of the MAPK family. Chen *et al.* observed an increase in the association of $\alpha_v\beta_3$ integrin with the adapter protein Shc and subsequent activation of ERK1/2 and JNK under shear conditions in endothelial cells [Chen *et al.*, 1999].

1. 4.5.1.1 Mitogen-Activated Protein Kinases and Tight Junction Regulation

Several studies have linked MAPK pathways to tight junction protein regulation. A study by Basuroy *et al.* shows that ERK interacts with the C-terminal region of occludin and mediates the prevention of H_2O_2-induced disruption of TJs by epithelial growth factor in Caco-2 cells [Basuroy *et al.*, 2006]. Another study by Pedram *et al.* reported that vascular permeability factor (VEGF) significantly enhances permeability in aortic endothelial cells via a linked signaling pathway, sequentially involving Src, ERK, JNK, and phosphatidylinositol 3-kinase/AKT [Pedram *et al.*, 2002]. This led to the serine/threonine phosphorylation and redistribution of actin and the tight junction proteins, zonula occludens-1 and occludin, and the loss of the endothelial cell barrier architecture, thus indicating that MAPK pathways can play a very important role in TJ regulation.

P38 has also previously been implicated in tight junction regulation. Kelvil *et al.* observed that inhibition of p38 MAP kinase during oxidant challenge in HUVECs significantly attenuated actin stress fiber formation and prevented gap formation, thus attenuating the increase in permeability following addition of hydrogen peroxide [Kelvil

et al., 2001]. Another study using Sertoli cells has shown that TJ dynamics are regulated, at least in part, by TGF-beta3 via the p38 mitogen activated protein (MAP) kinase pathway. This in turn regulates the production of occludin, by Sertoli cells and a specific p38 MAP kinase inhibitor, could block occludin loss from the blood testis barrier [Lui *et al.*, 2003].

1.4.5.2. GTPases

GTPases are a large family of enzymes that can bind and hydrolyze GTP. The GTP binding and hydrolysis takes place in the highly conserved G domain common to all GTPases. GTPases play an important role in:

- Signal transduction at the intracellular domain of trans-membrane receptors, including recognition of taste, smell and light.
- Protein biosynthesis (translation) at the ribosome.
- Control and differentiation during cell division.
- Translocation of proteins through membranes.
- Transport of vesicles within the cell. (GTPases control assembly of vesicle coats).

One important function of this sub-set of proteins, are their involvement in signal transduction. The small GTPases cycle between inactive (GDP-bound) and active (GTP-bound) states (See Fig. 1.9). Under basal conditions, GTPases are GDP-bound and inactive. Upon stimulation, for example by cyclic strain, GTPases release GDP and bind to GTP, a reaction accomplished by guanine nucleotide exchange factors (GEFs). In

inactive. Upon stimulation, for example by cyclic strain, GTPases release GDP and bind to GTP, a reaction accomplished by guanine nucleotide exchange factors (GEFs). In their active GTP-bound state, GTPases interact with a variety of effector proteins to promote cellular responses. The active state of GTPases is transient because of their intrinsic GTPase activity, which is stimulated further by GTPase activating proteins (GAPs).

Fig. 1.9. Signal Transduction and Small GTPases. [http://www.piercenet.com]

1.4.5.2.1 Rho GTPase Family

The Rho family of small GTPases consists of at least 20 members, and three sub-families, **Rho** (RhoA, RhoB and RhoC), **Rac** (Rac-1, Rac-2 and Rac-3) and **Cdc42** (Cdc42Hs and G25K). Cdc42, Rac-1 and RhoA are the most extensively characterized members of the Rho family [Mackay *et al.*, 1998].

al., 1998]. The Rho GTPase family of proteins control the organization of the actin cytoskeleton in all eukaryotic cells and also playing a major role in controlling the stability and integrity of the tight junction [Kozma *et al.*, 1995]. The Rho GTPase family of proteins also mediates regulation of transcription, membrane trafficking and apoptosis. Rho GTPases are activated by many different stimuli including G-protein-coupled receptors, tyrosine kinase receptors, integrin clustering or engagement, cell–cell adhesion and cytokine receptors [Sah *et al.*, 2000].

Several targets have been identified for RhoA, including p160 Rho kinase (ROCK) and Rhotekin. The activity of ROCK is enhanced upon binding to GTP-Rho and leads to phosphorylation of the regulatory part of the myosin molecule, myosin light chain, which enables the myosin molecule to change conformation, interact with actin to cause F-actin bundling, thereby leading to stress fiber formation [Mackay *et al.*, 1998]. This is a key event in various models of barrier dysfunction [Dudek *et al.*, 2001; Wojciak-Stothard *et al.*, 2002]. Therefore, increased Rho kinase activity is detrimental to barrier function (See Fig. 1.10). As RhoA and ROCK are central to actin cytoskeletal rearrangement it is thought they may play a pivotal role in junctional disassembly when stimulated, as the tight junction proteins are intimately linked to the actin cytoskeleton.

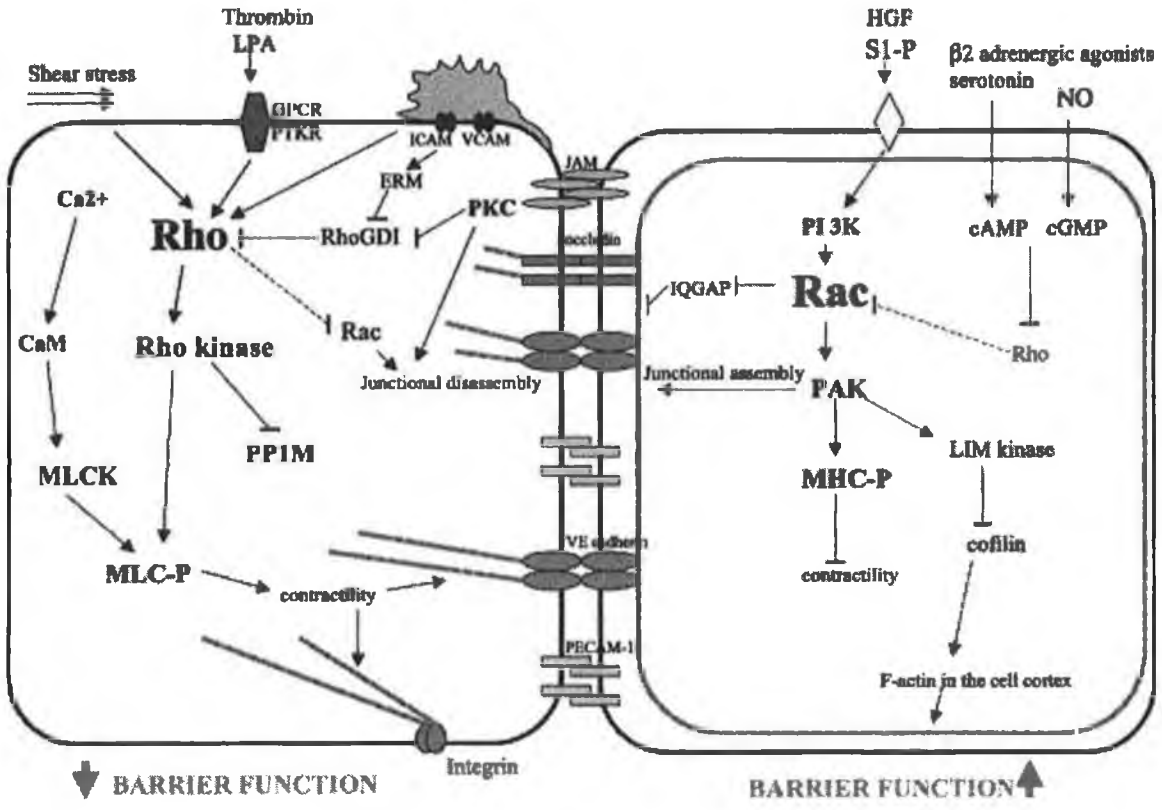

Fig. 1.10. Barrier Function: Rac and Rho [Wojciak-Stothard *et al.*, 2002]

Conversely Rac-1 activation leads to an increase in barrier function (See Fig. 1.10). In fact, Rac-1 is required for the assembly and maturation of epithelial and endothelial junctions and its activity increases during junction formation [Noren *et al.*, 2001]. Its activation induces actin polymerization to form lamellipodia (broad web-like extensions), [Kozma *et al.*, 1995]. Sphingosine 1-phosphate (S1-P) has been proposed to act via the activation of Rac-1. S1-P is a lysophospholipid stored and released by activated blood platelets that plays a role in improving endothelial barrier function. S1-P induces enhancement of the cortical F-actin ring, which is usually associated with stabilization of intercellular endothelial junctions [Garcia *et al.*, 2001]. One of the target proteins for Rac-1 is p21-activated kinase (Pak). It is necessary for S1-P-mediated

enhancement of endothelial barrier function and also plays a key role in the linkage of Rac-1 signaling pathways to Ras signaling pathways through the phosphorylation of Raf and MEK (See Fig. 1.11). LIM kinase is a Pak target, phosphorylating and inactivating cofilin, an actin severing protein, which could explain the observed accumulation of cortical F-actin. Rac-1 therefore stabilises tight junctions and counteracts the effects of Rho [Dudek *et al.*, 2001; Wojciak-Stothard *et al.*, 2002].

RhoA and Rac-1 have emerged as key permeability regulators acting antagonistically to regulate endothelial barrier function. Since paracellular transport through the epithelial and endothelial tight junction is suspected to be controlled by a 'purse-string' contraction of perijunctional F-actin [Madara *et al.*, 1987; Bement *et al.*, 1993], RhoA is strongly implicated as it is a potential mediator of actin. It increases stress fiber formation and actomyosin contractility to facilitate breakdown of intercellular junctions and loss of barrier function, whereas Rac-1 stabilises tight junctions through its effect on cortical F-actin [Dudek *et al.*, 2001; Wojciak-Stothard *et al.*, 2002]. The ability of this family of proteins to control the dynamics of cellular actin polymerisation and reorganisation has fundamental implications for the control of tight junction permeability [Hopkins *et al.*, 2000].

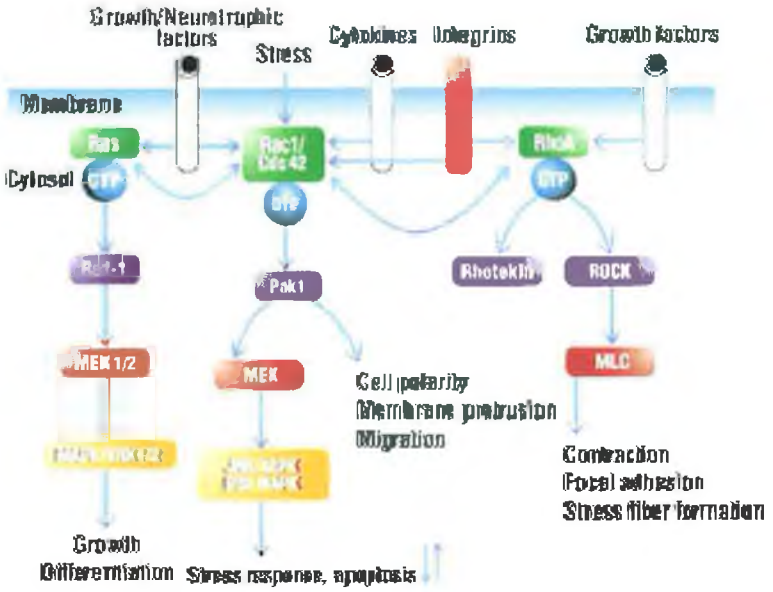

Fig. 1.11. Signaling pathways mediated by Ras, Rac1/Cdc42 or Rho GTPase. [http://www.piercenet.com].

1.4.5.3 Protein Kinase C

The protein kinase C (PKC) family consists of 11 members of serine/threonine kinases that are activated by diverse mechanisms including receptor tyrosine kinases, non-receptor tyrosine kinases, and G protein-coupled receptors [Newton, 1995]. PKC isoforms are classified into three groups based on their structure and particular substrate requirements: **conventional** (α, β1, β2, γ), **novel** (δ, ε, η, θ, μ), and **atypical** (ζ, λ, τ) [Newton, 1995].

Conventional PKC isoforms require diacylglycerol (DAG), calcium, and phosphatidylserine for their activation, whereas the novel isoforms lack the requirement for calcium, and atypical PKC isoforms require only phosphatidylserine. Treatment of

cells with phorbol esters such as PMA, activates PKC by mimicking DAG. PKC activity is regulated by its subcellular distribution as well as its phosphorylation state. Upon ligand binding and recruitment to the membrane, PKC becomes a substrate for kinases resulting in auto-phosphorylation and enhanced catalytic activity [Parekh *et al.*, 2000].

1.4.5.3.1 PKC and Tight Junction Regulation

Activation of PKC is directly linked to tight junction assembly [Stuart *et al.*, 1995]. Activation of PKC by 1,2-dioctanoylglycerol (diC8) stimulated the partial assembly of tight junctions in MDCK cells grown in low calcium conditions [Balda *et al.*, 1991]. It has also become apparent that multiple classes of PKC isoforms contribute to formation of the tight junction. The signaling events downstream of PKC that regulate cell permeability have not been completely elucidated; however, components of the tight junction complex may be directly phosphorylated by PKC. For instance, ZO-2 was phosphorylated *in vitro* by several PKC isoforms [Avila-Flores *et al.*, 2001]. Furthermore, a C-terminal region of mouse occludin was phosphorylated *in vitro* by a purified mixture of PKC isoforms [Andreeva *et al.*, 2001]. Therefore, PKC may directly modify components of the tight junction complex to regulate permeability.

1.4.5.4 Tyrosine Phosphatase

Protein tyrosine phosphatases are involved in a variety of cellular processes such as signal transduction, cell cycle regulation, and differentiation. This enzyme is a low

molecular weight cytosolic protein that is 157 amino acid residues long. Proteins can either be tyrosine phosphorylated by protein kinases or tyrosine dephosphorylated by protein phosphatases (See Fig. 1.12). Depending on the protein in question this may lead to activation or inactivation of the protein. Tyrosine specific protein phosphatases are enzymes that catalyse the removal of a phosphate group attached to a tyrosine residue. Tyrosine phosphatase has a beta alpha beta structural motif. This enzyme consists of a four-stranded parallel beta sheet, four alpha-helices, and a single 3_{10} helix.

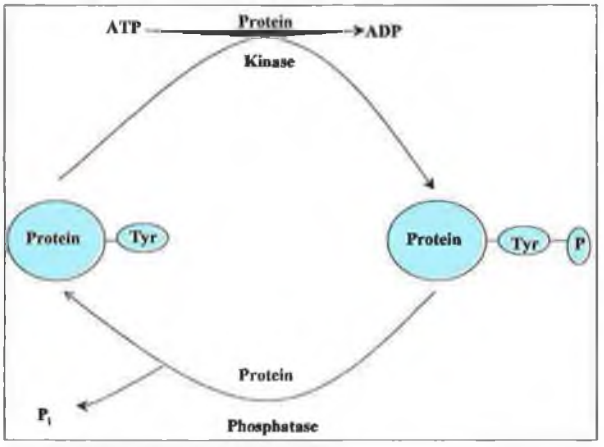

Fig. 1.12. Mode of action of tyrosine kinase/phosphatase.

1.4.5.4.1 Tyrosine Phosphatase and Tight Junction Regulation

The current literature can be quite conflicting when it comes to the effect of tyrosine phosphorylation on tight junction assembly. However tyrosine phosphorylation of occludin has been linked with increased TJ permeability in epithelial and endothelial cells [Staddon *et al.*, 1995; Gloor *et al.*, 1997]. Wachtel *et al.* investigated the effect of

inhibiting protein tyrosine phosphatases on the composition of endothelial TJs. In this study it was found that inhibition of protein tyrosine phosphatase resulted in the cleavage of occludin and subsequent loss of occludin localisation at the TJs via an MMP-dependent step [Wachtel *et al.*, 1999].

The tyrosine phosphorylation level of the intracellular C-terminal tail of occludin, C-occludin, has been described to be critical in determining the binding ability of occludin to other TJ proteins such as ZO-1, ZO-2, and ZO-3 [Kale *et al.*, 2003]. In this study, using Caco-2 cells, it was shown that the amounts of ZO-1, ZO-2, and ZO-3 bound to tyrosine phosphorylated C-occludin were several fold less than the amounts of these proteins bound to non-phosphorylated C-occludin. Thus, ample evidence in the literature exists to suggest that tyrosine phosphorylation of occludin can lead to a decrease in barrier integrity and conversely that activation of tyrosine phosphatases may lead to an increase in barrier function.

1.5 Cell-Cell Interactions in Endothelial Cells

There are four main types of intercellular junctional complexes found in ECs; adherens junctions, gap junctions, desmosomes and tight junctions (See Fig.. 1.13).

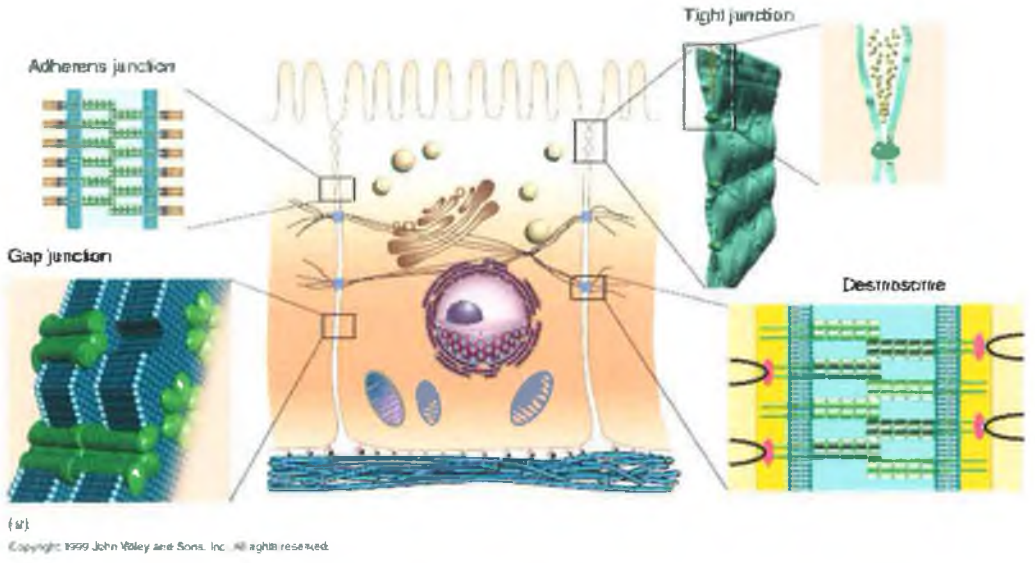

Fig. 1.13. Diagrammatic representation of the types of junctions found in ECs.

1.5.1 Adherens Junctions

Adherens junctions, located below the region of tight junctions, contain the trans-membrane proteins cadherins, whose extracellular segments bind to each other and their cytoskeletal linkers, catenins [Daniel *et al.*, 1997; Kaibuchi *et al.*, 1999]. They provide strong mechanical attachments between adjacent cells. For example, they hold cardiac muscle cells together as the heart expands and contracts. Although spatially and biochemically distinct, functional interaction between adherens junctions and tight junctions has been demonstrated [Rubin *et al.*, 1999].

1.5.2 Gap Junctions

Gap junctions, seen in virtually all cells that contact other cells in tissues, provide open channels through the plasma membrane, allowing ions and small molecules (less than approximately 1 kDa) to diffuse freely between neighboring cells. The gap junction's major physiological role therefore, is to couple both the metabolic activities and the electric responses of the cells they connect. An example of their importance in physiology is their role in electrical coupling. Gap junctions are abundant in cardiac and smooth muscle cells and depolarization of one group of muscle cells rapidly spreads to adjacent cells, leading to well-coordinated contractions of those muscles [Sohl *et al.*, 2005]. Gap junctions are constructed of trans-membrane proteins called connexins. Four or sometimes six connexins assemble to form a cylinder with an open aqueous pore in its center. Such an assembly of connexins in the plasma membrane of one cell then aligns with the connexins of an adjacent cell, forming an open channel between the two cytoplasmic compartments. The plasma membranes of the two cells are separated by a gap corresponding to the space occupied by the connexin extracellular domains—hence the term "gap junction," which was coined by electron microscopists [Sohl *et al.*, 2005].

1.5.3 Desmosomes

Desmosomes, a further junctional complex found in endothelial cells, and in epithelial cells such as skin cells, are specialized for cell-cell adhesions. Like adherens junctions, they are also cadherin-based. They anchor a second cytoskeletal filament network, intermediate filaments, which are strong elastic polymers, to the plasma

membrane. Coupled to the cytoplasmic surface of the desmosome, they form a supracellular network that strengthens tissues, protecting them against mechanical damage.

1.5.4 Tight Junctions

Tight junctions, or zonula occludens, are the most apical component of the intercellular junctional complex [Harhaj *et al.*, 2004]. Farquhar and Palade originally identified the tight junction by electron microscopy in 1963 [Farquhar *et al.*, 1963]. Tight junctions form a barrier to diffusion of ions and small molecules from the lumen to the tissue parenchyma (barrier function) and restrict the diffusion of lipids and proteins between the apical and basolateral plasma membranes (fence function) [Farquhar *et al.*, 1963; Van Meer *et al.*, 1986]. However, in a relatively short time, our knowledge of the tight junction has evolved from this relatively simple view of it being a permeability barrier in the paracellular space and a fence in the plane of the plasma membrane, to one of it acting as a multi-component, multi-functional complex that is involved in regulating numerous and diverse cell functions [Schneeberger *et al.*, 2004].

The tight junction is composed of anastamosing strands of 10 nm fibrils that completely encircle the apical region of the cell. These fibrils are composed of trans-membrane proteins that interact with proteins on adjacent cells [Staehelin 1973]. Tight junctions form in polarized epithelial and endothelial cells as well as in a variety of specialized epithelial cell types, including those of oligodendrocytes of the central nervous system, Sertoli cells in the testis, the choroid plexus, the stria vascularis in the

organ of Corti, and the collecting tubules of the kidney in some cases [Gow *et al.*, 1999].

Tight junctions are composed of multiple trans-membrane, scaffolding, and signaling proteins, including occludin, claudin family members, junctional adhesion molecules 1 to 3, cingulin, 7H6, spectrin, and linker proteins, such as the zonula occludens family members (ZO-1/2/3), the latter linking tight junction proteins to each other and the actin cytoskeleton [Furuse *et al.*, 1993; Furuse *et al.*, 1994; Gardner *et al.*, 1996; Hirase *et al.*, 1997]. (See Fig. 1.14).

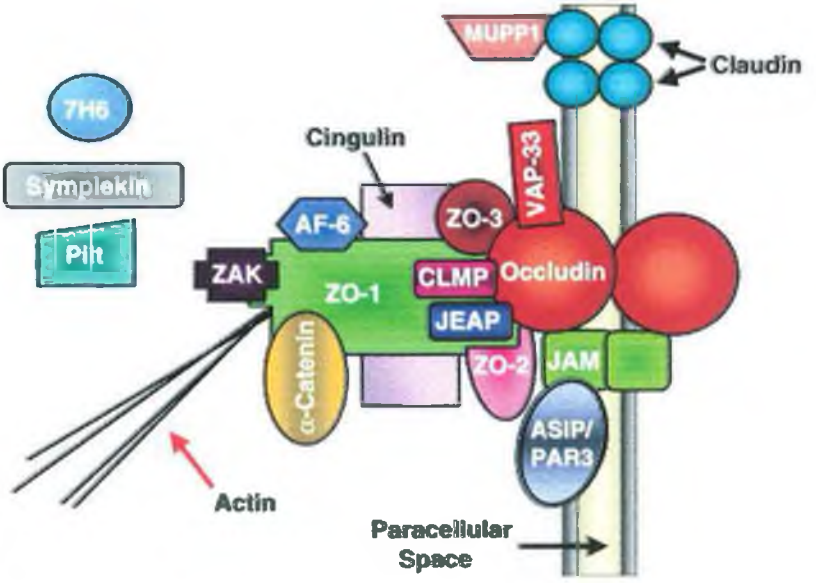

Fig. 1.14. The arrangement of tight junction proteins in endothelial cells [Feldman *et al.*, 2005].

1.5.4.1 Measurement of TJ permeability

Tight junction permeability in cell culture is generally measured using either of two techniques: trans-epithelial/trans-endothelial electrical resistance (TEER) or

paracellular tracer flux (See Fig. 1.15). TEER measurements are commonly used to assess the integrity of tight junctions. Electrical resistance across a monolayer represents the sum of the paracellular resistance, which consists of the resistance of the junction and the intercellular space, and the transcellular resistance, which consists of the resistances of the apical and basolateral cell membranes [Claude, 1978]. Paracellular tracer flux may also be used to measure tight junction integrity. In this method, permeability to hydrophilic, uncharged paracellular tracers is measured using radioactive or fluorescently conjugated tracers such as mannitol or dextran. Tight junction permeability to particular solutes depends on the size, shape, and charge of the solute [Dvorak, *et al.*, 1999]. Paracellular tracer flux measures permeability of a monolayer over a time course of several hours, whereas TER represents an instantaneous measurement.

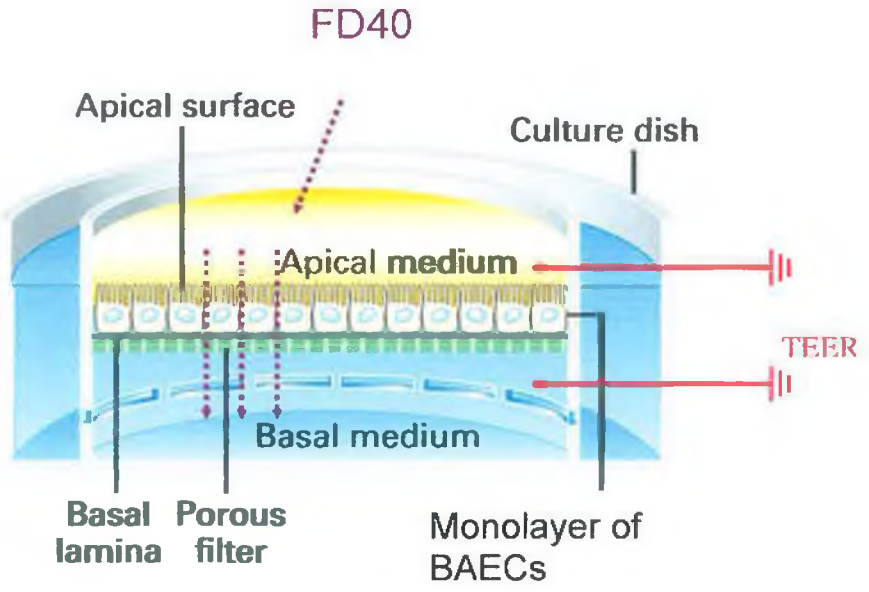

Fig. 1.15. Diagrammatic representation of measurement of TEER and paracellular tracer flux in BAECs.

1.6 Proteins of the Tight Junction

1.6.1 Occludin

1.6.1.1 Discovery and Structure

Occludin was the first tight junction integral membrane protein to be identified and was originally isolated from chick livers [Furuse *et al.*, 1993]. The nucleotide sequence of chicken occludin encodes a 504 amino acid polypeptide with a predicted molecular mass of 55.9 kDa. The amino acid sequence predicted four putative membrane-spanning segments, two 44-amino acid extracellular loops and two intracellular domains (See Fig. 1.16). Furuse *et al.* established that the amino acid sequence of the first extracellular loop is highly concentrated with tyrosine and glycine residues (~60%) [Furuse *et al.*, 1993]. Further cDNA cloning and sequencing disclosed more definitive information about the structure of occludin [Furuse *et al.*, 1994]. It was determined that there were four transmembrane domains in the NH_2-terminal half of occludin, which divide the protein into five separate domains and were referred to as domains A–E. The COOH-terminal half (domain E) is comprised of ~250 amino acid residues and is located in the cytoplasm. These early observations also revealed that charged amino acids were strongly concentrated in COOH-terminal cytoplasmic domain (domain E) and there was a very high content of tyrosine and glycine residues in the extracellular domains (domains B and D) [Furuse *et al.*, 1994].

It wasn't confirmed that occludin was present in mammals until work in 1996 by Ando-Akatsuka *et al.* Chicken occludin knowledge was limiting when it came to gene

targeting experiments and therefore the identification of mammalian occludin genes was another important discovery. Ando-Akatsuka first reported the nucleotide sequences of cDNAs encoding rat–kangaroo (potoroo), human, mouse, and dog occludin. As found in chicken occludin, all of the occludins expressed in these species contained four trans-membrane domains. The amino acid sequences of human, murine, and canine occludin are approximately 90% homologous but have significantly deviated from the avian and marsupial homologues. Each of the species is capable of forming a common α-helical coiled structure. It was postulated that this segment of the amino acid sequence was retained in order to interact with occludin's binding partner ZO-1 [Ando-Akatsuka *et al.*, 1996].

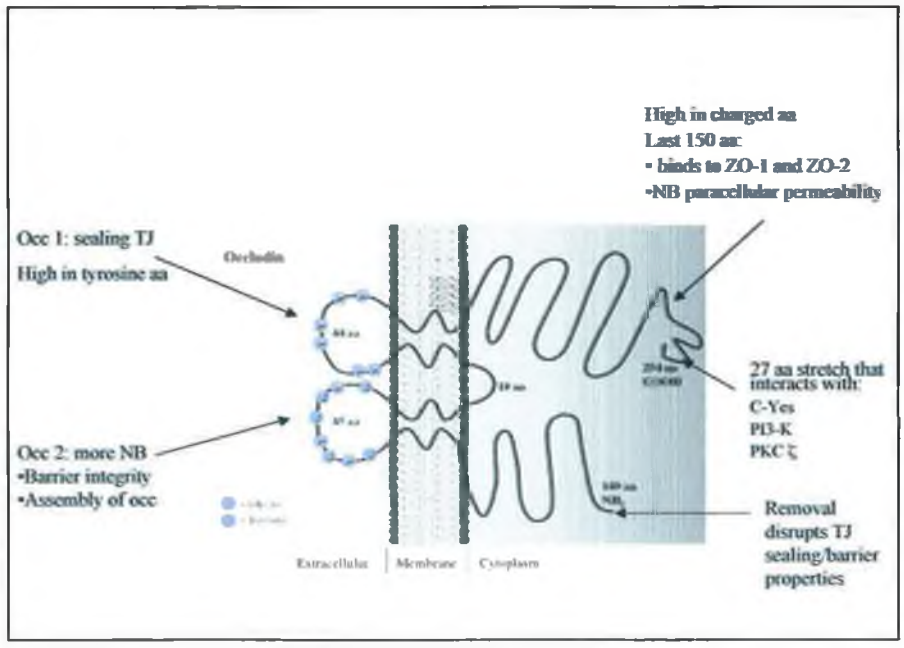

Fig. 1.16. Schematic representation of occludin structure. Adapted from Feldman *et al.*, 2005.

1.6.1.2 Function of Occludin

The function of occludin was initially investigated by introducing chick occludin cDNA into MDCK cells in a Lac-inducible vector [McCarthy *et al.*, 1996]. In this study occludin expression was induced by the addition of isopropyl-h-d-thiogalactoside (IPTG) to the medium. 5 h after addition of IPTG, the TER across the MDCK monolayer increased progressively, relative to control level. The TER level across the transfected cells was 30–40% higher than the TER across the control monolayer at 31-h post-induction, indicating that the expression of occludin correlates with barrier properties. This can also be observed in various other tissues. For example, arterial endothelial cells express 18-fold greater occludin protein levels than venous endothelial cells and form a tighter solute barrier [Kevil *et al.,* 1998]. Similarly, occludin is highly expressed in brain endothelium which forms a very tight barrier, but occludin is expressed at much lower levels in endothelial cells of non-neuronal tissue, which have lower barrier properties than brain endothelium [Hirase *et al.*, 1997]. Clearly, occludin is a key tight junction protein whose expression levels dictate tissue barrier properties. However, somewhat surprisingly, occludin is not required to maintain the structural integrity of tight junctions, since structurally intact tight junctions were formed in the absence of occludin [Saitou *et al.*, 1998]. These findings indicate that there are as yet unidentified TJ integral membrane proteins which can form strand structures, recruit ZO-1, and function as a barrier without occludin.

1.6.1.3 Unique Roles of Specific Occludin Domains (See Fig. 1.16)

COOH-terminus

The COOH-terminus of occludin is necessary for correct TJ assembly and function [Chen *et al.*, 1997]. In this study, *Xenopus* embryo were used to show that full-length and COOH-terminally truncated occludin localize at the TJ. However, it was reported that the TJs containing the mutant occludin proteins were leaky and this leakage induced by the COOH-terminally-truncated occludin could be corrected by transfection with full-length occludin mRNA. The COOH-terminus of occludin binds to ZO-1 (See section 1.6.3).

N-terminus

A study by Bamforth *et al.* investigated the localisation of N-terminally truncated occludin in the murine epithelial cell line CSG 120/7. An N-terminally truncated occludin construct, was correctly targeted to the TJ and co-localised with ZO-1. However, the truncated construct generated a detrimental effect on the barrier function of the TJs, as demonstrated by electrophysiological measurements [Bamforth *et al.*, 1999]. Overall, the result of removing the N-terminus was a disruption of the TJ's sealing/barrier properties.

Extracellular and Transmembrane Domains

Wong and Gumbiner have previously employed synthetic peptides (Occ-1 and Occ-2) corresponding to the two putative extracellular domains of occludin and studied their ability to modulate TJs in A6 cells (*Xenopus* kidney epithelial cell line). The second extracellular domain peptide (Occ-2) reversibly disrupted the integrity of the TJ barrier. A subsequent study discovered that the second extracellular loop was also required for occludin localisation at the TJ [Medina *et al.*, 2000].

It has also been reported in a recent study that multiple extracellular and trans-membrane domains of occludin are implicated in the regulation of the TJ barrier [Balda *et al.*, 2000]. It was observed that the extracellular domains and at least one of the transmembrane domains were crucial in maintaining selective paracellular permeability.

Coiled-coil domain of COOH-terminal region

Studies by Ando-Akatsuka *et al.* showed that occludin's coiled-coil domain appears to function as a site for specific interactions of potential regulatory proteins [Ando-Akatsuka *et al.*, 1996]. Photoactivation studies demonstrated that c-Yes, the regulatory (p85) subunit of PI 3-kinase, PKC-ζ and the gap junction component, connexin 26, interacted with this domain. These findings provide the first evidence to support a highly specific interaction between occludin and regulatory proteins that had been previously implicated in TJ modulation.

1.6.2 ZO-1

1.6.2.1 Discovery and Structure

The first protein identified as a TJ constituent was ZO-1 [Stevenson *et al.*, 1989; Anderson *et al.*, 1988]. ZO-2 and ZO-3 were later isolated as proteins that co-associate with ZO-1. ZO-1 (210–225 kDa), ZO-2 (180 kDa), and ZO-3 (130 kDa) are peripheral membrane-associated components of the cytoplasmic plaque of tight junctions and are found ubiquitously within tight junctions of epithelial and endothelial cells [Harhaj *et al.*, 2003]. In addition, ZO-1 and ZO-2 are also localised to adherens junctions in cells that do not form tight junctions, such as fibroblasts and cardiac myocytes [Itoh, *et al.*, 1999]. ZO proteins are members of the membrane-associated guanylate kinase (MAGuK) homologue family, containing three PDZ domains, an SH3 domain, and a non-catalytic guanylate kinase (GuK) homology domain [Woods *et al.*, 1993]. ZO family members also contain an acidic domain, a basic domain, a leucine zipper dimerisation motif, and a proline-rich C-terminus [Haskins *et al.*, 1998; Jesaitis *et al.*, 1994; Willott *et al.*, 1993; Beatch, *et al.*, 1996]. ZO-1 expression may be regulated at the post-transcriptional level by alternative splicing. Specifically, ZO-1 contains an alternatively spliced 80 amino acid domain within its C-terminus, the α-motif [Willott *et al.*, 1993], likely explaining why researchers often see two bands when monitoring ZO-1 by Western blotting.

1.6.2.2 ZO-1 Function

Because ZO-1 is found in the TJ in all epithelial and endothelial cells, its proximity to the membrane bilayer as determined by immuogold electron microscopy [Stevenson *et al.*, 1986], and its close stoichiometry to junction fibril particles [Anderson *et al.*, 1988], suggests ZO-1 is an important component of the tight junction.

One of its most important functions arises form the fact that ZO-1 has binding sites for many cellular proteins via multiple protein-binding domains. As previously mentioned, occludin co-associates with ZO-1. This co-association occurs at specific domains within the N-terminal (MAGUK-like) half of ZO-1. The PDZ-1 domain of ZO family members also binds to the C-terminal regions of claudins-1 to -8 [Itoh, *et al.*, 1999]. ZO-1 binds to JAM *in vitro* and *in vivo* [Ebnet *et al.*, 2000]. ZO-1 also interacts with cingulin *in vitro*. [D'Atri, *et al.*, 2002].). In addition to tight junction proteins, ZO-1 also binds to the adherens junction protein, β-catenin, and the gap junction protein, connexin-43 [Itoh, *et al.*, 1999].

In addition to binding to many tight and adherens junction proteins, the SH3 domain of ZO-1 may also mediate binding to G-proteins such as $G_{\alpha 12}$ [Meyer, *et al.*, 2002]. Furthermore, ZO-1 interacts with the Ras target, AF-6, and its binding was disrupted by activated Ras *in vitro* [Yamamoto *et al.*, 1997].

Of utmost importance is the fact that the distinctive proline-rich C-terminal half of ZO-1 co-sediments with F-actin. These findings clarify that ZO-1 establishes a link

between occludin and the actin cytoskeleton [Fanning *et al.*, 1998]. One can therefore hypothesise a dynamic regulatory association between endothelial permeability and hemodynamic stimuli, as tight junction components are intimately coupled to the hemodynamically responsive actin cytoskeleton [McCue *et al.,* 2004]. It is plausible therefore that force-dependent modulation of tight junction assembly is a highly likely process, albeit very poorly understood.

It is clear that ZO-1 plays a pivotal role in organising and promoting interactions in the tight junction. This is evident from the large array of cellular proteins that it interacts with, through multiple binding sites and interaction domains.

1.6.3 Interaction of Occludin and ZO-1

As previously mentioned, the tight junction in endothelial cells consists of an intimate gathering of various proteins and signaling molecules which help control paracellular transport and apical and basolateral separation. It has been well documented that two important proteins, occludin and ZO-1, and more specifically their interaction, plays a crucial role in tight junction function. In fact, increased co-association of both proteins, as investigated via immunoprecipitation, can be used as a measure of tight junction stability. A study by Rao *et al.* showed that oxidative stress disrupts the tight junction in part by causing disassociation of the occludin-ZO-1 complex [Rao *et al.*, 2002].

To find out more about the interaction of the two proteins, occludin's Domain E

(the COOH-terminal cytoplasmic domain) was expressed in *Escherichia coli* fused with glutathione-S-transferase and this COOH-terminal fusion protein specifically bound to a complex of ZO-1, ZO-2 and a number of other peripheral membrane proteins (see Fig. 1.17). Subsequent *in vitro* binding experiments using glutathione-S-transferase fusion proteins with a range of deletion mutants of Domain E narrowed down the sequence necessary for the ZO-1/ZO-2 association [Furuse *et al.*, 1994]. It was established that the same sequence is required for occludin to localise at the TJ and to bind to the ZO-1/ZO-2 complex and is therefore central to tight junction functioning. Furuse's 1994 study concluded that ZO-1 is directly bound to the Domain E of occludin, and that ZO-2 may be associated with occludin through ZO-1.

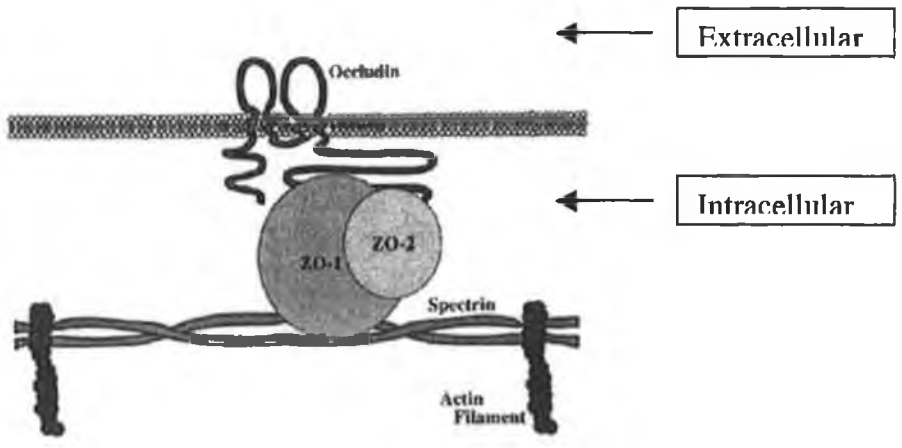

Fig. 1.17. Schematic drawing of the possible molecular architecture of tight junctions; occludin interaction with ZO-1 and ZO-2 [Furuse *et al.*, 1994].

1.6.4. Claudins

Similar to occludin, the claudins are a family of membrane spanning proteins comprised of four trans-membrane domains, two extracellular loops and cytoplasmic N-

and C-termini (see Fig. 1.18). They are also involved in the formation of tight junctions in epithelial and endothelial cells. There are 24 members of the claudin family, all of which share a YV motif in the final two amino acids of the C-terminal that is required for binding to the ZO family [Furuse *et al.*, 2001]. Claudins are the primary regulators of the formation of tight junctions [Furuse *et al.*, 1998]. They also regulate permeability and barrier function of tight junctions, for example, claudin-1 over-expression in MDCK cells increased TER by four-fold and reduced flux of 4 and 40 kDa dextran compared with control cells [Inai, *et al.*, 1999].

1.6.5 JAM

Another well documented TJ protein is JAM or Junctional Adhesion Molecule. It is a 36–41 kDa, single pass trans-membrane protein found in epithelial and endothelial tight junctions [Martin-Padura *et al.*, 1998] (see Fig. 1.18). It is thought to play an important part of TJ permeability as over-expression of JAM in CHO cells reduced paracellular permeability to 40 kDa dextran by 50% in a calcium-dependent manner [Martin-Padura *et al.*, 1998]. JAM has been shown to co-precipitate with ZO-1, which suggests that JAM may indirectly mediate the recruitment of occludin to the TJ via ZO-1 [Feldman *et al.*, 2005].

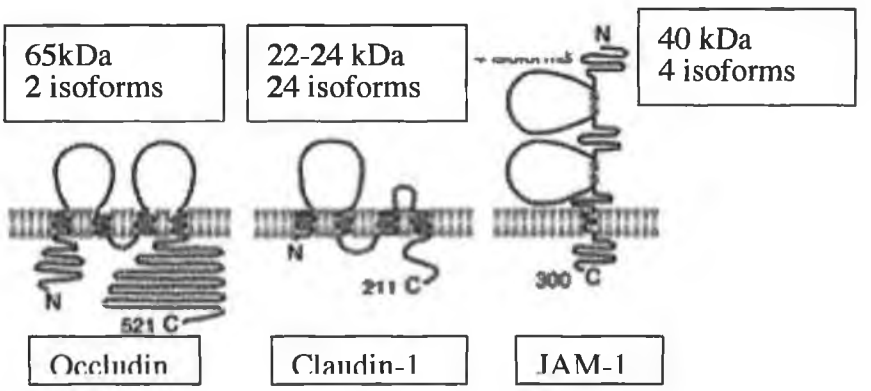

Fig. 1.18. Integral membrane TJ proteins [Schneeberger *et al.,* 2004].

1.7 Mechanical Forces and Tight Junctions

Because vascular pathologies exhibiting altered vessel hemodynamic loading with associated remodeling (e.g., atherosclerosis, restenosis, retinopathy, inflammatory lung disease, sepsis, edema, and systemic carcinomas) frequently correlate with compromised endothelial barrier integrity [Harhaj *et al.,* 2004; Van Nieuw-Armerongen *et al.,* 2002; Tinsley *et al.,* 2004; Koschinsky 2004], we hypothesise a direct link between mechanical forces and EC permeability.

In fact, mechanoregulation of vascular endothelial tight junction protein expression has also been confirmed in 2 recent, albeit contrasting, studies. In a report by DeMaio *et al.* exposure of BAECs to shear stress reduced occludin mRNA and protein expression in parallel with an increase in tyrosine phosphorylation and endothelial permeability (monitored as hydraulic conductivity) [DeMaio *et al.,* 2001]. In a more recent article,

Conklin *et al.* demonstrated shear stress–induced up regulation of occludin mRNA [Conklin *et al.*, 2002]. The contrast between these studies may reflect differences in the shearing paradigms used. Overall, however, these results show us that tight junction protein expression (and, therefore, permeability) in vascular endothelial cells are subject to regulation by hemodynamic forces. Consistent with this, a recent study by Shin *et al.* demonstrated reduced transendothelial permeability to albumin after exposure of human umbilical vein endothelial cells (HUVECs) to chronic pulse pressure (cyclic pressure), an important hemodynamic component of pulsatile blood flow (i.e., in addition to, but distinct from, cyclic strain) [Shin *et al.,* 2003].

1.7.1 Mechanical Forces and Atherosclerosis

The accumulation of atherogenic substances such as low density lipoprotein (LDL), growth factors, and fibrinogen in the intima of arteries is thought to be one of the initiating factors of atherosclerosis [Fry 1987; Curmi *et al.*, 1990; Nielson *et al.*, 1992]. It has been shown experimentally that the endothelium in regions of the arterial system that tend to collect LDL and other macromolecules may have compromised barrier function compared to areas which do not [Bell *et al.*, 1974; Kao *et al.*, 1995; Stemerman *et al.*, 1986]. Furthermore, these areas have been found to correspond *in vivo* to branches, bifurcations, and curves in the arterial system that may have certain common hemodynamic factors such as low shear stress and boundary layer separation [Kao *et al.*, 1995; Stemerman *et al.*, 1986; Herrmann *et al.*, 1994]. Data such as these lead us to assume that the endothelium is rendered dysfunctional by the aberrant hemodynamic stimuli in these areas, leading to increased permeability and the build up of atherogenic

assume that the endothelium is rendered dysfunctional by the aberrant hemodynamic stimuli in these areas, leading to increased permeability and the build up of atherogenic particles in the intima [Kao *et al.*, 1995; Penn *et al.*, 1992; Fry 1987].

Fig. 1.19 readily illustrates the fact that atherosclerotic plaques do not occur at random locations in the vasculature but rather at areas where there are unstable hemodynamic features. In this diagram the aorta has been stained with Oil-Red-O, which shows lipid-rich atherosclerotic lesions in the arterial wall. Despite the systemic nature of the hyperlipidemia, the lesions in this animal are largely confined to areas around curvatures and branch points, suggesting that patterns of blood flow are important in localising this disease [Topper *et al.*, 1999].

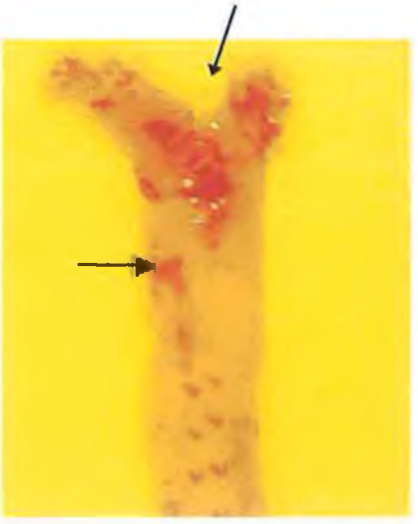

Fig. 1.19. Non-random localisation of early atherosclerotic lesions in a Watanabe heritable hyperlipidemic (WHHL) rabbit. The upper arrow indicates the arch of the thoracic aorta, which has been opened up to display the intimal lining en face. The lower arrow indicates the localised nature of the atherosclerotic lesions adjacent to the paired

1.8. Tight Junctions and Disease

An intact tight junction is central to normal cell function and disruption of this junction can lead to many pathophysiological conditions such as the development of atherosclerosis which has been previously discussed. Enteropathogenic *E. coli,* an important cause of third world infant mortality, produces a protein, EspF, that can increase TJ permeability and redistribute occludin [McNamara *et al.*, 2001]. *V. cholera* has also been found to produce a cytotoxin, which can function as a protease and has been shown to digest occludin bands.

Decreased occludin expression is observed in parallel with diminished barrier function, in a wide variety of tissues and inflammatory conditions. Collagenous colitis occurs with decreased trans-epithelial resistance and decreased expression of occludin and claudin-4 in the colon [Burgel *et al.*, 2002]. The best investigated inflammatory model with regard to epithelial barrier function is however inflammatory bowel disease (IBD), a condition that has long been associated with altered epithelial barrier function [Hollander 1988]. The actively inflamed tissue component is observed to be leaky. Occludin has been observed to be down regulated even in non-actively inflamed tissue in ulcerative colitis [Kucharzik *et al.*, 2001].

Another disease state that sees changes to tight junctions is diabetes. Occludin expression is significantly reduced in ECs of the Blood Retinal Barrier (BRB) of diabetic rats. This occurred simultaneously with increased permeability to macromolecules [Antonetti *et al.,* 1998; Barber *et al.*, 2003]. Elevated VEGF is thought

to be responsible for these changes as well as for altered occludin phosphorylation state. Similarly, occludin content of brain capillaries was sharply reduced in diabetic rats, even though ZO-1 content was unchanged [Antonetti *et al.*, 1998].

Tight junction integrity is also compromised in epithelial and endothelial cancers. Although the undermining of TJ barriers occurs early in the process of cancer, it is unclear yet whether the process is in any way causal or more precisely, promotional, to the process of cancer [Feldman *et al.*, 2005]. Processes and agents known to augment the promotional stage of cancer induce TJ leakiness [Mullin *et al.,* 1997]. Oncogenes are likewise known to induce TJ leakiness [Li *et al.,* 2000]. Occludin expression has been shown to decrease progressively with the carcinoma grade in human endometrial cancer [Tobiok *et al.,* 2004], moreover occludin distribution and expression are similarly altered in increasing Gleason grades of prostate cancer [Busch *et al.,* 2002]. In addition, microvessels of human brain tumors, whose TJ leaks are responsible for cerebral edema in certain types of brain cancer, are another example of downregulation of occludin in cancer, a phenomenon perhaps attributable to increased VEGF secretion [Davies 2002].

1.9 Relevance and Objectives of this Study

Loss of tight junction function has been implicated in many pathophysiological conditions such as atherosclerosis. The areas where permeability has been compromised are not random and frequently correlate to areas of unstable hemodynamic stimulation and endothelial dysfunction. It is therefore the aim of this study to examine how hemodynamic force, in particular cyclic strain, regulates vascular endothelial tight

junction assembly and barrier function at the molecular and cellular level.

To this end the tight junction proteins occludin and ZO-1 have been investigated. In particular the effect of physiological levels of cyclic strain on occludin and ZO-1 protein and mRNA expression, subcellular localisation and co-association has been examined. Phosphorylation of these proteins is known to be a key event in TJ regulation and as such has been examined in-depth. These changes have also been correlated to changes in TJ function by means of a permeability assay. The mechanotransduction pathway by which the TJ proteins detect and respond to cyclic strain has also been investigated.

To our knowledge, this is the first in-depth investigation of this physiologically significant phenomenon, and as such, it enhances our overall understanding of how hemodynamic forces regulate tight junctions within the vascular endothelium.

The main aim of this thesis therefore is to examine the effect of cyclic strain on the TJ proteins occludin and ZO-1. The findings of this research project have been divided into three results chapters with the following objectives:

Chapter 3:

The aim of this chapter is firstly to examine the regulatory effects of cyclic strain on occludin and ZO-1 protein expression, mRNA levels, subcellular localisation and co-association. The effect on actin organisation is also examined, as is the effect of strain on transendothelial permeability.

Chapter 4:

The aim of this chapter is firstly to determine any changes in the phosphorylation states of occludin and ZO-1 following strain. Secondly, the pathways by which occludin and ZO-1 are phosphorylated will be examined using pharmacological inhibitors. Thirdly the relationship between these phosphorylation events and the downstream changes reported in the previous chapter will be examined.

Chapter 5:

The specific aim of this chapter is to investigate the signaling mechanisms involved in cyclic strain-regulation of occludin and ZO-1 in vascular ECs, with specific emphasis on G-proteins, integrins and PTK-mediated pathways.

Chapter 2

Materials and Methods

All reagents used in this study were of the highest purity commercially available and were of cell culture standard when applicable.

2.1 Materials

AGB Scientific (Dublin, Ireland)

Whatmann chromatography paper

Amersham Pharmacia Biotech (Buckinghamshire, UK)

Anti-mouse 2° antibody, HRP-conjugated

Anti-rabbit 2° antibody, HRP-conjugated

ECL Hybond nitrocellulose membrane

ECL Hyperfilm

Rainbow molecular weight marker, broad range (6-175kDa)

Protein-A Sepharose beads

Protein-G Sepharose beads

Bachem UK Ltd. (St. Helens, UK)

Linear RGD peptide

Cyclic RGD peptide

Bio Sciences Ltd (Dun Laoghaire, Ireland)

DMEM

dNTP's

DEPC-treated water

Trizol® reagent

Calbiochem (San Diego, CA)

Dephostatin-Tyrosine phosphatase inhibitor

Genistein-Protein tyrosine kinase inhibitor

NSC23766-Rac-1 inhibitor

Pertussis toxin- Gi-protein inhibitor

PD98059-MEK inhibitor

PD169316-p38 inhibitor

PMA-PKC activator

ROCK (Y-27632)-Rho kinase inhibitor

Rottlerin-PKC inhibitor

Corriell Cell Repository (NJ, USA)

Bovine Aortic Endothelial Cells

Cells derived from the thoracic aorta of a one-year-old male Hereford cow

Passages 7-15 used

Costar (Buckinghamshire, UK)

Transwell®-Clear plates (6 well format, 0.4 μm pore size, 24 mm filter diameter)

Dako Cytomation (UK)

Dako mounting media

Dunn Labortechnik GmBH (Asbach, Germany)

6-well Bioflex® plates

Flexcell International Corp. (Hillsborough, NC)

Flexercell® Tension Plus™ FX-4000T™ system

Scientific Imaging Systems (Eastman Kodak Group, Rochester, NY)

Kodak 1D image analysis software

Molecular Probes (Oregon, USA)

Alexia-conjugated anti-rabbit IgG

Alexia-conjugated anti-mouse IgG

Alexia-Phalloidin 568

MWG Biotech (Milton Keynes, UK)

Occludin primer set

ZO-1 primer set

GAPDH primer set

PALL Corporation (Dun Laoghaire, Ireland)

Biotrace nitrocellulose membrane

Pierce Chemicals (Cheshire, UK)

BCA protein assay kit

Supersignal West Pico chemilumescent substrate

Promega (Madison, WI)

Taq DNA Polymerase

MLV-RT

RNase H

Oligo-dT

Santa Cruz Biotechnology (CA, USA)

Anti-occludin rabbit polyclonal IgG

Sarstedt (Drinagh, Wexford, Ireland)

T25 tissue culture flasks

T75 tissue culture flasks

T175 tissue culture flasks

6-well tissue culture plates

5,10 and 25 ml serological pipettes

15 and 50 ml falcon tubes

Sigma Chemical Company (Poole, Dorset, England)

2-mercaptoethanol

Acetic Acid

Acetone

Agarose

Ammonium Persulphate

Bisacrylamide

Bovine Serum Albumin

Brightline Haemocytometer

Bromophenol blue

Chloroform

Dapi Nuclear Stain

EDTA

EGTA

Ethidium Bromide

Fibronectin

Foetal Calf Serum

FITC-Dextran 40kDa

Tris Acetate

Glycerol

Glycine

Hanks Balanced Salt Solution

Hydrochloric acid

Isopropanol

Leupeptin

Methanol

Penicillin-Streptomycin (100x)

Ponceau S

Potassium Chloride

Potassium Iodide

Potassium Phosphate (Dibasic)

Phosphatase Inhibitor Cocktail

Protease Inhibitor Cocktail

RPMI-1640

Sodium Chloride

Sodium Hydroxide

Sodium Orthovanadate

Sodium Phosphate

Sodium Pyrophosphate

SYBER green Jump Start Taq Ready Mix

SDS

TEMED

Tris Base

Tris Cl

Triton X-100

Trypsin-EDTA solution (10x)

Tween 20

Qiagen (West Sussex, U.K.)

SYBR Green® PCR Kit

Zymed Laboratories Inc., (San Francisco)

Anti-ZO-1 rabbit polyclonal IgG

Anti-occludin rabbit polyclonal IgG

Anti-ZO-1 mouse monoclonal IgG

Anti-occludin mouse monoclonal IgG

Phosphoprotein Antibody Sampler Pack: phospho-serine, threonine and tyrosine antibodies

2.2 Cell Culture Methods

All cell culture techniques were carried out in a clean and sterile environment using a Bio Air 2000 MAC laminar flow cabinet. Cells were visualized using an Olympus CK30 phase contrast microscope unless otherwise stated.

2.2.1 Culture of Bovine Aortic Endothelial Cells

Differentiated BAECs were obtained from Coriell Cell Repository, New Jersey, USA. (CAT NO: AG08500). The cells were derived from a one-year-old male Hereford cow. The thoracic aorta was removed immediately post-mortem on 22/10/85. Cells were maintained in RPMI-1640 supplemented with 10% (v/v) fetal bovine serum (FBS), 100 U/ml penicillin and 100 μg/ml streptomycin. Cells were cultured in T175 cm^2, T75 cm^2, T25 cm^2 flasks and 6 well plates. In the case of cyclic strain experiments, cells were grown on Bioflex® series culture plates which have a flexible, pronectin bonded growth surface. In the case of dextran permeability studies cells were grown on 24 mm round polyester filters, (0.4 μm pore size, 6 well format). Cells between passages 7–15 were used in these experiments.

BAECs are a strongly adherent cell line forming a confluent contact-inhibited monolayer, with a distinct cobblestone morphology. As such, trypsinisation was necessary for sub-culturing or harvesting of cells. For trypsinisation, growth media was removed from the flask and the cells were gently washed two times in Hanks buffered saline solution (HBSS) to remove α-macroglobulin, a trypsin inhibitor present in FBS. A

suitable volume of trypsin / ethylenediamine tetracetic acid (EDTA) (10% v/v trypsin EDTA in HBSS) was added to the flask and incubated for 5 min or until all the cells were removed from the flask surface. Trypsin was inactivated by the addition of growth medium and the cells were removed from suspension by centrifugation at 2500 x g for 6 min (Centrifuge EBA8 [Hettich, Germany]). Cells were then resuspended in culture medium and typically diluted 1:5 into culture flasks, or cryogenically preserved. Cells were incubated in a humidified atmosphere 5% v/v CO_2 at 37°C.

2.2.2 Cell counting

Cells counts were performed using a Sigma brightline hemocytometer slide. Trypan blue exclusion dye was routinely used to determine cell viability. 20 µl of trypan blue was added to 100 µl of cell suspension, the mixture was left to incubate for two min. 20 µl of this mixture was loaded to the counting chamber of the hemocytometer and cells visualized by light microscopy. Viable cells excluded the dye whilst dead cells stained blue. The number of cells was calculated using the following equation:

Average Cell No. x dilution factor x $1x10^4$ (area under cover slip mm^3) = Viable cells/ml

2.2.3 Cryogenic preservation and recovery of cells

For long-term storage of cells, BAECs were maintained in liquid nitrogen in a cryofreezer unit. Cells to be stored were centrifuged following trypsinization and the resultant pellet was resuspended in 20% (v/v) FBS containing dimethylsulphoxide

(DMSO) at a final concentration of 10% (v/v). 1 ml aliquots were transferred to sterile cryovials and frozen in a –80°C freezer at a rate of –1°C/minute using a Nalgene cryo-freezing container. Following overnight freezing at –80°C, the cryovials were transferred to a cyrofreeze unit (Thermoylen locator jr. cryostorage system). Cells were recovered from long-term storage by rapid thawing at 37°C and resuspended in 10 ml of growth medium followed by centrifugation at 3500 rpm for 5 min. The resultant cell pellet was resuspended in fresh medium and transferred to a culture flask. The following day, media was removed and cells were washed in HBSS and fresh culture media added.

2.2.4 Cyclic Strain

For cyclic strain studies, BAECs were seeded into 6-well Bioflex® plates (Dunn Labortechnik GmBH - Asbach, Germany) at a density of approximately $6x10^5$ cells/well. Bioflex® plates contain a pronectin-coated silicon membrane bottom which enables precise deformation of cultured cells by microprocessor-controlled vacuum (Banes *et al.*, 1985). When cells were two days post-confluent, a Flexercell® Tension Plus™ FX-4000T™ system (Flexcell International Corp. - Hillsborough, NC) was subsequently employed to apply a physiological level of cyclic strain to each plate (0-5% strain, 60 cycles/min, 0-24 h). Control plates with static endothelial cell cultures were placed in the same incubator.

Following 24 h of cyclic strain, cells were either harvested for (i) Western blotting and Immunoprecipitation (IP) purposes; (ii) for analysis of mRNA expression by Real-Time PCR; (iii) for measurement of transendothelial permeability; (iv) or fixed

in situ for immunocytochemical analysis.

2.2.5 Treatment with pharmacological inhibitors:

Cells were routinely cultured for at least 2 passages prior to treatment with pharmacological inhibitors. For these experiments, BAECs were grown until 2 days post confluent, after which the growth media was removed and cells were washed 3 times in HBSS. Inhibitors were diluted in RPMI-1640 supplemented with antibiotics. For DMSO-soluble inhibitors, a suitable stock concentration was prepared so that the final concentration of DMSO in working solutions was less than 0.5%.

For pharmacological inhibition studies with PKC and Tyrosine Phosphatase inhibitors, cells were incubated in complete media containing Rottlerin (20 μM), PMA (100 nM) or Dephostatin (20 μM) approximately 1 hour prior to initiation of cyclic strain (and subsequently for the duration of the experiment). For cycloheximide studies, cells were incubated in complete media containing 20 μg/ml cycloheximide.

For pharmacological inhibition of signal transduction pathways, the following inhibitors were used; pertussis toxin (100 ng/ml), which inhibits Gi-proteins, linear RGD peptide (0.5 mM) and cyclic RGD (0.1 μM), that inhibit integrins, genistein (50 μM), which inhibits protein tyrosine kinases, ROCK inhibitor Y-27632 (10 μM), which inhibits Rho kinase, NSC23766 (50 μM), which inhibits Rac GTPase, PD98059 (10 μM), which inhibits MEK and PD169316 (10 μM), which inhibits p38. Inhibitors were applied for at least 1 h prior to initiation of cyclic strain (and subsequently for the

duration of the experiment) with the exception of pertussis toxin, which was applied for 4 h before commencement of cyclic strain to insure full inhibition of Gi-proteins.

Concentrations used were taken from current literature and based on manufacturers recommendations in tandem with an inhibitor concentration gradient.

2.2.6 Preparation of whole cell lysates

For purposes of Western blotting and IP, cells were washed twice in phosphate buffered saline (PBS) before being harvested using a cell scraper. Pelleted cells were subsequently lysed in a modified RIPA buffer (50 mM HEPES, 150 mM NaCl, 10 mM EDTA, 10 mM sodium pyrophosphate, 1 mM sodium orthovanadate, 100 mM NaF and 1% Triton X-100), supplemented with protease/phosphatase inhibitor cocktails (1/100 dilution of stock, Sigma-Aldrich), Following gentle rotation at 4°C for 60 min, lysates were centrifuged at 13,000 rpm for 20 min at 4°C to sediment any triton soluble material and generate a supernatant fraction. Pelleted triton-insoluble material was subsequently resuspended, according to the method of Sakakibara *et al.* in SDS-IP buffer (25 mM HEPES, 4 mM EDTA, 25 mM NaF, 1% SDS and 1 mM sodium orthovanadate) and homogenised using a Kontes homogeniser before being passed 10 times through a 27-guage needle [Sakakibara *et al.,* 1997]. Following gentle rotation at 4°C for 30 min, lysates were centrifuged at 13,000 rpm for 30 min at 4°C to generate an SDS-soluble supernatant fraction which was combined with the triton-soluble fraction to yield a total BAEC lysate (used for all Western Blotting and IP studies).

Samples were stored at –20°C for short-term storage or –80°C for long-term storage.

2.2.7 Bicinchoninic Acid (BCA) protein microassay

In this assay Cu^{2+} reacts with the protein under alkaline conditions to produce Cu^{+}, which in turn reacts with BCA to produce a coloured product [Pierce, 1997]. Two separate reagents were supplied in this commercially available assay kit (Pierce Chemicals), **A;** an alkaline bicarbonate solution and **B;** a copper sulphate solution. 1 part solution B is mixed with 50 parts solution A; 200 µl of this mixture is added to 10 µl of protein lysate or BSA standards (standard curve in the range 0-2 mg/ml). The 96-well plate is incubated at 37°C for 30 min and the absorbance read at 560nm using a microtitreplate reader.

2.3 Western Blotting Studies

2.3.1 Western Blots

SDS-PAGE was conducted on BAEC lysates (8 µg {ZO-1} or 10 µg {occludin} of protein) according to the method of Laemmli *et al.* under reducing conditions using 6% (ZO-1) or 12% (occludin) polyacrylamide gels [Laemmli *et al.,* 1970]. Samples were mixed with 4X loading buffer (8% SDS, 20% β-mercatoethanol, 40% glycerol, Brilliant Blue R in 0.32M Tris pH6.8) and boiled at 95°C for 5 minutes, then immediately placed on ice. The gel was electrophoresed in resevior buffer (0.025M Tris pH 8.3; 0.192M

Glycine; 0.1% (w/v) SDS) at 40 milliamps (mA) per gel using an Atto vertical mini-electrophoresis system until the dye front reached the bottom of the gel.

6% and 12% resolving gels and 5% and 3% stacking gels were prepared as follows:

Occludin Resolving Gel (12%):

3.94 ml Buffer A (1.5M Tris pH 8.8)
4.73 ml 40% acrylamide stock
6.92 ml distilled water
157 µl 10% (w/v) SDS
79 µl 10% (w/v) ammonium persulphate
24 µl TEMED

ZO-1 Resolving Gel (6%):

3.94 ml Buffer A (1.5M Tris pH 8.8)
2.36 ml 40% acrylamide stock
9.3 ml distilled water
157 µl 10% (w/v) SDS
79 µl 10% (w/v) ammonium persulphate
24 µl TEMED

Occludin Stacking Gel (5%):

1 ml Buffer B (0.5M Tris pH 6.8)
0.5 ml 40% acrylamide stock
2.46 ml distilled water
40 µl 10% (w/v) SDS
20 µl 10% (w/v) ammonium persulphate
6 µl TEMED

ZO-1 Stacking Gel (3%):

1 ml Buffer B (0.5M Tris pH 6.8)
300 µl 40% acrylamide stock
2.66 ml distilled water
40 µl 10% (w/v) SDS
20 µl 10% (w/v) ammonium persulphate
6 µl TEMED

The resolved proteins were electroblotted onto a nitrocellulose membrane (Amersham Pharmacia Biotech - Buck, UK) in a tris/glycine buffer system (25 mM tris, 0.192 mM glycine, 10% methanol pH 8.3) using a wet blotter (ZO-1) or semi-dry blotter (Occludin), (HorizBlot, Atto Corporation - Tokyo, Japan). Membranes were subsequently blocked for 1.5 h at room temperature in PBS-T (phosphate buffered saline, 0.1% Tween-20) containing 5% w/v BSA, before being immunostained with a specific primary antibody overnight at 4°C. Primary antibodies used were anti ZO-1 and anti-occludin rabbit polyclonal IgG (1:1500 and 1:500) (Zymed Laboratories Inc, San Francisco) or anti-occludin rabbit polyclonal IgG (1;1000) (Santa Cruz Biotechnology CA, USA). Membranes were subsequently given 3 washes with PBS-T prior to a 1.5 h incubation with a horse radish peroxidase-conjugated goat anti-rabbit secondary IgG (1:3000 and 1:2000) (Amersham Pharmacia Biotech - Buck, UK).

Anti-occludin rabbit polyclonal IgG is highly purified from rabbit antiserum by epitope chromatography using a GST-occludin coupled gel. The reactivity of this antibody with the occludin protein has been confirmed by Western blotting using total cell lysate derived from MDCK cells, human T84 cells, and rat fibroblasts by the manufacturers. With regards to Anti-ZO-1 rabbit polyclonal IgG, this antibody was purified from rabbit antiserum by antigen-affinity chromatography. In addition, its reactivity with ZO-1 protein has also been confirmed by the manufacturers.

Western immunoblots were developed with West Pico SuperSignal reagent (Pierce - Cheshire, UK) with subsequent visualization by exposure to X-ray film (Hyperfilm™ ECL™, Amersham Pharmacia Biotech - Buck, UK). For semi-

quantitative comparisons between bands, scanning densitometry was performed using NIH Image v1.61 software. Membranes were routinely stained with Ponceau S to normalize for protein loading/transfer.

Intra-assay Western blotting variability values ranged from 5% to 25%. With regards to inter-assay variability, data is normalized for baseline variability which can result from changes in protein expression and phosphorylation levels according to passage cell number etc. It also allows us to normalize for variability in absolute values or readings between experiments, which can arise from the detection method. This is particularly pertinent with gel densitometry. Variations in protein transfer, chemiluminescence efficiency, development conditions and film quality can all contribute significantly to the densitometric value assigned by NIH Image to any given protein band. As such, one frequently observes different absolute values (raw numbers) 'between' separate experiments, whilst observing the same densitometric trend between bands 'within' each experiment.

2.3.2 Immunoprecipitation (IP)

Following strain experiments, total BAEC lysate (combined triton- and SDS-soluble material) were prepared as described in section 2.2.6 and monitored by IP analysis for force-mediated changes in both occludin/ZO-1 phosphorylation state and occludin/ZO-1 co-association. IP was performed according to the method of Ferguson *et al.* with minor modifications [Ferguson *et al.*, 2000]. Cell lysate containing 200 μg protein was incubated with 5 μg of "pull down" antibody (anti-occludin / ZO-1 rabbit

polyclonal IgG – Zymed Laboratories Inc.), 20 μL of 10% BSA and 20 μL of protein-A Sepharose beads (ZO-1) or protein-G Sepharose beads (occludin) (Amersham Biosciences) to give a final reaction volume of 500 μL. Incubation proceeded overnight at 4°C or for 2 h at room temperature with continuous eppendorf rotation. Following incubation, beads were washed 4 times, twice with homogenization buffer containing 1% triton X-100 and twice with homogenization buffer alone. Beads were pelleted and resuspended in 50 μL of SDS-PAGE sample solubilisation buffer and heated for 10 min at 90°C. Beads were again pelleted [Capsulefuge (Tomy, Freemont, CA)] and the resulting solubilised proteins (supernatant) resolved by SDS-PAGE with Western blotting as described in section 2.3.1. For monitoring occludin/ZO-1 phosphorylation state, immunoblots were probed and visualized in the normal manner with phospho-serine, -threonine and –tyrosine antibodies (Phosphoprotein Antibody Sampler Pack, Zymed Laboratories Inc.) and densitometric data normalised to changes in occludin/ZO-1 protein expression. For monitoring occludin/ZO-1 co-association, immunoblots were probed and visualized in the normal manner with polyclonal anti-ZO-1 rabbit IgG as the 'pull down' antibody and polyclonal anti-occludin rabbit IgG as the primary antibody for Western Blot.

Occludin and ZO-1 Western blots respectively reveal doublets when the protein in question was immunoprecipitated from unstimulated endothelial cell lysates and resolved by SDS-PAGE. When gels were probed for phospho –Tyr, -Ser and –Thr, only one band was revealed. In each instance, the lower of the two ZO-1 bands, and the lower of the two occludin bands was seen to be phosphorylated. Moreover, interestingly we only obtained a single occludin band when we IP and immunoblot for occludin, despite

the observation of two bands when we simply Western blot for occludin. The molecular weight of this single band (50-55kDa) appears to concur with the molecular weight of the lower band in the occludin Western blot. Moreover, it also appears to be the phosphorylated species. The reason for this is unknown at present, although it likely stems from technical characteristics of the antisera and/or IP protocol employed.

2.4 Measurement of Transendothelial Permeability

Following strain experiments, cells in Bioflex® wells were trypsinized and re-plated at high density ($5x10^5$ cells/well) into Transwell®-Clear plates with polyester membrane inserts (6-well format, 0.4 μm pore size, 24 mm filter diameter - Costar, Buckinghamshire, UK). When cells had reached confluency (within 24 h), transendothelial permeability was monitored as previously described [Zink *et al.*, 1995], Briefly, RPMI 1640 complete media (supplemented with 10% serum and antibiotics) was added to the upper and lower chambers of the trans-well plate (upper: 1.5 ml, lower: 2.6 ml). At t=0, FITC-labelled dextran [FD40] (40 kDa, Sigma-Aldrich) was added to the abluminal chamber (to give a final concentration of 250 □g/ml) and diffusion of dextran across the monolayer allowed to proceed at 37°C for 2 h. Media samples (7 μl) were collected every 15 min from the subluminal compartment and monitored for FITC-dextran fluorescence at excitation and emission wavelengths of 490 and 520 nm, respectively (Perkin-Elmer Luminescence Spectrometer LS50B with micro-plate reader attachment). Results are expressed as total subluminal fluorescence at a given time point

(from 0-120 min) expressed as a percentage of total abluminal fluorescence at t=0 min (i.e. %TEE of FD40 or % Trans Endothelial Exchange).

2.5 Immunocytochemistry

2.5.1 Standard and Confocal Immunocytochemistry

Following strain experiments, cells were prepared for immunocytochemical analysis according to the method of Groarke *et al.* with minor modifications [Groarke *et al.*, 2001]. Cells in Bioflex® wells were washed twice with PBS and fixed with 3% formaldehyde for 15 min. Cells were then permeabilised with 0.2% Triton X-100 for 5 min, blocked in 5% BSA for 30 min and incubated with primary antibody (8 μg/ml polyclonal anti-occludin rabbit IgG or 1 μg/ml polyclonal anti-ZO-1 rabbit IgG – Zymed Laboratories Inc.), or anti -occludin rabbit polyclonal IgG (1:100) (Santa Cruz Biotechnology CA, USA) for 2 h. This was followed by incubation for 1 h with secondary antibody (1:400 Alexia-conjugated goat anti-rabbit IgG - Molecular Probes, Eugene, OR). Bioflex® well membranes were subsequently excised by scalpel, mounted onto slides and sealed using Dako mounting media (Dako Cytomation, Cambridgeshire, UK) and cover slips for visualisation by both standard fluorescent microscopy (Olympus BX50) and confocal microscopy (Zeiss LSM-510 META Axiplan 2 Upright Confocal Microscope). Suitable controls were included in all experiments. These included exclusion of primary antibody and staining of cell nuclei with DAPI (Sigma-Aldrich).

2.6 RNA Preparation Methods

2.6.1 RNA isolation

Trizol® is a ready-to-use reagent for the isolation of total RNA, DNA and/or protein from cells and tissues. Trizol® reagent maintains the integrity of the RNA while disrupting the cells and dissolving the cell components. Cells were lysed directly in culture plates by the addition of 1 ml of Trizol® per 10 cm^2. A volume less than this can result in contamination of the RNA with DNA. To ensure complete homogenisation, cells were lysed by passing through a pipette a number of times. The samples were then incubated for 5 min at room temperature to allow complete dissociation of nucleo-protein complexes. 0.2 ml of chloroform was added per ml of Trizol® reagent used and was then mixed vigorously for 15 sec before being incubated for 5 min at room temperature. Samples were then centrifuged at 12,000 x g for 15 min at 4°C. The mixture separated into a lower red, phenol-chloroform phase, an interphase and an upper colourless aqueous phase. RNA remains exclusively in the aqueous phase. The aqueous phase was carefully removed and transferred to a fresh, sterile tube. The RNA was precipitated out of solution by the addition of 0.5 ml of isopropanol per 1 ml of Trizol® used. Samples were incubated for 15 min at room temperature and then centrifuged at 12,000 x g for 10 min at 4°C. The RNA precipitate forms a gel-like pellet on the side of the tube. The supernatant was removed and the pellet washed in 1 ml of 75% ethanol per ml of Trizol® used followed by centrifugation at 7,500 x g for 5 min at 4°C. The resultant pellet was air-dried for 5-10 min before being resuspended in 50 µL DEPC-treated water. The sample was then stored at –80°C until used

2.6.2 Spectrophotometric analysis of nucleic acids:

DNA or RNA concentrations were determined by measuring the absorbance (using a quartz cuvette) at 260 nm, the wavelength at which nucleic acids absorb light maximally. A 50 μg/ml solution of DNA or 40 μg/ml solution of RNA (diluted with TE buffer- buffer -10mM Tris-HCL, 1mM EDTA, pH8.0) has an absorbance reading of 1.0 at this wavelength. In order to calculate the concentration of DNA/RNA in samples the following calculations were used;

For DNA: Abs @ 260nm x 50 x dilution factor = μg/ml

For RNA: Abs @ 260nm x 40 x dilution factor = μg/ml

The purity of the DNA or RNA samples was established by reading the absorbance at 260 nm and the absorbance at 280 nm and then determining the ratio between the two (A_{260}/A_{280}). Pure DNA which has no protein impurities has a ratio of 1.8 whereas pure RNA has a ratio of 2.0. Lower ratios indicate the presence of proteins, higher ratios imply the presence of organic reagents.

2.6.3 Reverse Transcription Polymerase Chain reaction (RT-PCR)

PCR has over the last 20 years proved to be an important and powerful tool for amplifying small quantities of DNA for analysis. RT-PCR is a modification of this technique in which small quantities of specific messenger RNA (mRNA) are analysed.

Total RNA is isolated using oligo-dT primers, which is subsequently converted

to copy DNA (cDNA) using the enzyme reverse transcriptase. cDNA of interest was amplified by PCR using gene-specific primers. Expression of GAPDH (a house-keeping gene) was monitored in tandem with the gene of interest, to serve as a loading control and to facilitate semi-quantitative analysis of expression of genes of interest. The ratio of gene X to GAPDH typically served as a means of determining relative amounts of the target genes in each reaction.

2.6.4 Reverse Transcription

Reverse transcription was preformed using Moloney Murine Leukemia Virus Reverse Transcriptase (M-MLV RT) in accordance with manufacturers specifications with some minor modifications [Roth *et al.,* 1985; Sambrook *et al.,* 1989]. 0.5 μg of total RNA (isolated as described in section 2.6.1) was mixed with 0.125 μg oligo-dT primers and the reaction mixture brought to a final volume of 12 μl with DEPC water. This mixture was heated for 10 min at 70°C to allow annealing of oligo-dT primers to the polyA tail of mRNA. Following this, PCR tubes were immediately cooled on ice and the remaining components of the reaction were added as follows:

MLV 5X Reaction Buffer 5 μl

10 mM dNTP 3 μl

MLV-RT 200 units

The mixture was then made up to a final volume of 25 μl using DEPC water and incubated for 60 min at 42°C. Contaminating RNA was subsequently removed by the

addition of 1 µl of RNase H (2 units/µl) at 37°C for 20 min. cDNA samples were then either used immediately or stored at –80°C until required.

2.6.5 Polymerase Chain Reaction

A. Standard PCR

A 50 µl PCR reaction mixture was prepared as follows;

RNase free water 36.5 µl

10X reaction buffer 5 µl

10 mM dNTP 1 µl

25 mM MgCl 3 µl

10 µM Forward primer 1 µl

10 µM Reverse primer 1µl

Taq Polymerase 0.5 µl

cDNA sample 2 µl

The mixture was overlaid with 50µl of mineral oil and then placed in a Hybaid PCR Thermocycler (SPRT 001). Samples were subjected to an initial incubation of 92°C for 2 min followed by 30 cycles comprising of the following steps: 92°C for 1 min, annealing temperature for 2 min and 72°C for 3 min. PCR products were removed from beneath the mineral oil and placed in fresh tubes before being subjected to agarose gel electrophoresis (see section 2.6.6).

Bovine Primers:

Occludin (220bp): 5'-agtggctcaggagctgccattgacttcacc-3' (For)

5'-aggtggatattccctgatccagtcgtcgtc-3' (Rev)

GeneBank accession number: Human, NM002538 [Ando-Akatsuka *et al.*, 1996].

ZO-1 (281bp): 5'-agcctcatctccaggcccttacctt-3' (For)

5'-cgtggttctgtctcatcatctcctc-3' (Rev)

GeneBank accession number: Human, NM003257 [Willott *et al.*, 1993]

GAPDH (337 bp) 5'aggtcatccatgaccacttt 3' (For)

5'ttgaagtcgcaggagacaa 3' (Rev)

Table 1 Primers used with standard and Real Time PCR

B. Real-Time PCR

Quantitative PCR was also carried out using a Real-Time Rotor-GeneRG-3000TM lightcycler (Corbett Research). The principle of Real-Time amplification detection is that the amount of fluorescence is proportional to the concentration of product in a reaction. Higher fluorescence indicates a higher concentration of a product. Reactions were set up in Real-Time PCR reaction tubes on ice. A master mix containing the following for each tube was prepared:

2 µl cDNA

12.5 µl SYBR green

8.5 µl Dnase-free water

23 µl of master mix was added to each tube along with 1 µl forward and reverse primers (see table 1). Tubes were spun down for 6 sec and placed in RealTime PCR machine Select RotorGene-5 program.

Each sample was assayed in triplicate, and the program used for the different primer sets was as follows;

Denaturing Phase: 95°C ;15 min	⎡	
Annealing Phase: 55°C ; 30 sec	⎢	45 cycles
Elongation Phase: 72°C ; 30 sec	⎣	

When the run had finished, a control GAPDH sample value was set to 1 and results graphed.

2.6.6 Agarose gel electrophoresis

Agarose gels were prepared by boiling the appropriate quantity of agarose in 100 ml of 1X TAE buffer (40 mM Tris-Acetate pH 8.2, 1 mM EDTA), gels were generally 1-2% (w/v) depending on the size of the DNA being visualised. Gels contained 0.5 µg ethidium bromide per 1 ml of agarose for visualization of DNA within the gel. When the

gel was hand-hot the gel was cast in a GibcoBRL Horizon gel electrophoresis apparatus. Samples were mixed with 6X gel loading buffer (40% w/v sucrose, 0.25% w/v bromophenol blue). 12.5 µl of PCR product was mixed with 3 µl of loading buffer and subsequently loaded. The gel was run at 100V in 1X TAE buffer until the blue dye front was approximately 0.5 cm from the end of the gel. DNA was visualized on a transilluminator and photographed for densitometric analysis using the Kodak 1D gel documentation system (Scientific Imaging Systems, Eastman Kodak Group, Rochester, NY). Standard PCR and the subsequent separation of products using agarose gel electrophoresis were implemented as a method to ensure primer specificity and to rule out the possibility of non-specific binding and primer dimerisation. PCR products that yielded a single band confirmed the suitability of primers sets for use with the more sensitive method of Real-Time PCR.

2.7 Statistical analysis

Results are expressed as mean ± SEM of a minimum of three independent experiments (n=3) unless otherwise stated. Statistical comparisons between groups of normalised densitometric data were performed using unpaired Student's *t*-test. For permeability assays, Two-Way ANOVA was used for comparison of samples with control (unstrained) groups. A value of $P \leq 0.05$ was considered significant.

Chapter 3

Examination of the effect of cyclic strain on the tight junction proteins occludin and ZO-1 and subsequent effects on transendothelial permeability in BAECs.

3.1 Introduction

3.2 Results

3.3 Discussion

3.4 Conclusion

3.1 Introduction

Among the physiological stimuli that impact upon the endothelium, mechanical or hemodynamic forces associated with blood flow are of central importance. These include cyclic circumferential strain, caused by a transmural force acting perpendicularly to the vessel wall, and shear stress, the frictional force of blood dragging against cells. (See section 1.3). These forces can have a profound impact on endothelial cells and in particular EC barrier function. The vascular endothelium constitutes a highly effective barrier that regulates fluid and solute balance in addition to movement of molecular/cellular components between bloodstream and tissues [Alexander *et al.*, 2002; Rubin *et al.*, 1999]. As such, regulation of endothelial barrier integrity (or permeability) is crucial for vascular homeostasis.

Barrier function is maintained by the regulated apposition of tight junctions, composed of occludin, ZO-1 and a complex network of additional proteins, in addition to adherens protein complexes, between adjacent endothelial cells. The organisation of these protein complexes is controlled by a number of physiological/pharmacological mediators. The link between barrier function and hemodynamic forces has been tantalising due to the evidence of compromised barrier function in many disease states with altered hemodynamic profile. In support of this, two earlier studies have indicated that shear stress may putatively regulate endothelial occludin expression and phosphorylation [DeMaio *et al.*, 2001; Conklin *et al.*, 2002], thereby implicating hemodynamic force as a putative physiological (and pathological) regulator of vascular endothelial permeability.

The aim of this chapter is to examine the regulatory effects of cyclic strain on occludin and ZO-1 protein expression, mRNA levels, subcellular localisation and co-association. The effect on actin organisation is also examined, as is the effect of strain on transendothelial permeability. The effect of cycloheximide is also examined in order to more thoroughly define the role of protein expression in barrier function.

3.2 Results

3.2.1. Cyclic strain regulation of occludin and ZO-1 protein and mRNA levels.

The regulatory effect of 5% cyclic strain on occludin and ZO-1 protein expression in BAECs was determined by measuring protein levels in cell lysate. Following exposure of BAECs to cyclic strain, (5%, 24 h), occludin protein expression increased significantly by 2.3 ± 0.1 fold compared to unstrained control (Fig. 3.1A). Immunoblots indicate that occludin migrates as two major bands (50 and 65 kDa), a finding reflected in other studies [DeMaio *et al.*, 2001]. Moreover, expression of both bands increased in response to strain. Following exposure of BAECs to cyclic strain, (5%, 24 h), ZO-1 protein expression increased significantly by 2.0 ± 0.3 fold compared to unstrained control (Fig. 3.1B). Immunoblots indicate that ZO-1 also migrates as two major bands (210 and 225 kDa), a finding also reflected in other studies [DeMaio *et al.*, 2001]. Moreover, expression of both bands increased in response to strain.

Following the observation that cyclic strain increased occludin and ZO-1 protein levels, the regulatory effect of cyclic strain was examined at the level of mRNA. Real-Time PCR was used to determine mRNA levels. GAPDH, a housekeeping gene was used as an internal control for normalising purposes. Following exposure of BAECs to cyclic strain (5%, 24 h), occludin mRNA levels increased significantly by 2.6 ± 0.4 fold compared to unstrained control (Fig. 3.2A). No significant change in mRNA levels for ZO-1 was evident following strain (Fig. 3.2B).

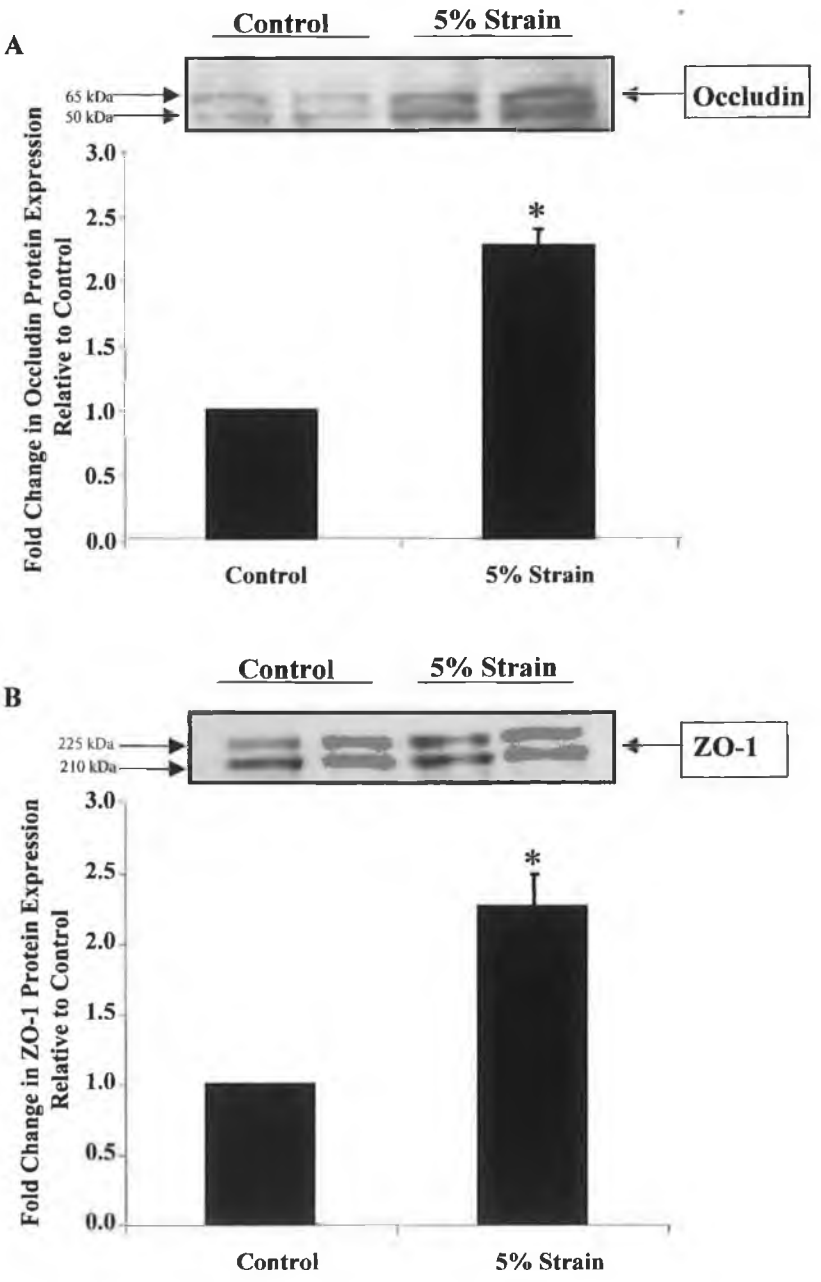

Fig. 3.1. Increased protein expression of occludin and ZO-1 following 5% strain for 24 h. BAECs were exposed to cyclic strain (5%, 24 h) and monitored for (A) occludin or (B) ZO-1 protein expression by Western blotting. Representative blots are shown above each graph. Densitometric intensity of both bands has been combined. Histograms represent fold change in band intensity relative to unstrained controls and are averaged from three independent experiments ±SEM; $*P \leq 0.05$ versus unstrained controls.

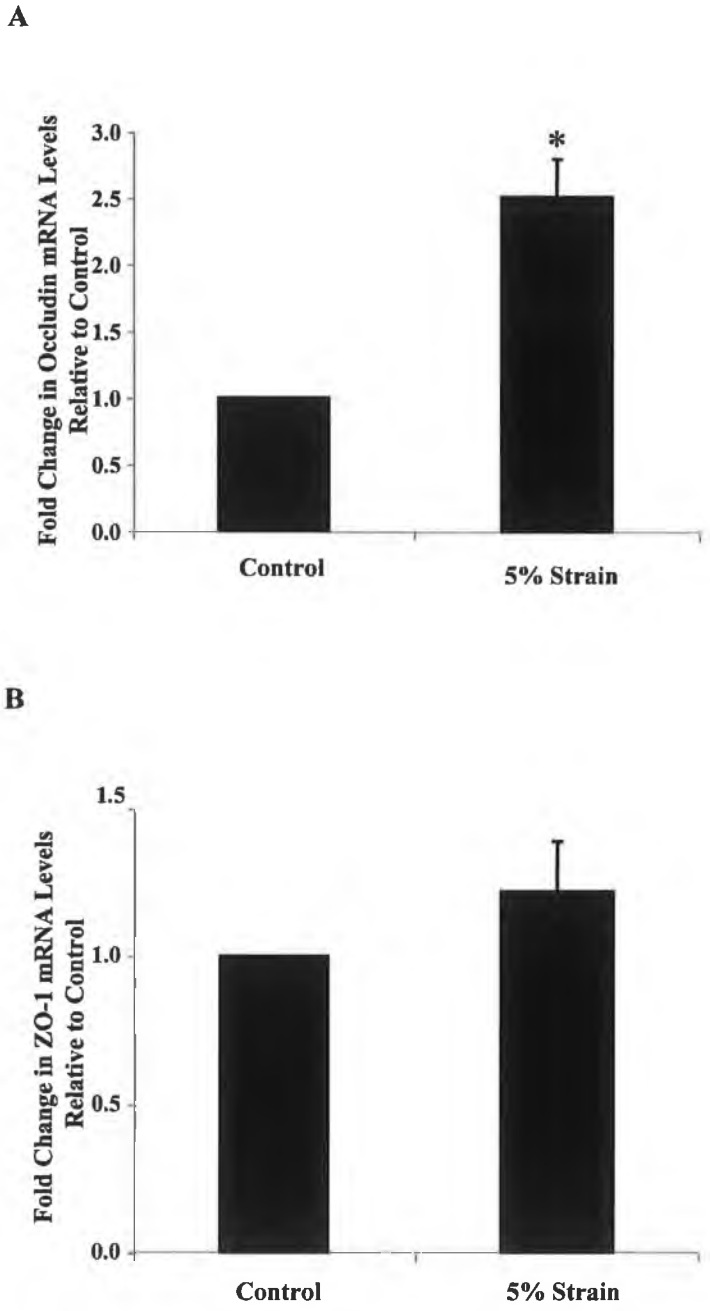

Fig. 3.2. Change in mRNA expression of occludin and ZO-1 following 5% Strain for 24 h. BAECs were exposed to cyclic strain (5%, 24 h) and monitored for (A) occludin or (B) ZO-1 mRNA using Real-Time PCR. Histograms represent fold change mRNA in strained samples to unstrained controls and are averaged from three independent experiments ±SEM; *$P \leq 0.05$ versus unstrained controls.

3.2.2. Cyclic strain affects subcellular localisation of occludin, ZO-1 and actin.

Subcellular localisation of occludin and ZO-1 were monitored by immunocytochemistry. Occludin: (i) control versus (ii) strain. ZO-1: (iii) control versus (iv) strain. Both proteins were monitored using standard fluorescence microscopy (1000x). Images are representative of at least three individual sets of experiments (Fig. 3.3). Occludin immunoreactivity was observed within the cell nucleus and cytosol (i) but became more concentrated along the cell border in response to chronic strain (ii). Moreover, ZO-1 immunoreactivity, which exhibited a discontinuous and jagged localisation pattern at the cell-cell border in unstrained cells (iii), became more continuous and well defined following strain (iv). White arrows indicate cell-cell border localisation.

Following exposure of BAECs to cyclic strain (5%, 24 h), subcellular arrangement of actin was also monitored by immunocytochemistry. Alexia Phalloidin was used to counterstrain F-actin filaments (Fig. 3.4). Actin: (i) control versus (ii) strain (200x) and (iii) control versus iv) strain (1000x). Actin was monitored using confocal fluorescence microscopy. Control cells expressed actin mainly in an unorganised fashion. However after exposure of cells to cyclic strain, actin localised at areas of cell-cell contacts and we observe actin cortical ring formation around the cell periphery.

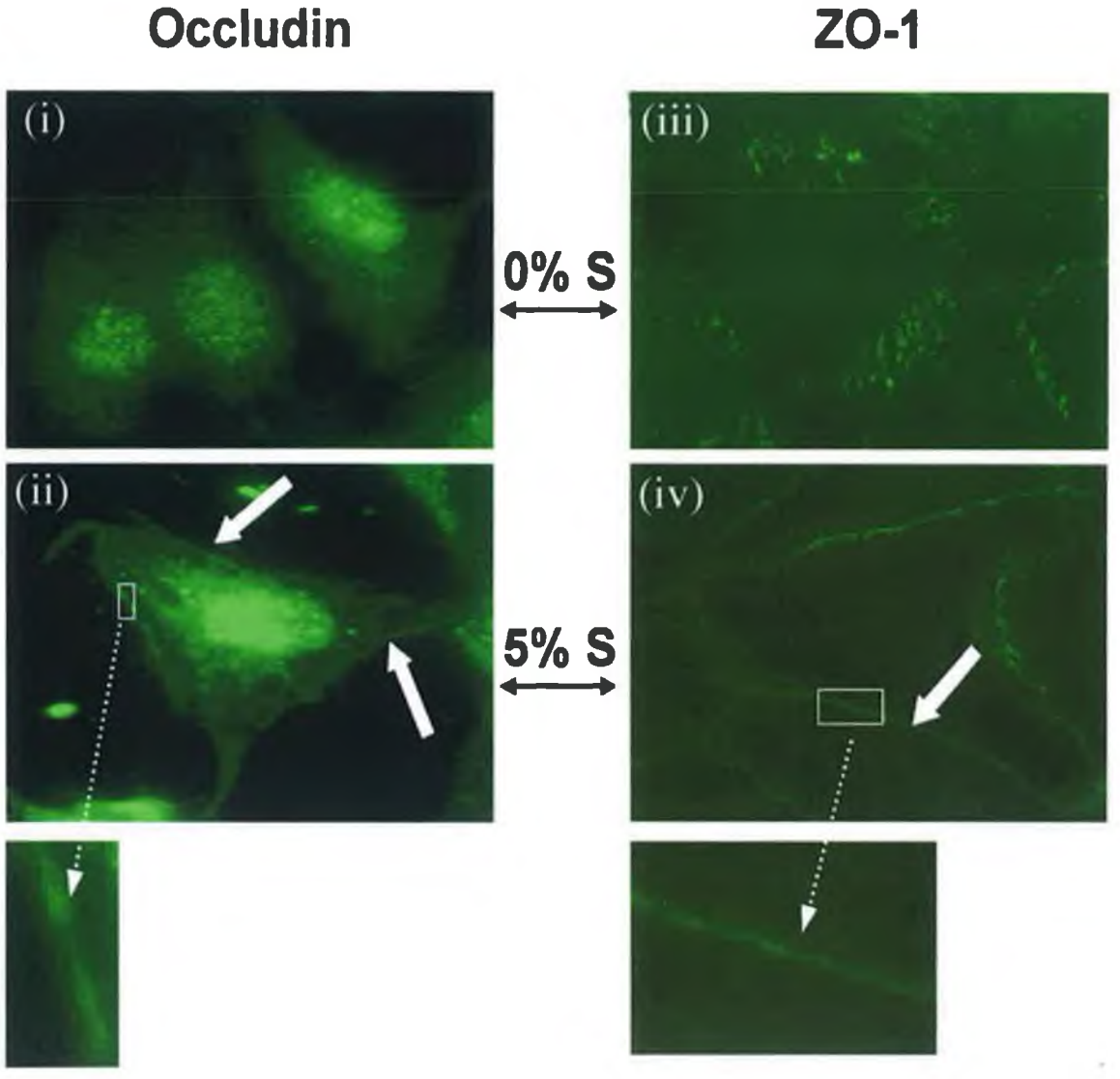

Fig. 3.3. Effect of cyclic strain on occludin and ZO-1 subcellular localization in BAECs. Following exposure of BAECs to cyclic strain (5%, 24 h), subcellular localisation of occludin and ZO-1 were monitored by immunocytochemistry. Occludin: (i) control versus (ii) strain. ZO-1: (iii) control versus (iv) strain. Both proteins were monitored using standard fluorescence microscopy (1000x). White arrows indicate cell-cell border localization. Images are representative of at least three individual sets of experiments.

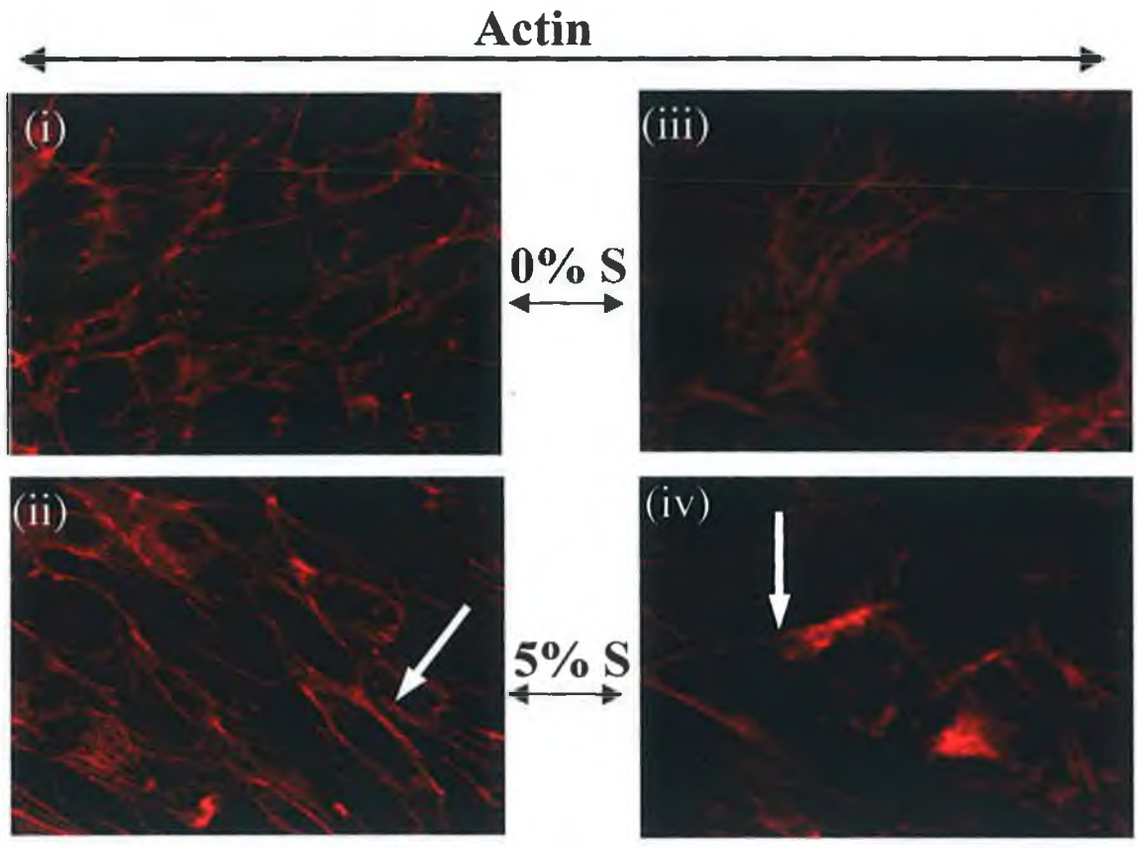

Fig. 3.4. Effect of cyclic strain on actin arrangement in BAECs. Following exposure of BAECs to cyclic strain (5%, 24 h), subcellular arrangement of actin was monitored by immunocytochemistry. Actin: (i) control 200x versus (ii) strain 200x (iii) control 1000x and iv) strain 1000x. Actin was monitored using confocal fluorescence microscopy. Images are representative of at least three individual sets of experiments White arrows indicate cell-cell border localisation.

3.2.3. Cyclic strain-dependent co-association of occludin/ZO-1.

Following exposure of BAECs to cyclic strain (5%, 24 h), co-association of occludin and ZO-1 was monitored in total BAEC lysates by IP. In response to strain, the level of occludin detected in anti-ZO-1 immunoprecipitates was seen to increase significantly by 2.0 ± 0.1 fold compared to unstrained control (Fig. 3.5).

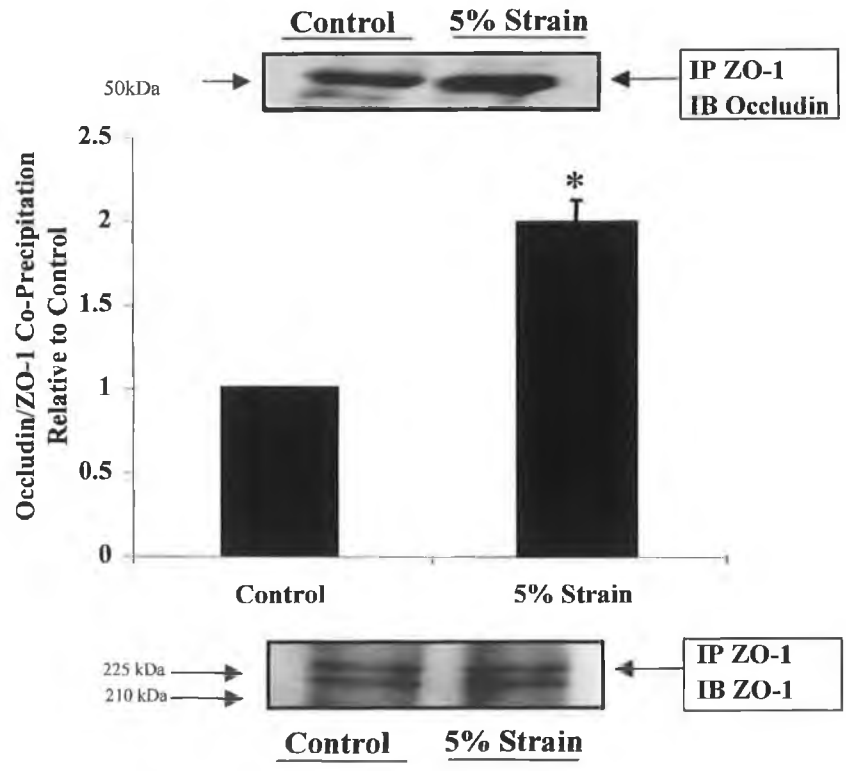

Fig. 3.5. Effect of cyclic strain on occludin and ZO-1 co-association in BAECs. Following exposure of BAECs to cyclic strain (5%, 24 h), co-association of occludin and ZO-1 was monitored by IP and Western blotting. Representative blot is shown above graph. Also included is a control blot measuring strain-dependent change in total ZO-1 protein i.e. lower blot. Histogram represents fold change in band intensity relative to unstrained control and is averaged from three independent experiments ±SEM; *$P \leq 0.05$ versus unstrained controls.

3.2.4. Effect of cyclic strain on transendothelial permeability.

As transendothelial permeability could not be directly monitored in Bioflex® plates post-strain, both control and "strain-conditioned (5%, 24 h)" BAECs were trypsinized and re-plated into Transwell®-Clear plates at a density sufficient to reach confluency within 24 h. BAECs were subsequently immunocytochemically monitored for localization of occludin and ZO-1 after 24 h (Fig. 3.6). Membrane localization of occludin in (i) unstrained versus (ii) strained BAECs, and of ZO-1 in (iii) unstrained versus (iv) strained BAEC is shown 24 h after re-plating. Both proteins were monitored using standard fluorescence microscopy (1000x). White arrows indicate cell-cell border localisation, confirming that strain-induced changes in ZO-1 and Occludin localisation fully persist 24 h after passage from Bioflex® plates into Transwell®-Clear plates.

BAEC monolayer permeability to 40 kDa FITC-dextran was subsequently monitored as described in section 2.4. Briefly, media samples (7 μl) were collected every 15 min from the subluminal compartment and monitored for FITC-dextran fluorescence at excitation and emission wavelengths of 490 and 520 nm, respectively. Cyclic strain significantly reduces BAEC permeability to FITC-dextran, with control cells showing a 3.5 ± 1.0 fold higher level of FITC-dextran in the subluminal chamber after 2 h relative to strained BAEC (Fig. 3.7).

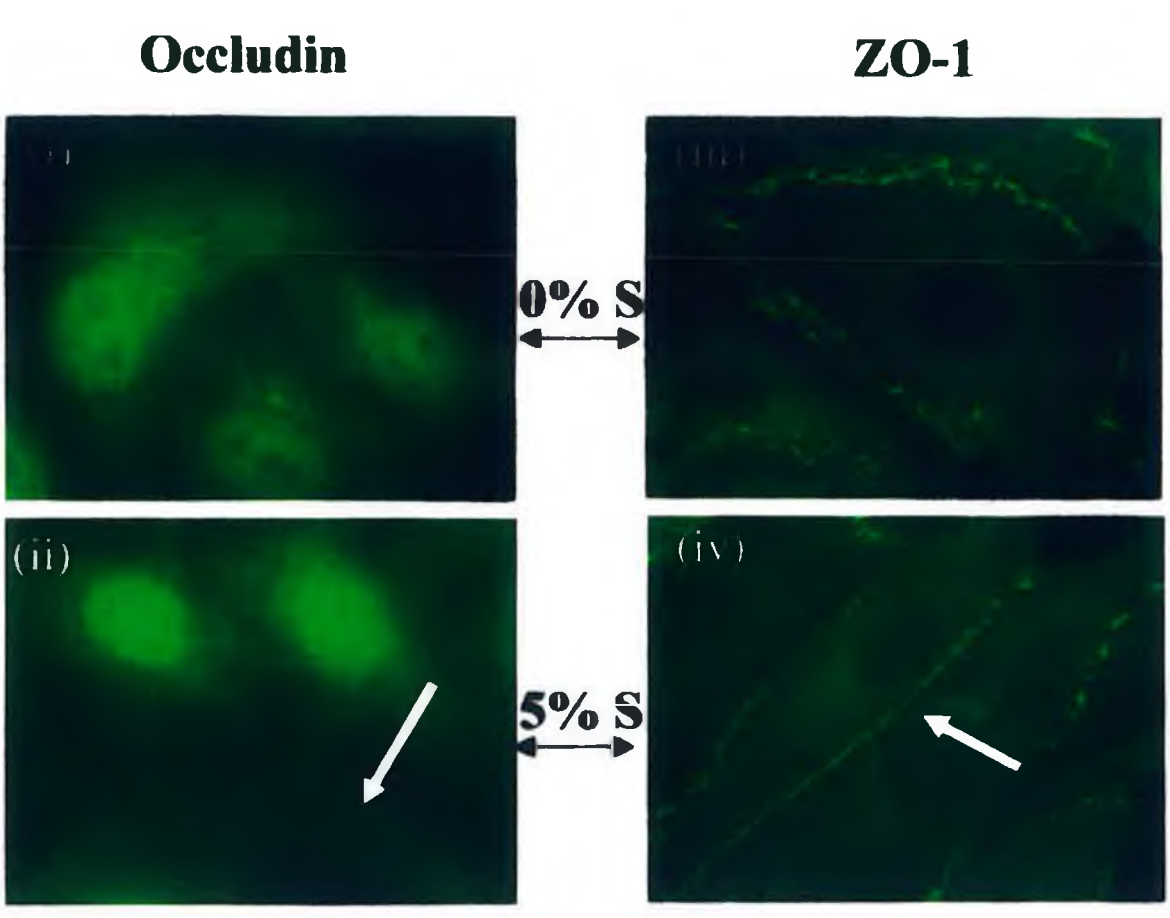

Fig. 3.6. Effect of cyclic strain on subcellular localisation of occludin and ZO-1 24 h post-strain. Membrane localization of occludin in (i) unstrained and (ii) strained BAECs and of ZO-1 in (iii) unstrained and (iv) strained BAECs is shown 24 h after re-plating. Both proteins were monitored using standard fluorescence microscopy (1000x). White arrows indicate cell-cell border localisation.

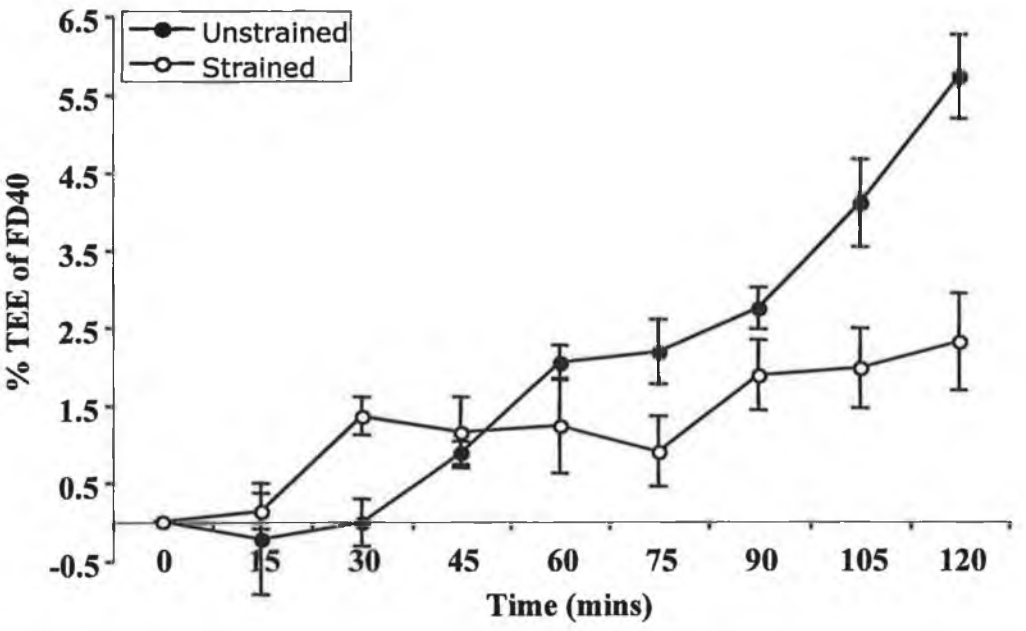

Fig. 3.7. Effect of cyclic strain on BAEC transendothelial permeability. Following exposure of BAECs to cyclic strain (5%, 24 h), control and "strain-conditioned" BAECs were trypsinized and re-plated into Transwell®-Clear plates and monitored for permeability to 40 kDa FITC-dextran. Data points are shown as total subluminal fluorescence at a given time point (from 0-120 min) expressed as a percentage of total abluminal fluorescence at t=0 min (i.e. %TEE of FD40 or % Trans Endothelial Exchange).

3.2.5. Effect of cycloheximide on cyclic strain regulation of occludin and ZO-1

The effect of cycloheximide (20 μg/ml) on cyclic strain-induced changes to occludin and ZO-1 protein expression in BAECs was determined by measuring protein levels in cell lysates. Addition of cycloheximide is known to halt new protein production and as such was employed to look at the proportion of strain-induced changes that were due to new protein synthesis. Following exposure of BAECs to cyclic strain (5%, 24 h), occludin protein expression increased significantly by 1.5 ± 0.3 fold compared to unstrained control. However, following strain in the presence of cycloheximide, occludin protein expression fell significantly to 0.8 ± 0.1 (Fig. 3.8A). Following cyclic strain, ZO-1 protein expression also increased significantly compared to unstrained controls (2.0 ± 0.2 fold), and fell significantly in the presence of cycloheximide to 0.75 ± 0.1 (Fig. 3.8B). Thus cycloheximide can block new protein synthesis and reduce occludin / ZO-1 protein levels to below pre-strain levels by 20-25%

Following exposure of BAECs to cyclic strain (5%, 24 h), co-association of occludin and ZO-1 was monitored in total BAEC lysates by IP. In response to strain, the level of occludin detected in anti-ZO-1 immunoprecipitates was seen to increase significantly by 2.0 ± 0.1 fold compared to unstrained control. However, following strain in the presence of cycloheximide the level of occludin detected in anti-ZO-1 immunoprecipitates fell significantly to 1.6 ± 0.1 (Fig. 3.9). Following BAEC exposure to cyclic strain (5%, 24 h) in the absence or presence of cycloheximide subcellular

localisation of occludin and ZO-1 was monitored by immunocytochemistry. Results indicated that cyclic strain-induced localisation of either occludin (Fig. 3.10) or ZO-1 (Fig. 3.11) to the cell-cell border was not effected by cycloheximide treatment

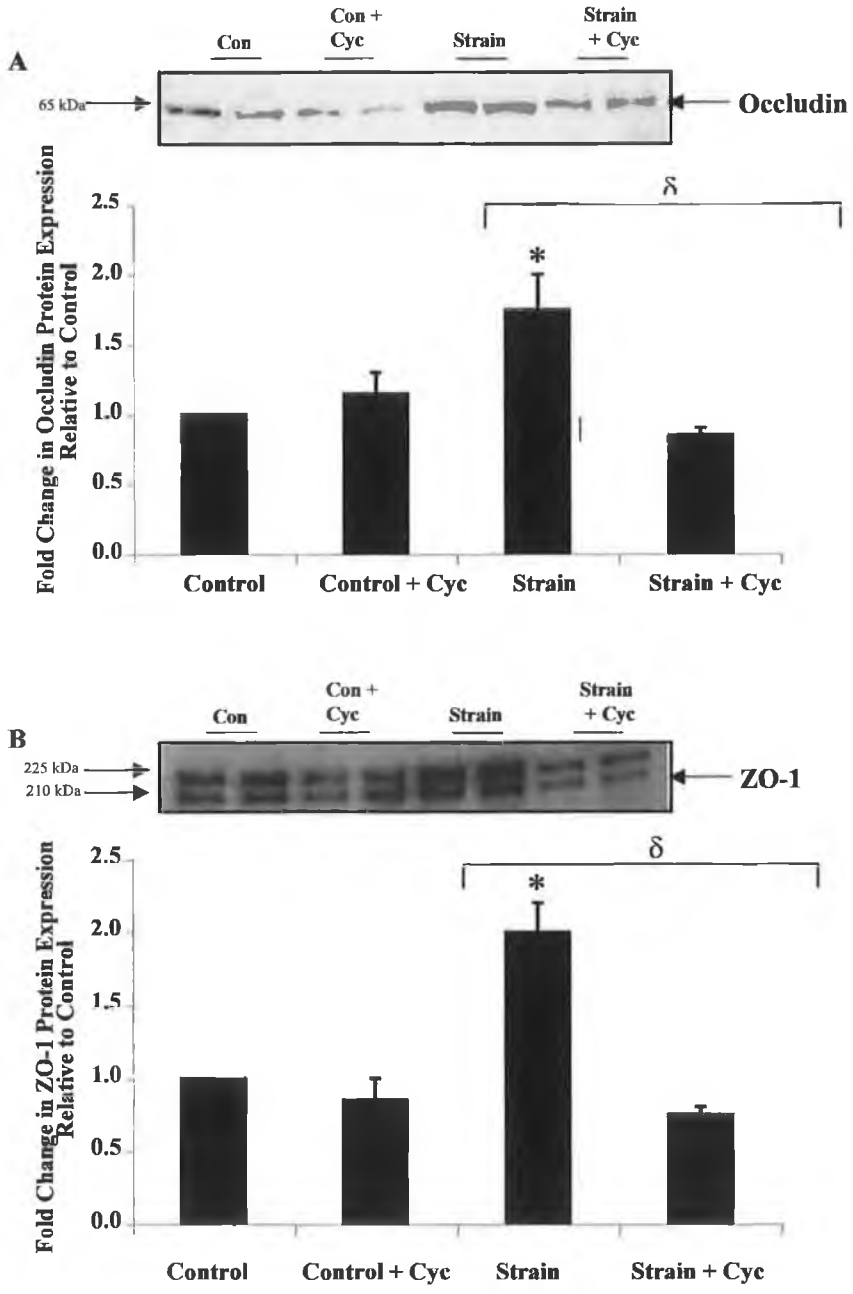

Fig. 3.8. Increased protein expression of occludin and ZO-1 following 5% Strain for 24 h can be attenuated by addition of cycloheximide. BAECs were exposed to cyclic strain (5%, 24 h) ± cycloheximide and monitored for (A) occludin or (B) ZO-1 protein expression by Western blotting. Representative blots are shown above each graph. Densitometric intensity of both bands has been combined. Histograms represent fold change in band intensity relative to unstrained controls and are averaged from three independent experiments ±SEM; $^{*}P \leq 0.05$ versus unstrained controls. $^{\delta}P \leq 0.05$ versus untreated strain.

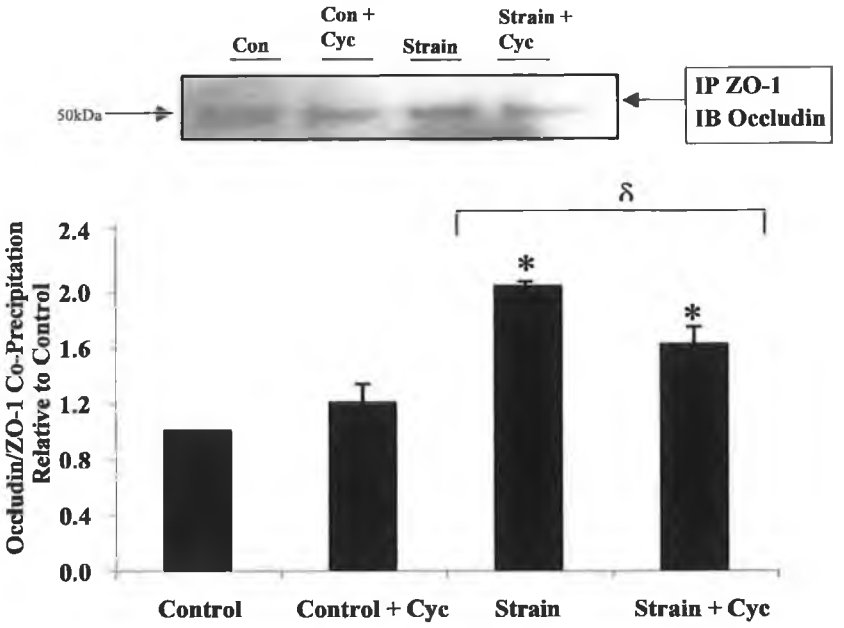

Fig. 3.9. Effect of 5% Strain + cycloheximide for 24 h on occludin and ZO-1 co-association in BAECs. Following exposure of BAECs to cyclic strain (5%, 24 h), co-association of occludin and ZO-1 were monitored by IP and Western blotting. Histogram represents fold change in band intensity relative to unstrained control and is averaged from three independent experiments ±SEM; $^{*}P \leq 0.05$ versus unstrained controls. $^{\delta}P \leq 0.05$ versus untreated strain.

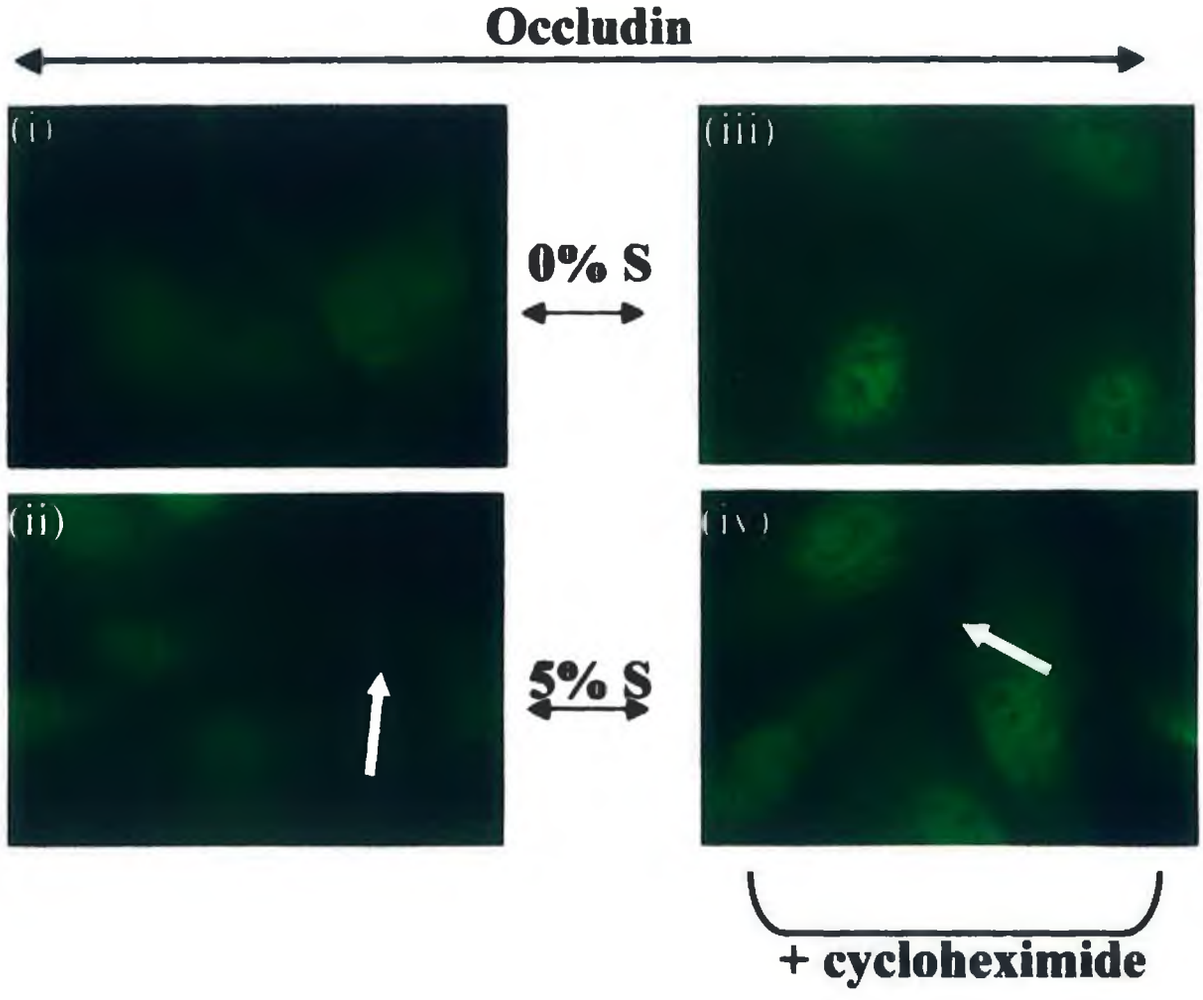

Fig. 3.10. Effect of cycloheximide on cyclic strain-induced occludin subcellular localisation in BAECs. Following exposure of BAECs to cyclic strain (5%, 24 h), localisation of occludin was monitored by immunocytochemistry. Occludin: (i) untreated control versus (ii) strain and (iii) cycloheximide-treated control versus (iv) strain. Occludin was monitored using standard fluorescence microscopy (1000x). White arrows indicate cell-cell border localisation. Images are representative of at least three individual sets of experiments.

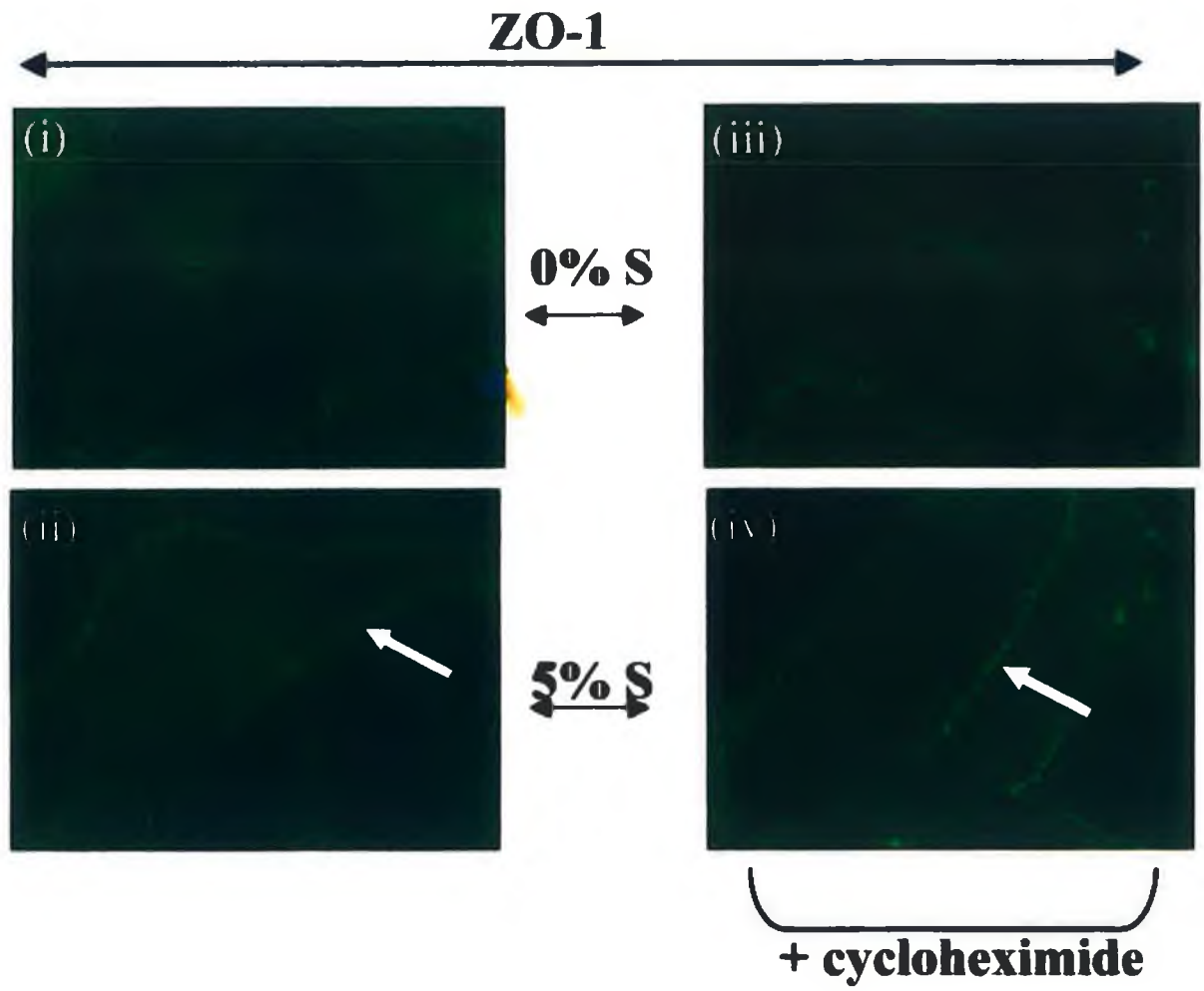

Fig. 3.11. Effect of cycloheximide on cyclic strain-induced ZO-1 subcellular localisation in BAECs. Following exposure of BAECs to cyclic strain (5%, 24 h), localisation of ZO-1 was monitored by immunocytochemistry. ZO-1: (i) untreated control versus (ii) strain and (iii) cycloheximide-treated control versus (iv) strain. ZO-1 was monitored using standard fluorescence microscopy (1000x). White arrows indicate cell-cell border localisation. Images are representative of at least three individual sets of experiments.

3.3 Discussion:

This chapter deals with the effect of cyclic strain on the tight junction proteins occludin and ZO-1. Given the importance of mechanical forces in regulating vascular hemeostasis, the ability of cyclic strain to alter the permeability of endothelial cells was investigated.

Firstly, the effect of cyclic strain on TJ protein expression and mRNA levels was investigated. Mechanoregulation of vascular endothelial tight junction protein expression has also been confirmed in recent studies [Demaio *et al.*, 2001: Conklin *et al.*, 2002]. Our investigations clearly demonstrate that chronic cyclic strain (5%, 24 h) of BAECs significantly up-regulates protein expression of occludin and ZO-1 in parallel with an increase in mRNA levels for occludin (but not ZO-1). This latter result suggests that the strain-dependent increase in ZO-1 protein levels is most likely due to increased protein translation and/or decreased ZO-1 protein turnover. It may also allude to the importance of post-translational modification of ZO-1 in the tight junction process (see Chapter 4). Conklin *et al.* also demonstrated that mechanical forces, in this case shear stress, induced up regulation of occludin mRNA [Conklin *et al.*, 2002].

In conjunction with the changes in protein and mRNA we then examined the location of both proteins in the cell before and after exposure to cyclic strain using standard fluorescent microscopy. A considerable change in the localisation of both proteins within the cell following strain can be seen. Occludin is mainly distributed in the nucleus and cytoplasm of the static cell and ZO-1 can be observed in a discontinuous

and jagged fashion along the cell border, both patterns of staining indicative of a poorly formed junction. In response to strain both proteins relocate. Occludin distribution becomes more focused at the cell-cell border and ZO-1 transforms into a more linear distribution at the cell-cell junction. A similar change in ZO-1 localisation was observed by Shin *et al.* in HUVECs exposed to chronic pulse pressure [Shin *et al.*, 2003]. This change in subcellular localisation indicates that both proteins are now in a more favourable location to function as a barrier to paracellular permeability.

ZO-1 contains many binding sites and is known to link tight junction proteins such as occludin to each other and the actin cytoskeleton [Alexander *et al.*, 2002; Furuse *et al.*, 1993; Furuse *et al.*, 1994; Gardner *et al.*, 1996; Hirase *et al.*, 1997]. Therefore actin staining was examined in cells exposed to hemodynamic forces as it is entirely plausible to link the changes seen in actin realignment with the localisation of occludin and ZO-1 following hemodynamic stimulation [McCue *et al.*, 2004]. From Fig. 3.4 we can see that there are changes in the distribution of actin following strain. The actin cytoskeleton becomes more localised to areas of cell-cell contacts, presumably with increased binding to ZO-1. This is consistent with the important role the cytoskeleton plays in modulating inter-endothelial junctions and endothelial permeability.

The immunocytochemical evidence putatively indicates that occludin and ZO-1 are localised together at the cell-cell border following exposure to strain. Use of IP techniques has proved conclusively that following strain there is significantly more occludin bound to ZO-1, another index of tight junction upregulation. Therefore, in conjunction with the increase in occludin and ZO-1 protein expression following strain,

we can see an increase in binding of both proteins to each other. As mentioned previously, disruption of tight junctions can occur in part by dis-association of the occludin -ZO-1 complex, and conversely increased occludin/ZO-1 co-association leads to an increase in endothelial barrier integrity.

Junctional integrity is regulated by cytoskeletal tension, alterations in junctional protein co-association, and linkage between junctional proteins and the actin cytoskeleton, all of which help to govern intercellular cleft size and degree of fluid/solute permeability [Rubin *et al.*, 1999; Balda *et al.*, 1998; Rubin *et al.*, 1999]. One would therefore expect changes in subcellular localisation of occludin and ZO-1 at the cell-cell border where tight junctions form, coupled with parallel changes in occludin/ZO-1 association (i.e. co-IP), to accompany a change in endothelial barrier function, as previously evidenced by numerous studies [Furuse *et al.*, 1994; Rao *et al.*, 2002; Wang *et al.,* 2002; Lee *et al.*, 2004; Fischer *et al.,* 2004; Sheth *et al.*, 2003]. The changes discussed so far have all indicated that cyclic strain induces barrier properties in BAECs. However it is the transendothelial permeability assay that provides the most compelling evidence. Cyclic strain significantly reduces BAEC transendothelial permeability to FITC-dextran (i.e. a measurable index of barrier function). Immunocytochemistry carried out in tandem confirms that strain-induced changes in ZO-1 and occludin localisation fully persist 24 h after passage from Bioflex® plates into Transwell®-Clear plates, indicating that the changes in transendothelial permeability are critically linked to the effect of strain on occludin and ZO-1. When viewed collectively, these data lead us to conclude that cyclic strain up-regulates endothelial occludin/ZO-1 expression and tight junction assembly, putatively leading to increased barrier integrity.

Consistent with this conclusion, a recent study by Shin *et al.* demonstrated reduced transendothelial permeability to albumin following exposure of HUVECs to chronic pulse pressure (cyclic pressure), an important hemodynamic component of pulsatile blood flow (i.e. in addition to, but distinct from, cyclic strain) [Shin *et al.,* 2003]. To our knowledge, this is the only other existing study reporting on the putative role of pulsatile force in the modulation of endothelial barrier function.

In order to determine if the strain-dependent increases in occludin and ZO-1 protein expression were causative of increases in co-association and subcellular localisation of both proteins, cycloheximide was employed. Cycloheximide is an inhibitor of protein biosynthesis in eukaryotic organisms, produced by the bacterium *Streptomyces griseus*. It exerts its effect on protein synthesis by blocking translational elongation. The addition of cycloheximide successfully blocked the increases in occludin and ZO-1 protein expression that were observed following strain. However, although protein levels are reduced to control levels with the addition of cycloheximide, there is still a significant increase in occludin/ZO-1 co-association, suggesting that only approximately 40% of the co-association is a direct consequence of new protein synthesis and that other regulatory mechanisms are occurring in these events. These may include phosphorylation and post-translational modifications of occludin and ZO-1, each of which will be discussed in the following chapters. Moreover, the increases in occludin and ZO-1 protein levels following strain are not necessary for the subcellular localisation of occludin and ZO-1. This is evident because even with the addition of cycloheximide, the changes in localisation of both proteins following strain still exists. This adds further evidence to the case for post-translational modifications of occludin

and ZO-1 following strain.

3.4 Conclusion

It has been demonstrated that physiological levels of cyclic strain act to promote a healthy and functioning tight junction in BAECs. We have clearly shown that in BAECs, occludin and ZO-1 protein expression are up-regulated following strain, in addition to an up-regulation of occludin but not ZO-1 mRNA. We have also demonstrated that cyclic strain leads to increased localisation of occludin, ZO-1 and actin to the cell-cell border, which is indicative of a functioning tight junction. In addition to this, we see an increase in the amount of occludin bound to ZO-1 following strain. These changes are very importantly linked to a functionally relevant model that demonstrates a decrease in transendothelial permeability following strain (See Fig. 3.12). We have also demonstrated that the increase in the amount of occludin bound to ZO-1 following strain is only partially reliant on new protein synthesis, and the increased localisation of occludin and ZO-1 to the cell-cell border is not dependent on the increase in protein levels observed, suggesting post-translational regulation of tight junction proteins following strain.

Thusfar, our data suggests that hemodynamic forces play an important role in dictating the permeability of BAECs via its effect on occludin and ZO-1, two proteins of pivotal importance in the TJ. This relationship is to be investigated in more detail in the following chapters.

Overview of Results Presented in Chapter 3

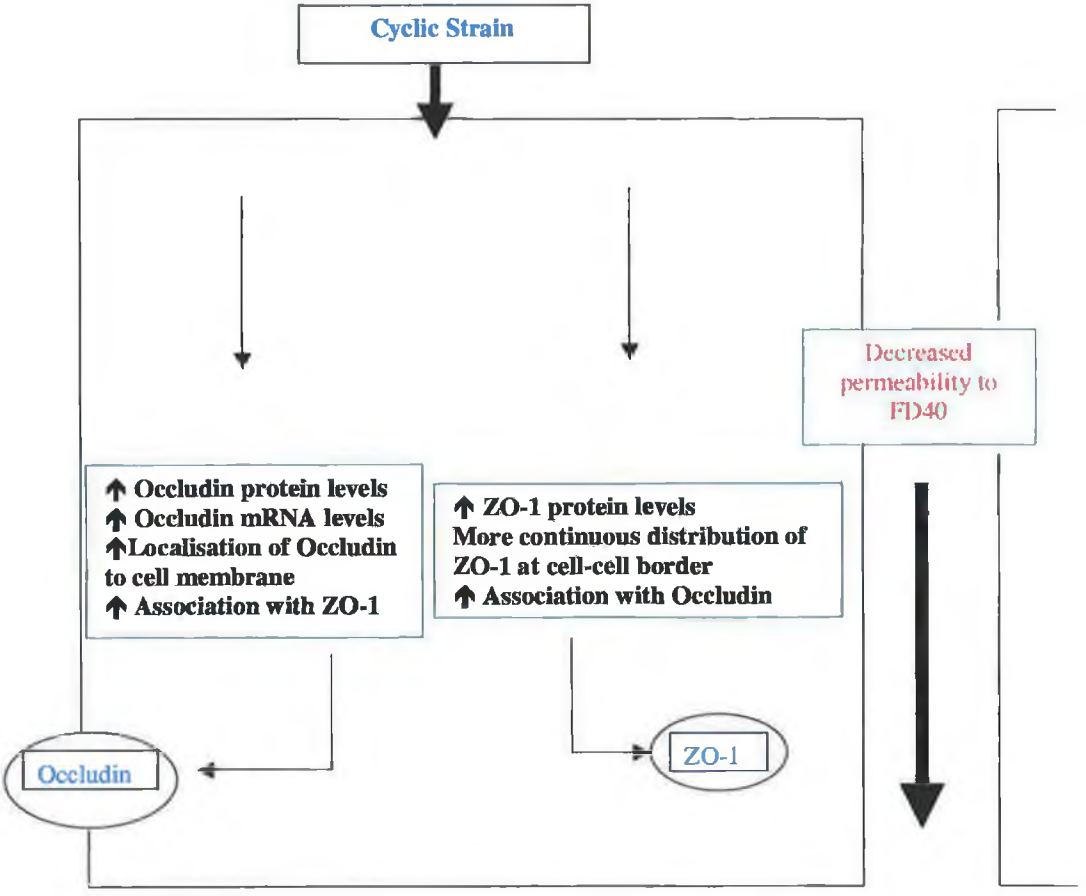

Fig. 3.12. The effect of cyclic strain (5%, 24h) on occludin and ZO-1 in BAECs.

Chapter 4

Examination of the phosphorylation changes in occludin and ZO-1 following strain and their subsequent effects on TJ permeability in BAECs.

4.1 Introduction

4.2 Results

4.3 Discussion

4.4 Conclusion

4.1 Introduction

As the increases in co-association and sub-cellular localisation of occludin and ZO-1 observed following strain are likely due, in part, to post translational modifications of both proteins, it was decided to look at phosphorylation changes in occludin and ZO-1 following exposure of BAECs to cyclic strain.

In this regard, much attention has focused on the role of phosphorylation in the assembly and function of tight junctions. Numerous studies have identified kinase/phosphatase-dependent mechanisms leading to modulation of junctional protein association and subcellular distribution [Alexander *et al.*, 2002; Rubin *et al.*, 1999; Balda *et al.*, 1998]. Recent papers by Rao *et al.* and Sheth *et al.* for example, demonstrate that oxidative stress-induced disruption of tight junctions results from increased tyrosine phosphorylation of occludin and ZO-1 leading to their subsequent dissociation from the actin cytoskeleton, reduction in occludin/ZO-1 co-association, and cellular redistribution of both proteins [Rao *et al.*, 2002; Sheth *et al.*, 2003]. However the processes by which these changes take place are poorly understood, although they are believed to be central to tight junction regulation and therefore highly worthy of investigation.

The aim of this chapter is firstly to determine any changes in the phosphorylation states of occludin and ZO-1 following strain. Secondly, the pathways by which occludin and ZO-1 are phosphorylated will be examined using pharmacological inhibitors. Thirdly the relationship between these phosphorylation events and the downstream changes reported in the previous chapter will be examined.

4.2 Results

4.2.1. Cyclic strain-dependent occludin and ZO-1 phosphorylation in BAECs.

Following exposure of BAECs to cyclic strain (5%, 24 h), phosphorylation of occludin was monitored in total lysate by IP using phospho-specific antibodies. In response to strain, tyrosine phosphorylation of occludin decreased significantly by 75.7 ± 8% compared to unstrained control (Fig. 4.1A). This is in distinct contrast to relatively small decreases in threonine (Fig. 4.1B) and serine (Fig. 4.1C) phosphorylation of occludin observed in response to strain (16.3 ± 6% and 30 ± 5%, respectively). Results were normalised to changes in occludin protein expression.

Following exposure of BAECs to cyclic strain (5%, 24 h), phosphorylation of ZO-1 was also monitored in total lysate by IP using phospho-specific antibodies. In response to strain, threonine (Fig. 4.2A) and serine (Fig. 4.2B) phosphorylation of ZO-1 increased significantly (51.7 ± 9% and 82.7 ± 25%, respectively) compared to control. By contrast, tyrosine phosphorylation of ZO-1 remained unchanged (Fig. 4.2C) in response to strain. Results were normalised to changes in ZO-1 protein expression.

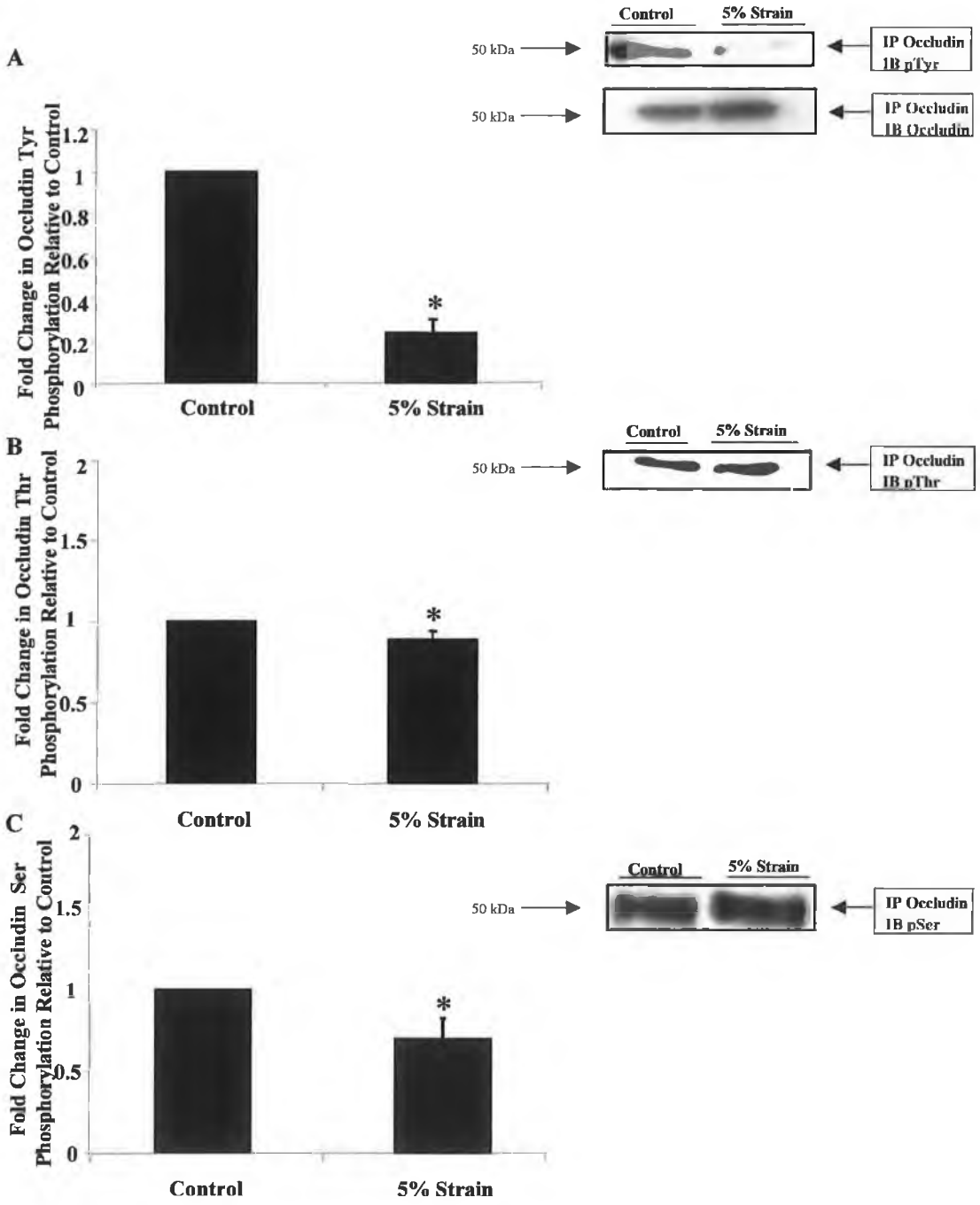

Fig. 4.1. Effect of cyclic strain on occludin phosphorylation in BAECs. BAECs were exposed to cyclic strain (5%, 24 h) and monitored for phosphorylation of occludin in control and strained BAECs by IP and Western blotting using (A) phospho-tyrosine, (B) -threonine and (C)-serine -specific antibodies. Representative blots are shown above

each graph. Histograms represent fold change in band intensity relative to unstrained controls and are averaged from three independent experiments ±SEM; $*P \leq 0.05$ versus unstrained controls. With respect to (A), (B), and (C), histograms are normalised to changes in occludin protein expression (See Fig. 4.1.A lower blot).

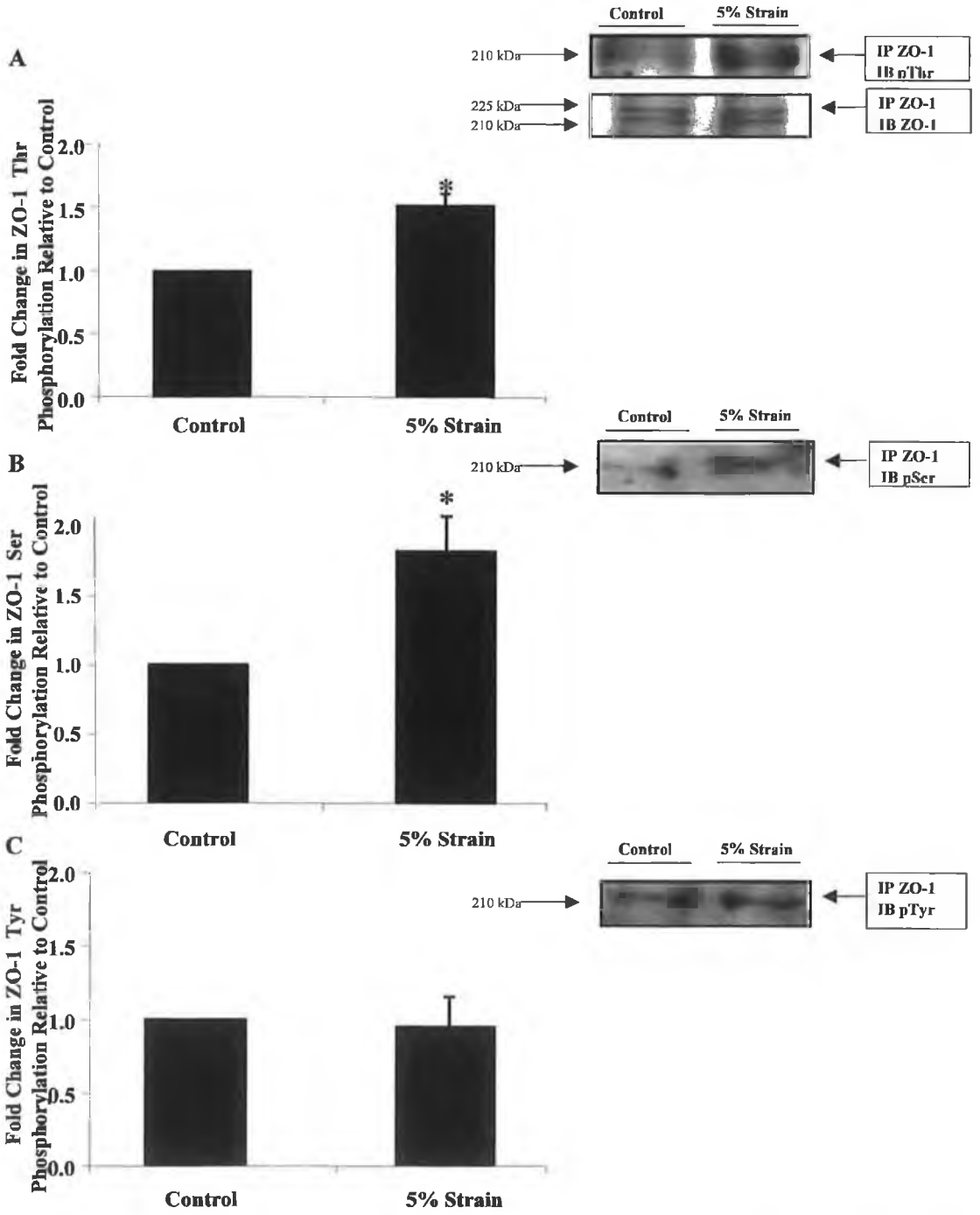

Fig. 4.2. Effect of cyclic strain on ZO-1 phosphorylation in BAECs. BAECs were exposed to cyclic strain (5%, 24 h) and monitored for phosphorylation of ZO-1 in control and strained BAECs by IP and Western blotting using (A) phospho-threonine, (B)-serine and (C) tyrosine-specific antibodies. Representative blots are shown above each graph. Histograms represent fold change in band intensity relative to unstrained controls and are averaged from three independent experiments ±SEM; *$P \leq 0.05$ versus

unstrained controls. With respect to (A), (B), and (C), histograms are normalised to changes in ZO-1 protein expression (See Fig. 4.2.A lower blot).

4.2.2. Effect of Dephostatin on strain-induced occludin tyrosine dephosphorylation.

Investigation confirmed that strain-induced decreases in occludin tyrosine phosphorylation could be completely blocked by treatment of BAECs with dephostatin (tyrosine phosphatase inhibitor), (Fig. 4.3). Tyrosine phosphorylation of occludin decreased significantly to 0.57 ± 0.2 fold when cells were strained. Following strain + dephostatin, tyrosine phosphorylation of occludin increased significantly to 1.17 ± 0.1 fold. Therefore dephostatin prevents tyrosine dephosphorylation of occludin. Dephostatin addition had no effect on unstrained samples.

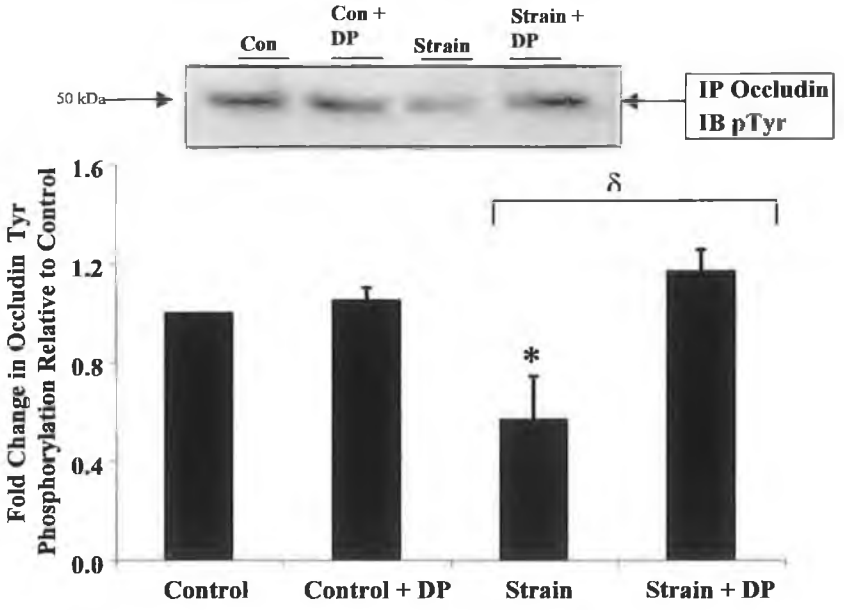

Fig. 4.3. Effect of Dephostatin on Strain-induced occludin tyrosine phosphorylation. Graph illustrates the ability of DP to ablate strain-induced occludin tyrosine dephosphorylation. Representative blot is shown above the graph. Histogram represent fold change in band intensity relative to unstrained control is averaged from three independent experiments ±SEM; $^{*}P \leq 0.05$ versus unstrained controls. $^{\delta}P \leq 0.05$ versus untreated strain.

4.2.3. Effect of Rottlerin on occludin serine/threonine phosphorylation.

The effect of rottlerin on occludin serine/threonine phosphorylation was monitored to show that the inhibitory effects of rottlerin were due specifically to PKC inhibition and subsequent blockade of ZO-1 ser/thre phosphorylation and therefore to rule out PKC as a modulator of occludin phosphorylation state changes in response to strain (Fig. 4.4). PMA was included as a positive control because it activates PKC by mimicking DAG. It was found that the addition of rottlerin had no effect on occludin threonine phosphorylation. However addition of PMA, caused threonine phosphorylation to increase significantly by 2.6 ± 0.2 fold, compared to control. It was also found that the addition of rottlerin had no effect on occludin serine phosphorylation. However addition of PMA, caused serine phosphorylation to increase significantly by 2.1 ± 0.1 fold, compared to control.

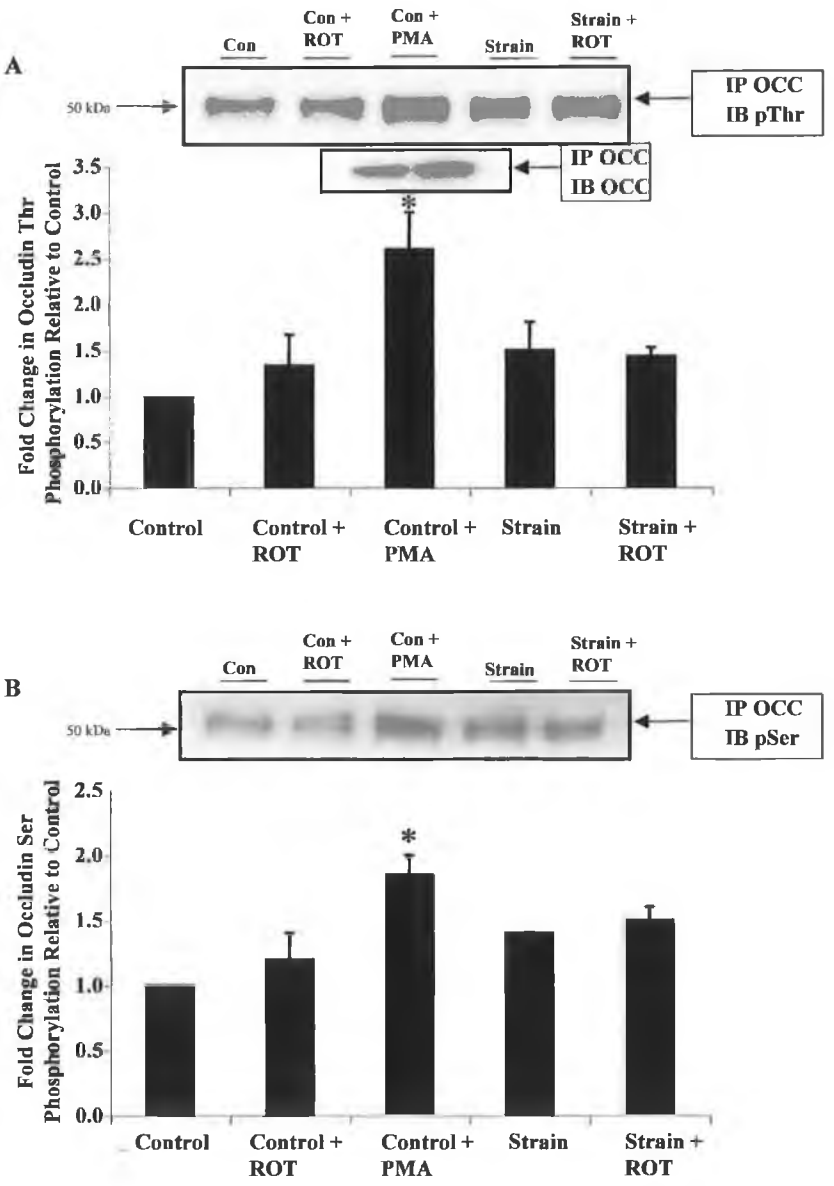

Fig. 4.4. Effect of Rottlerin on strain-induced occludin threonine and serine phosphorylation. Graph A illustrates the effect of ROT on threonine phosphorylation, of occludin. Graph B illustrates the effect of ROT on occludin serine phosphorylation, Representative blots are shown above the graph. Histograms represent fold change in band intensity relative to unstrained control and are averaged from three independent experiments ±SEM; *$P \leq 0.05$ versus unstrained controls. (ROT, rottlerin, PMA phorbol 12-myristate 13-acetate). With respect to (A) and (B) histogram is normalised to changes in occludin protein expression (See Fig. 4.A. lower blot).

4.2.4 Effect of Dephostatin on occludin co-association with ZO-1.

BAECs were exposed to cyclic strain (5%, 24 h) in the absence or presence of dephostatin and occludin/ZO-1 co-association monitored in total BAEC lysates by IP as described. Results indicated that the significant increase in strain-induced co-association of occludin/ZO-1 could be blocked significantly following treatment with dephostatin, compared with unstrained control (Fig. 4.5). However, it is of interest to note that co-association following treatment with dephostatin in unstrained cells is also significantly increased from unstrained control.

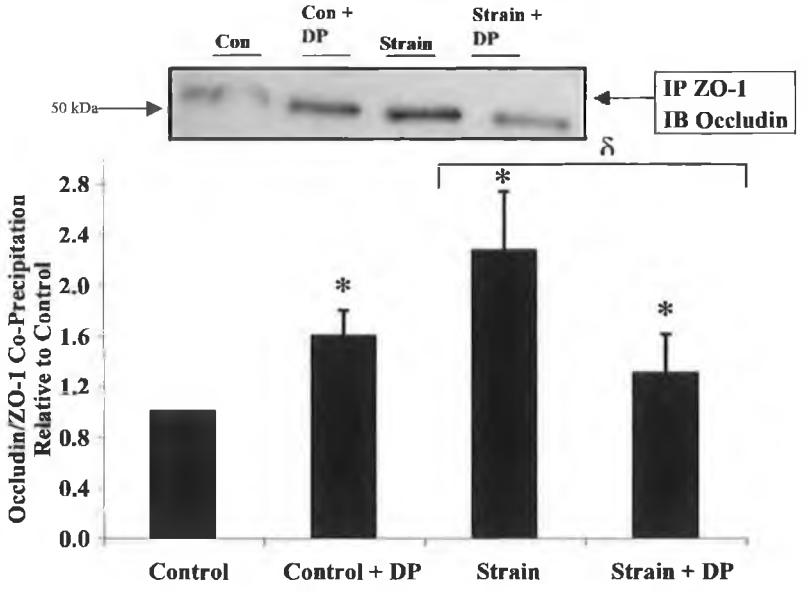

Fig. 4.5. Effect of Dephostatin on cyclic strain-induced occludin/ZO-1 co-association. Following exposure of BAECs to cyclic strain (5%, 24 h), co-association of occludin was monitored by IP and Western blotting. The effect of dephostatin on strain-induced occludin/ZO-1 co-association is shown. Representative blots are shown above the graphs. Histograms represent fold change in band intensity relative to unstrained control and are averaged from three independent experiments ±SEM; $^{*}P \leq 0.05$ versus unstrained controls. $^{\delta}P \leq 0.05$ versus untreated strain.

4.2.5. Effect of Dephostatin and Rottlerin on cyclic strain-dependent subcellular localisation of occludin.

Following BAEC exposure to cyclic strain (5%, 24 h) in the absence or presence of dephostatin, subcellular localisation of occludin was monitored by immunocytochemistry. Results indicated that cyclic strain-induced localisation of occludin to the cell-cell border was completely abated by dephostatin treatment (Fig. 4.6 i-iv).

Following BAEC exposure to cyclic strain (5%, 24 h) in the absence or presence of rottlerin, a PKC inhibitor, subcellular localisation of occludin was also monitored by immunocytochemistry. Results indicated that cyclic strain-induced localisation of occludin to the cell-cell border was not effected by rottlerin treatment, in correlation with the fact that rottlerin had no effect on occludin serine/threonine phosphorylation (Fig. 4.7 i-iv).

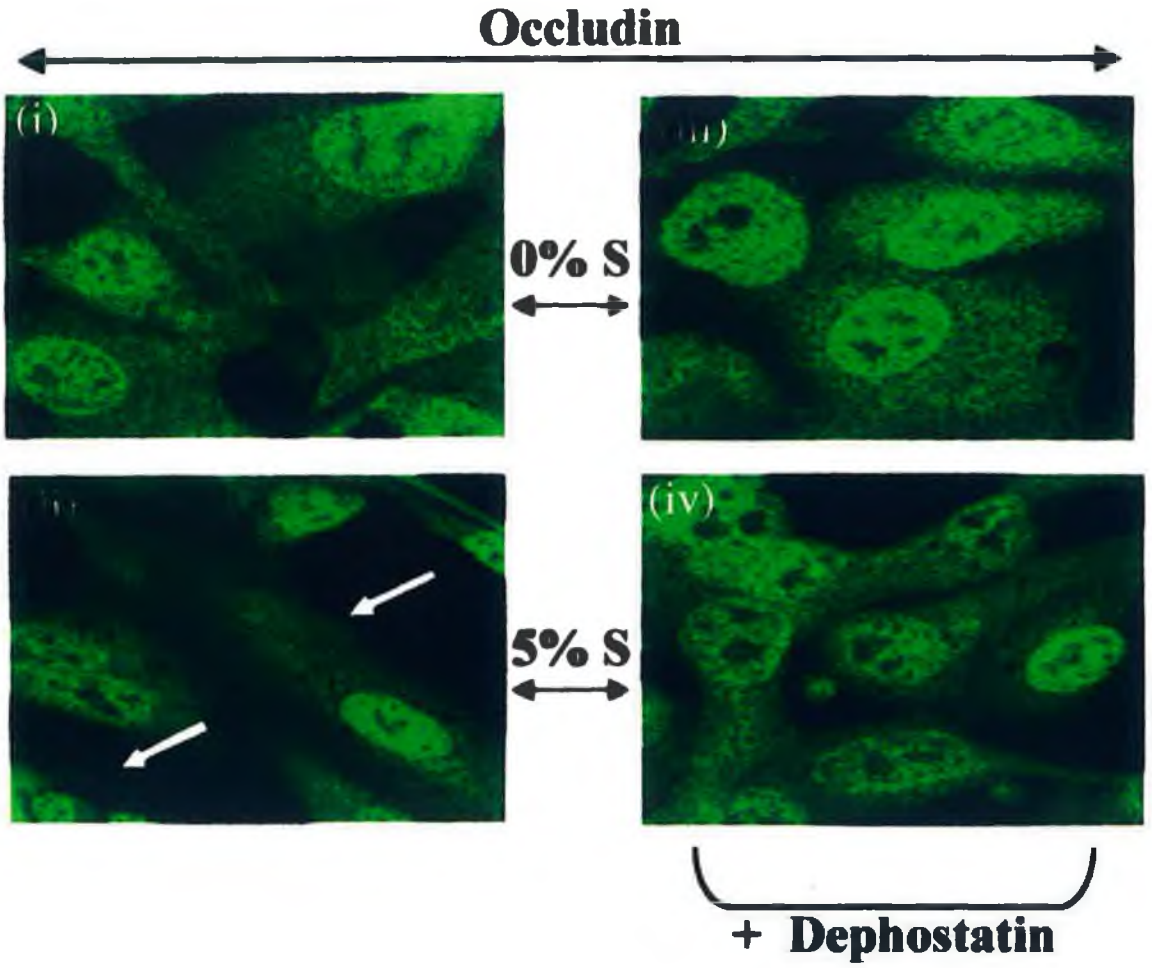

Fig. 4.6. Effect of Dephostatin on cyclic strain-induced occludin subcellular localisation in BAECs. Following exposure of BAECs to cyclic strain (5%, 24 h), localisation of occludin was monitored by immunocytochemistry. Occludin: (i) untreated control versus (ii) strain and (iii) dephostatin-treated control versus (iv) strain. Occludin was monitored using confocal fluorescence microscopy (1200x). White arrows indicate cell-cell border localisation. Images are representative of at least three individual sets of experiments.

4.2.6 Effect of Rottlerin on strain-induced ZO-1 serine phosphorylation.

Investigation confirmed that strain-induced increases in ZO-1 serine phosphorylation could be completely blocked by treatment of BAECs with rottlerin (PKC inhibitor). Serine phosphorylation of ZO-1 increased significantly following strain, compared to control, by1.83 ± 0.2 fold. However on addition of rottlerin, strain-induced serine phosphorylation of ZO-1 was significantly reduced to 0.5 ± 0.1 fold compared to strained samples (Fig. 4.8). Serine phosphorylation of ZO-1 was also reduced in non-strained samples following addition of rottlerin.

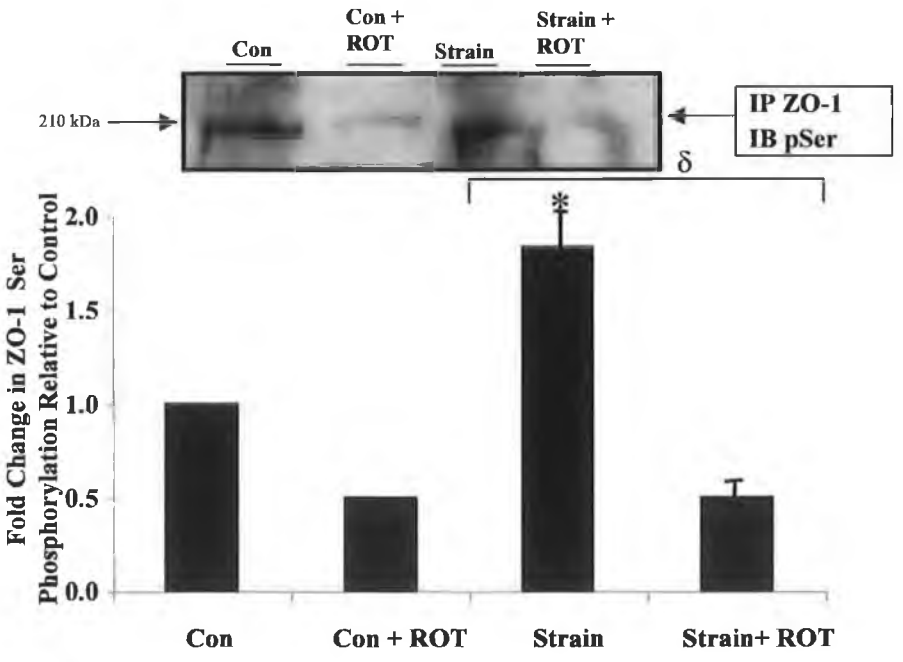

Fig. 4.8. Effect of Rottlerin on strain-induced serine ZO-1 phosphorylation. Graph illustrates the ability of ROT to ablate strain-induced serine phosphorylation, of ZO-1. Representative blot is shown above the graph. Histogram represent fold change in band intensity relative to unstrained control is averaged from three independent experiments ±SEM; $^{*}P \leq 0.05$ versus unstrained controls. $^{\delta}P \leq 0.05$ versus untreated strain.

4.2.7 Effect of Dephostatin on ZO-1 tyrosine phosphorylation.

Results showed that there was no change in ZO-1 tyrosine phosphorylation following strain. However changes in ZO-1 tyrosine phosphorylation were also monitored in the presence of dephostatin to illustrate that the inhibitory effects of dephostatin were due specifically to tyrosine phosphatase inhibition and subsequent blockade of ZO-1 tyrosine dephosphorylation. It was found that the addition of dephostatin had no effect on ZO-1 tyrosine phosphorylation in control or strained samples (Fig. 4.9).

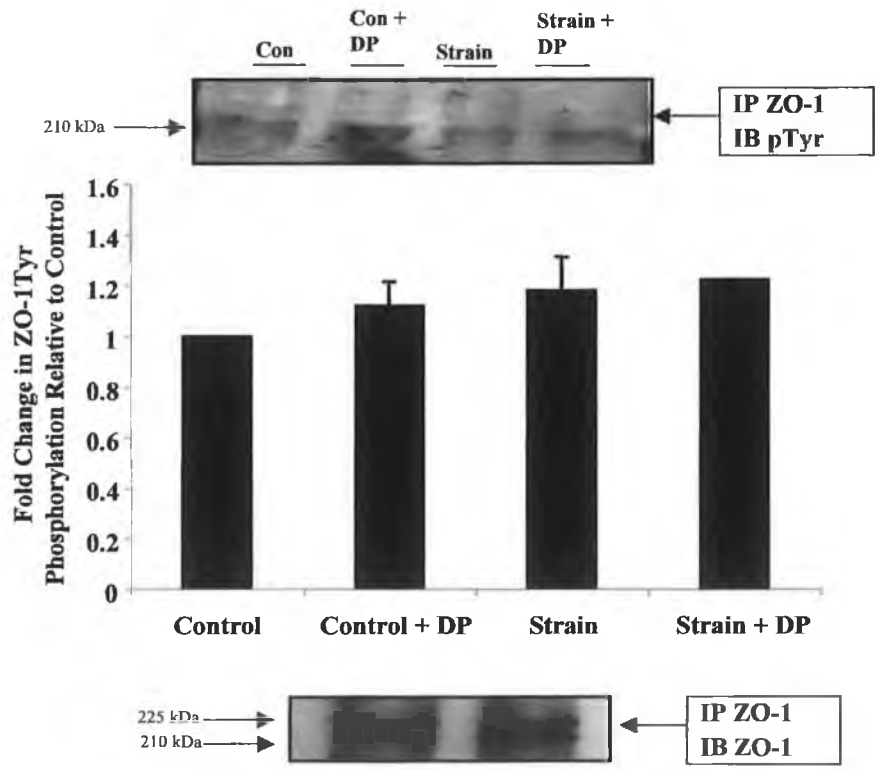

Fig. 4.9. Effect of Dephostatin on ZO-1 tyrosine phosphorylation. Graph illustrates the effect of DP on ZO-1 tyrosine phosphorylation. Representative blot is shown above the graph. Histogram represent fold change in band intensity relative to unstrained control and is averaged from three independent experiments ±SEM. With respect to (A) histogram is normalised to changes in ZO-1 protein expression (See Fig. 4.9 lower blot).

4.2.8 Effect of Rottlerin on strain-induced occludin/ZO-1 co-association.

BAECs were exposed to cyclic strain (5%, 24 h) in the absence or presence of rottlerin and occludin/ZO-1 co-association was monitored in total BAEC lysates by IP as described. Results indicated that strain-induced co-association of occludin/ZO-1 could be blocked significantly by 87.7 ± 30% following treatment with rottlerin (Fig. 4.10). Is it of interest to note that co-association increased significantly in non-strained samples following treatment with rottlerin.

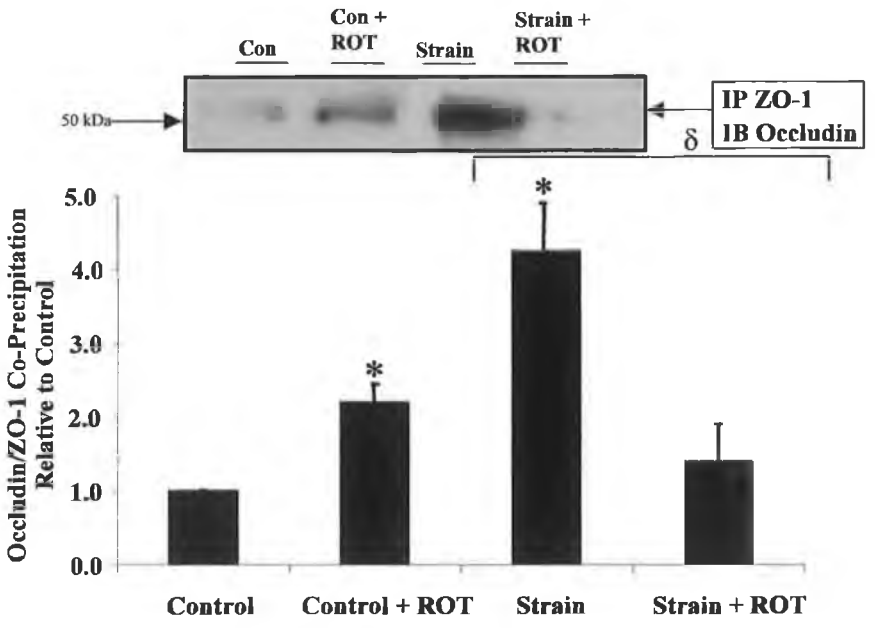

Fig. 4.10. Effect of ROT on cyclic strain-induced occludin/ZO-1 co-association. Following exposure of BAECs to cyclic strain (5%, 24 h), co-association of occludin was monitored by IP and Western blotting. The effect of rottlerin on strain-induced occludin/ZO-1 co-association is shown. Representative blots are shown above the graphs. Histograms represent fold change in band intensity relative to unstrained control and are averaged from three independent experiments ±SEM; *$P \leq 0.05$ versus unstrained controls. $^{\delta}P \leq 0.05$ versus untreated strain.

4.2.9. Effect of Rottlerin and Dephostatin on cyclic strain-dependent subcellular localisation of ZO-1.

Following BAECs exposure to cyclic strain (5%, 24 h) subcellular localisation of ZO-1 was monitored in the absence or presence of rottlerin. Results indicated that the continuous and well-defined organisation of ZO-1 immunoreactivity initially observed along the plasma membrane in response to cyclic strain was completely abated by rottlerin treatment, reverting to a discontinuous and jagged localisation pattern along the cell-cell border (Fig. 4.11 i-iv).

Following BAECs exposure to cyclic strain (5%, 24 h) in the absence or presence of dephostatin, subcellular localisation of ZO-1 was monitored by immunocytochemistry. Results indicated that cyclic strain-induced localisation of ZO-1 to the cell-cell border in a more continuous fashion was not effected by dephostatin treatment (Fig. 4.12 i-iv).

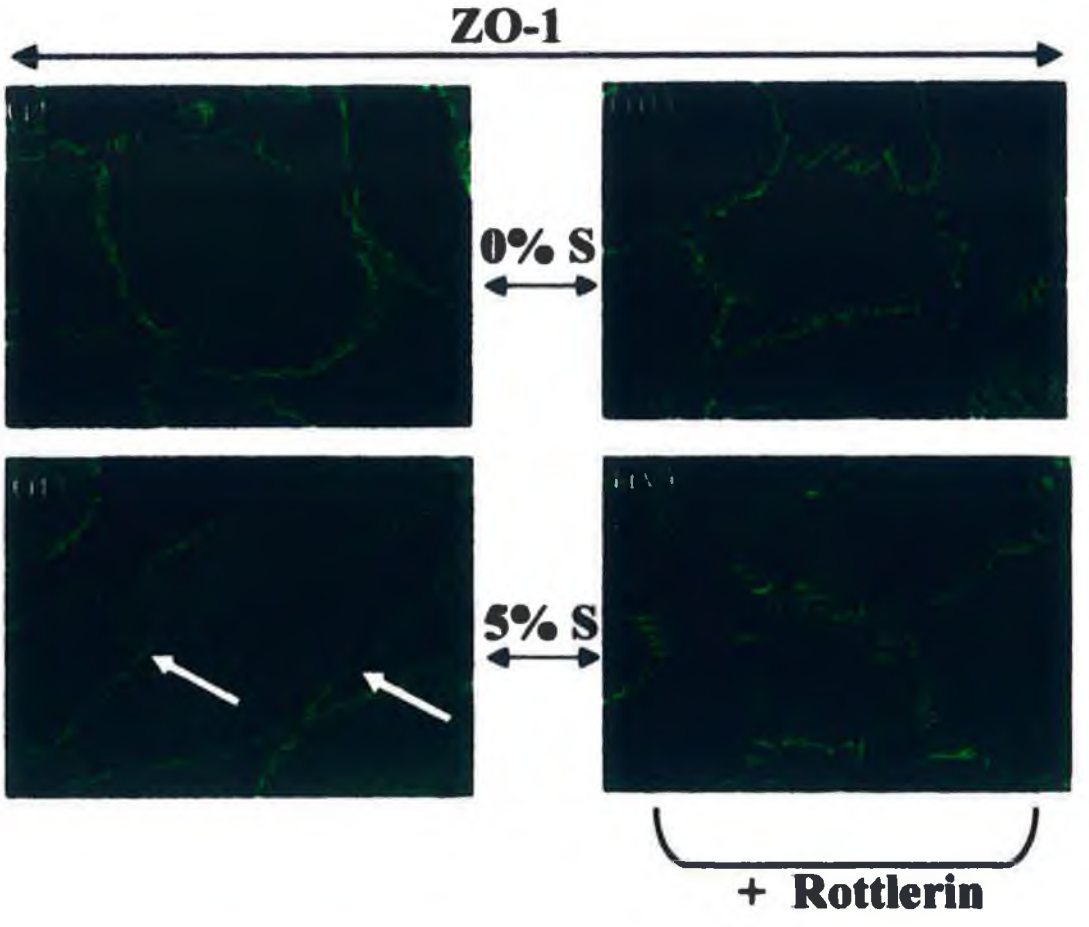

Fig. 4.11. Effect of PKC inhibition on cyclic strain-induced ZO-1 subcellular localisation in BAECs. Following exposure of BAECs to cyclic strain (5%, 24 h), localisation of ZO-1 was monitored by immunocytochemistry ZO-1: (i) untreated control versus (ii) strain and (iii) rottlerin-treated control versus (iv) strain. ZO-1 was monitored using confocal fluorescence microscopy (1200x). White arrows indicate cell-cell border localisation. Images are representative of at least three individual sets of experiments

4.2.10. Effect of Rottlerin and Dephostatin on cyclic strain-induced transendothelial permeability in BAECs.

Following exposure of BAECs to cyclic strain (5%, 24 h), control and strain-conditioned BAECs ± ROT were trypsinized and re-plated into Transwell®-Clear plates and monitored for permeability to 40 kDa FITC-dextran. As can be seen from Fig. 4.13.A, ROT-treated strain-conditioned cells had increased permeability to 40 kDa FITC-dextran compared with strain-conditioned cells.

A

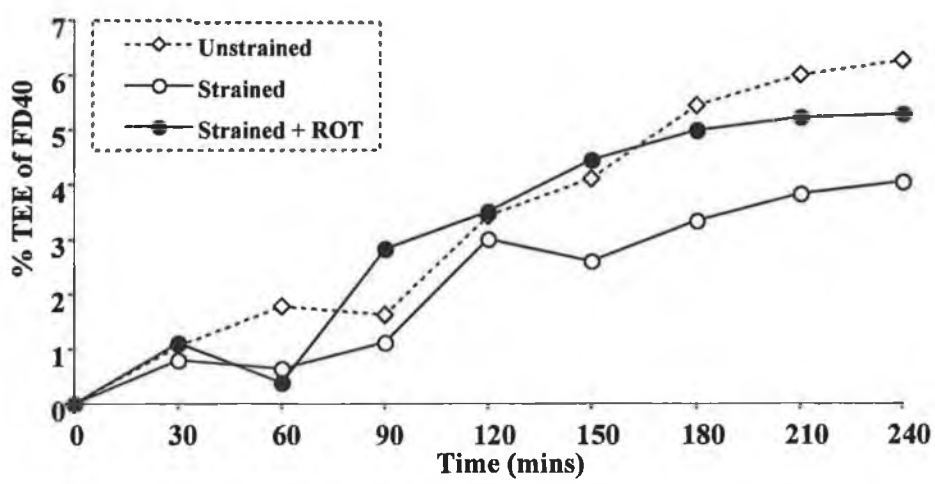

Fig. 4.13A. Effect of ROT on cyclic strain-induced transendothelial permeability in BAECs. Following exposure of BAECs to cyclic strain (5%, 24 h), control and strain-conditioned BAECs ± ROT (unstrained + ROT omitted from graph for clarity) were trypsinized and re-plated into Transwell®-Clear plates and monitored for permeability to 40 kDa FITC-dextran. Data points are shown as total subluminal fluorescence at a given time point (from 0-120 min) expressed as a percentage of total abluminal fluorescence at t=0 min (i.e. %TEE of FD40 or % Trans Endothelial Exchange). Representative graph shown averaged from two experiments.

Following exposure of BAECs to cyclic strain (5%, 24 h), control and strain-conditioned BAECs ± DP were trypsinized and re-plated into Transwell®-Clear plates and monitored for permeability to 40 kDa FITC-dextran. As can be seen from Fig. 4.13.B, DP-treated strain-conditioned cells had increased permeability to 40 kDa FITC-dextran compared with strain-conditioned cells.

B

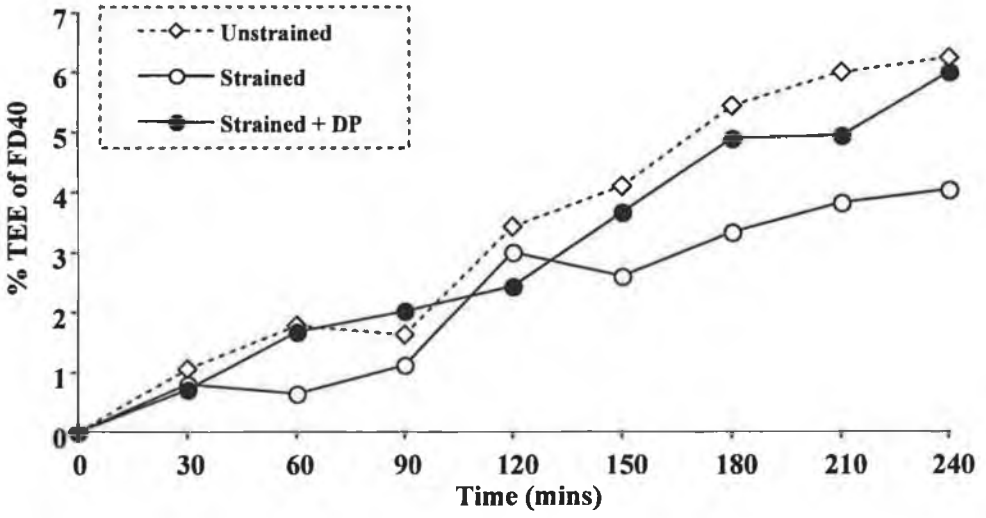

Fig. 4.13.B. Effect of DP on cyclic strain-induced transendothelial permeability in BAECs. Following exposure of BAECs to cyclic strain (5%, 24 h), control and strain-conditioned BAECs ± DP (unstrained + DP omitted from graph for clarity) were trypsinized and re-plated into Transwell®-Clear plates and monitored for permeability to 40 kDa FITC-dextran. Data points are shown as total subluminal fluorescence at a given time point (from 0-120 min) expressed as a percentage of total abluminal fluorescence at t=0 min (i.e. %TEE of FD40 or % Trans Endothelial Exchange). Representative graph shown averaged from two experiments.

4.3 Discussion

It has become clear that post-translational-modification of tight junction proteins via their phosphorylation, may be a pivotal step in the formation of functioning tight junctions. We therefore decided to reconcile the cyclic strain-dependent phosphorylation of occludin and ZO-1 observed in the present study with the strain-dependent modulation of occludin/ZO-1 co-association and plasma membrane localisation discussed in the previous chapter.

We first set out to examine baseline phosphorylation events in occludin and ZO-1 following strain. Experiments showed that a tyrosine dephosphorylation event was occurring in occludin following strain, in conjunction with increased serine/threonine phosphorylation of ZO-1.

We went on to confirm the role of tyrosine phosphatases in occludin tyrosine dephosphorylation and also to examine the consequences of this event in relation to tight junction assembly. Addition of dephostatin, a pharmacological inhibitor of tyrosine phosphatase, to the cells prior to strain prevents the tyrosine dephosphorylation of occludin. Moreover, when dephostatin was added to the cells prior to strain, the increase in occludin/ZO-1 co-association observed was attenuated, thereby linking the tyrosine dephosphorylation event in occludin to its ability to bind to ZO-1 This finding has been observed in other studies. A recent report by Kale *et al.* has shown that tyrosine phosphorylation of occludin reduces its co-association with ZO-1, implying that tyrosine dephosphorylation of occludin leads to an increase in co-association with ZO-1 [Kale *et*

al., 2003]. With respect to dephostatin addition, occludin/ZO-1 co-association was significantly elevated above baseline in unstrained cells following inhibitor treatment. This may reflect an indirect, albeit uncharacterized, increase in ZO-1 serine/threonine phosphorylation, which could putatively lead to an elevation in protein-protein co-association above untreated baseline. Irrespective of this, dephostatin treatment led to a clear reduction in strain-induced occludin/ZO-1 co-association to unstrained inhibitor-treated levels.

We next decided to investigate the effects of the inhibitor on occludin localisation. When tyrosine dephosphorylation of occludin is inhibited its ability to relocate to the cell membrane following strain is ablated. Therefore, tyrosine dephosphorylation of occludin, in addition to being necessary for increased occludin/ZO-1 co-association following strain, is also essential for its strain-dependent subcellular localisation.

The effect of rottlerin on occludin serine/threonine phosphorylation and sub-cellular localisation was also investigated to rule out any influence of PKC on occludin phosphorylation or localisation. Addition of rottlerin had no significant effect on occludin serine/threonine phosphorylation when levels were normalised to occludin protein. Moreover, addition of rottlerin also had no effect on the ability of occludin to localise to the cell membrane following strain, thereby excluding PKC from this process.

Conversely, we also began the process to confirm the role of PKC in the serine

/threonine phosphorylation of ZO-1 and also to examine the consequences of this event in relation to tight junction formation. Addition of rottlerin, a pharmacological inhibitor of PKC, (highest specificity for PKCδ [Gschwendt *et al.*, 1994]), prevents the serine phosphorylation of ZO-1 observed following exposure of BAECs to cyclic strain. In addition, rottlerin also decreased serine phosphorylation of ZO-1 in non-strained cells. The addition of rottlerin therefore, also provided a valuable tool in determining the function of serine/threonine phosphorylation events in tight junction assembly. In tandem with the occludin dephostatin results discussed previously, when rottlerin was added to the cells the increase in occludin/ZO-1 co-association observed following cyclic strain was attenuated, thereby linking the serine/threonine phosphorylation of ZO-1 to its ability to bind to occludin at the tight junction. With respect to rottlerin addition, occludin/ZO-1 co-association was significantly elevated above baseline in unstrained cells following inhibitor treatment. This may reflect an indirect, albeit uncharacterized, increase in occludin tyrosine dephosphorylation, which could putatively lead to an elevation in protein-protein co-association above untreated baseline. Irrespective of this, rottlerin treatment led to a clear reduction in strain-induced occludin/ZO-1 co-association to unstrained inhibitor-treated levels.

Due to the changes observed in occludin/ZO-1 co-association following strain with the addition of rottlerin we next sought to investigate the effects of the inhibitor on ZO-1 localisation. When serine/threonine phosphorylation of ZO-1 is inhibited, its ability to localise to the cell membrane in a continuous fashion in response to strain is abrogated. Therefore the serine/threonine phosphorylation of ZO-1 is necessary for its subcellular localisation following strain.

In view of the inhibitory effects of rottlerin, PKC likely plays a very important role in determining ZO-1 function at the tight junction in ECs in response to physiological levels of cyclic strain. In a contrasting study, PKC phosphorylation of ZO-1 was shown to increase paracellular permeability in response to poly-l-arginine stimulation [Ohtake *et al.*, 2003]. The difference observed in the role of ZO-1 phosphorylation in this study may be due to the fact that the stimulation was biochemical and also took place in different cell types (rabbit nasal epithelium cells), however it does emphasise the fact the PKC regulation of ZO-1 is important in TJ formation.

The effect of dephostatin on ZO-1 localisation and tyrosine phosphorylation were also monitored to illustrate that the inhibitory effects of dephostatin were due specifically to tyrosine phosphatase inhibition and subsequent blockade of occludin tyrosine dephosphorylation. Addition of dephostatin had no significant effect on ZO-1 tyrosine phosphorylation when levels were normalised to ZO-1 protein. Moreover, addition of dephostatin has no effect on the ability of ZO-1 to localise to the cell membrane in a continuous fashion following strain, thereby excluding tyrosine phosphatase from this process.

Thus far we have linked the occludin tyrosine dephosphorylation event and the ZO-1 serine/threonine phosphorylation event to an increase in the co-association of the two proteins and to changes in their subcellular localisation within the cell following strain. We next sought to determine if post translation modifications have any effect on the tightness of the paracellular barrier. We have demonstrated that addition of either

rottlerin or dephostatin, respectively, cause the decrease seen in transendothelial permeability following strain to be attenuated, clearly suggesting that there is a causal relationship between changes in occludin and ZO-1 biochemical properties and endothelial barrier function in response to strain. No statistical analysis was carried out on this data as each experiment was only repeated twice. However, as the two inhibitors respectively exhibit a similar effect on transendothelial permeability, and correlate to the biochemical data observed, there is a strong likelihood that the results are significant. A similar finding was reported in a study by DeMaio *et al.* which demonstrated that shear stress-induced increases in transendothelial permeability correlated with increased tyrosine phosphorylation of occludin [DeMaio *et al.,* 2001]. However, to the best of our knowledge, this is the first instance in which a direct link has been established.

4.4 Conclusion

In this chapter we have shown that phosphorylation changes in occludin and ZO-1 following strain contribute to increased barrier integrity in BAECs. More specifically we have observed a tyrosine dephosphorylation event in occludin that can be blocked by dephostatin and a serine/threonine phosphorylation of ZO-1 that can be attenuated by rottlerin suggesting involvement of tyrosine phosphatase and PKCδ, respectively in these events. Moreover the changes in phosphorylation of both proteins can be linked to the subcellular localisation of occludin and ZO-1 and also to the increase in their co-association following strain. Furthermore the phosphorylation changes can also be linked to the decrease in endothelial permeability seen following strain, as treating the cells with dephostatin and rottlerin respectively caused the junction to become more

permeable (See Fig. 4.14). In conclusion therefore, post-translation modification of occludin and ZO-1 following strain by tyrosine phosphatase and PKC leads to increased barrier integrity.

Overview of Results Presented in Chapter 4

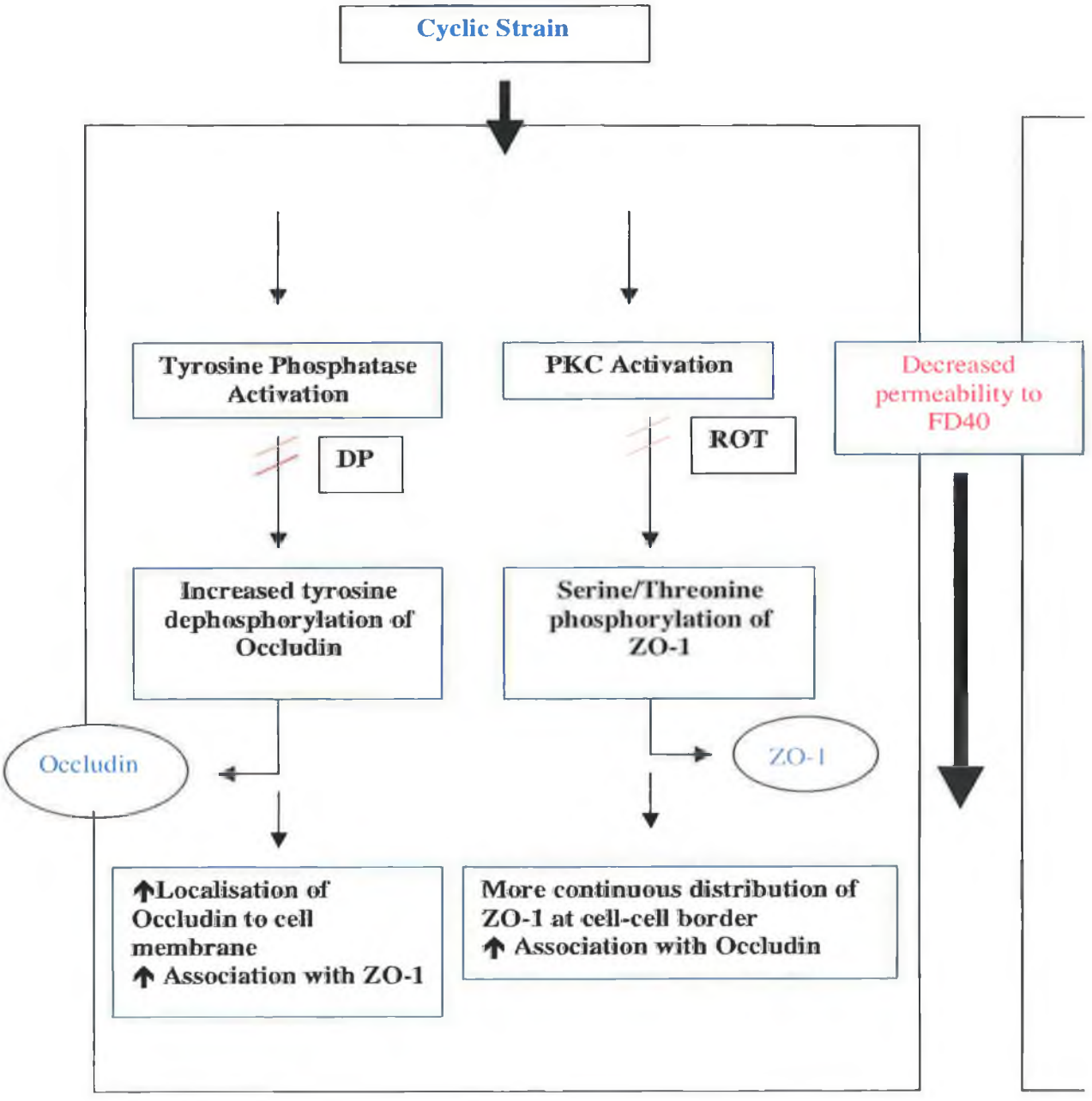

Fig. 4.14 Cyclic strain-induced phosphorylation events in BAECs

Chapter 5

Examination of the mechanotransduction pathway by which cyclic strain regulates tight junction permeability in BAECs.

5.1 Introduction

5.2 Results

5.3 Discussion

5.4 Conclusion

5.1 Introduction

Vascular cells have the ability to respond to mechanical stimuli, namely cyclic strain and shear stress. The ability to respond to these mechanical forces is facilitated by mechanically sensitive receptors or "mechanoreceptors" present in vascular cells. This process by which mechanical forces are detected and converted into a cell signal to elicit a response is referred to as mechanotransduction [Lehoux *et al.,* 1998]. This process allows changes in cellular phenotype and function in response to hemodynamic stimulation. Mechanotransduction requires the activation of mechanosensitive receptors, which may be activated directly by force, disruption of the ECM, or distortion of the cell membrane and cytoskeleton.

G-proteins, integrins and PTKs have all demonstrated mechano-sensitivity. As such they have formed the focus of this study as a means of elucidating the signaling pathway(s) involved in cyclic strain–mediated regulation of occludin and ZO-1. A number of studies have also demonstrated the downstream activation of a Rac-1/RhoA signaling cascade by a number of receptors in response to mechanical stimuli. This family of small GTPases are known to play a role in permeability and inflammation [Dudek *et al.*, 2001; Wojciak-Stothard *et al.*, 2002]. Thus, we have also examined the possible activation/inhibition of Rac-1/RhoA signaling in transducing the cyclic strain signal to ECs. We have also elected to examine the signaling molecules p38 and MEK, as the MAPK pathway is known to be activated in response to mechanical stimuli and have been linked to tight junction protein regulation.

To date there is very little reported about the mechanotransduction pathway that leads to activation of occludin and ZO-1 and ultimately to a tighter endothelial cell barrier.

The specific aim of this chapter is to investigate the signaling mechanisms involved in cyclic strain-regulation of occludin and ZO-1 in vascular ECs, with specific emphasis on G-proteins, integrins and PTK-mediated pathways.

5.2 Results

5.2.1. Effect of Genistein on the cyclic strain regulation of occludin and ZO-1.

The regulatory effect of genistein (50 μM), a receptor tyrosine kinase inhibitor, on cyclic strain-induced changes of occludin and ZO-1 protein expression in BAECs was determined by measuring total protein levels in cell lysates (by IP). Following exposure of BAECs to cyclic strain, (5%, 24 h), occludin protein expression increased significantly by 2.0 ± 0.1 fold compared to unstrained control. Following strain + genistein, occludin protein expression levels did not alter significantly from strain values, 2.0 ± 0.1 fold (Fig. 5.1A). Following exposure of BAEC to cyclic strain, (5%, 24 h), ZO-1 protein expression increased significantly by 2.0 ± 0.4 fold compared to unstrained control. Following strain + genistein, ZO-1 protein expression was reduced slightly although not significantly from strain values 1.7 ± 0.1 (Fig. 5.1B).

BAECs were exposed to cyclic strain (5%, 24 h) in the absence or presence of genistein and occludin/ZO-1 co-association monitored in total BAEC lysates by IP as described. Results indicated that the strain-induced increase in co-association of occludin/ZO-1 was not significantly effected following treatment with genistein (Fig. 5.2).

Following BAEC exposure to cyclic strain (5%, 24 h) subcellular localisation of occludin and ZO-1 was monitored in the absence or presence of genistein. Results

indicated that cyclic strain-induced localisation of occludin to the cell-cell border was not effected by genistein treatment (Fig. 5.3 i-iv). Furthermore, cyclic strain-induced localisation of ZO-1 to the cell-cell border in a more continuous fashion was not effected by genistein treatment (Fig. 5.4 i-iv).

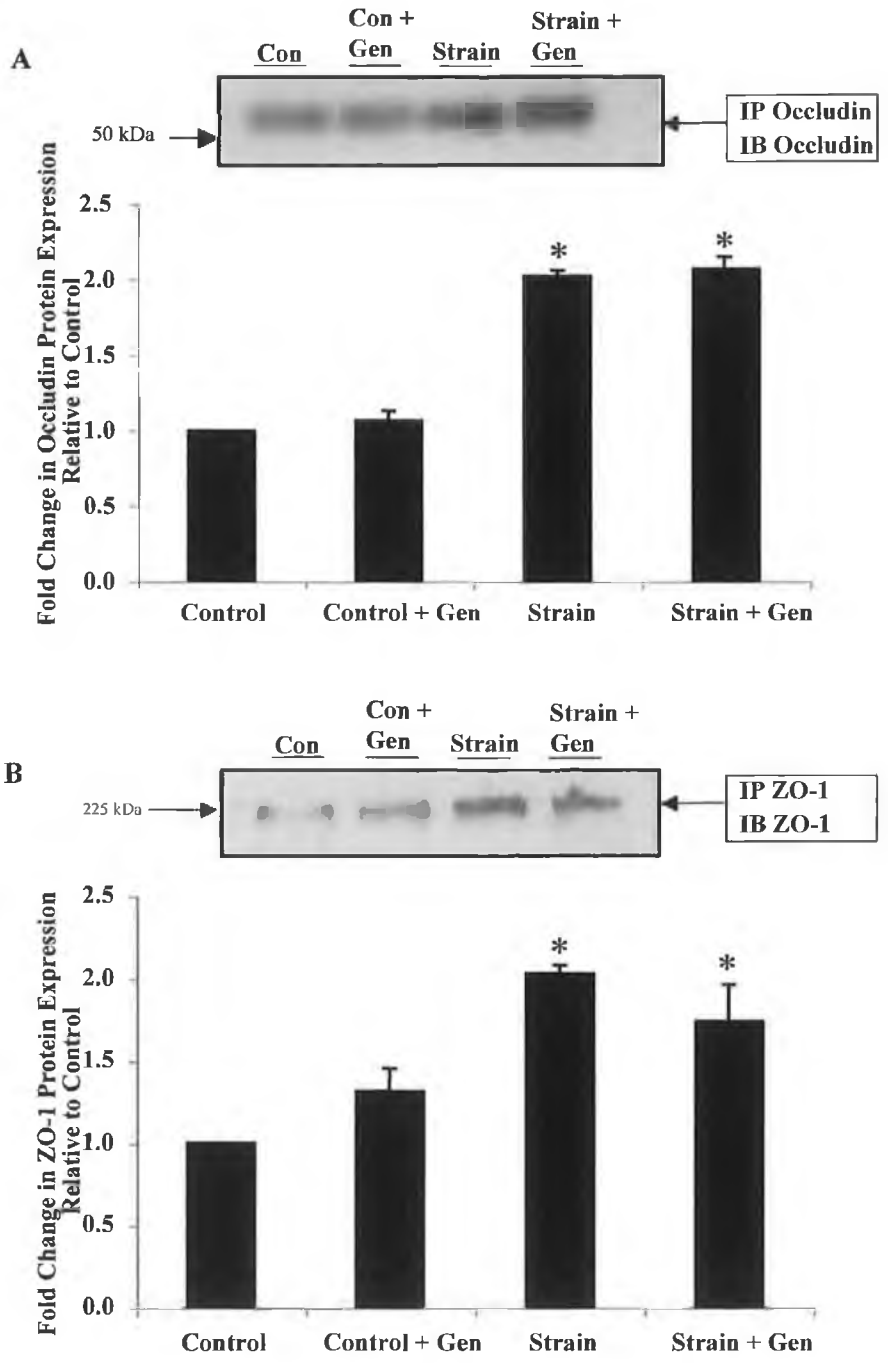

Fig. 5.1. The effect of Genistein on protein expression of occludin and ZO-1 following 5% Strain for 24h. BAECs were exposed to cyclic strain (5%, 24 h) ± genistein and monitored for (A) occludin or (B) ZO-1 protein expression by Western blotting. Representative blots are shown above each graph. Histograms represent fold change in band intensity relative to unstrained controls and are averaged from three independent experiments ±SEM; *$P \leq 0.05$ versus unstrained controls. Gen, Genistein.

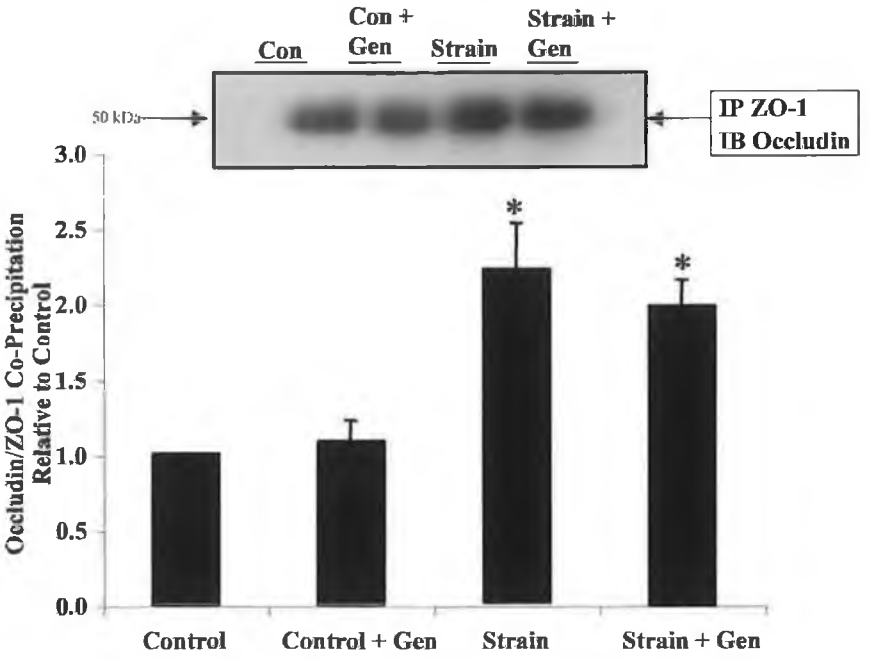

Fig. 5.2. Effect of Genistein on strain-induced occludin and ZO-1 co-association. Following exposure of BAECs to cyclic strain (5%, 24 h), co-association of occludin and ZO-1 were monitored by IP and Western blotting. Histogram represents fold change in band intensity relative to unstrained control and is averaged from three independent experiments ±SEM; *$P \leq 0.05$ versus unstrained controls.

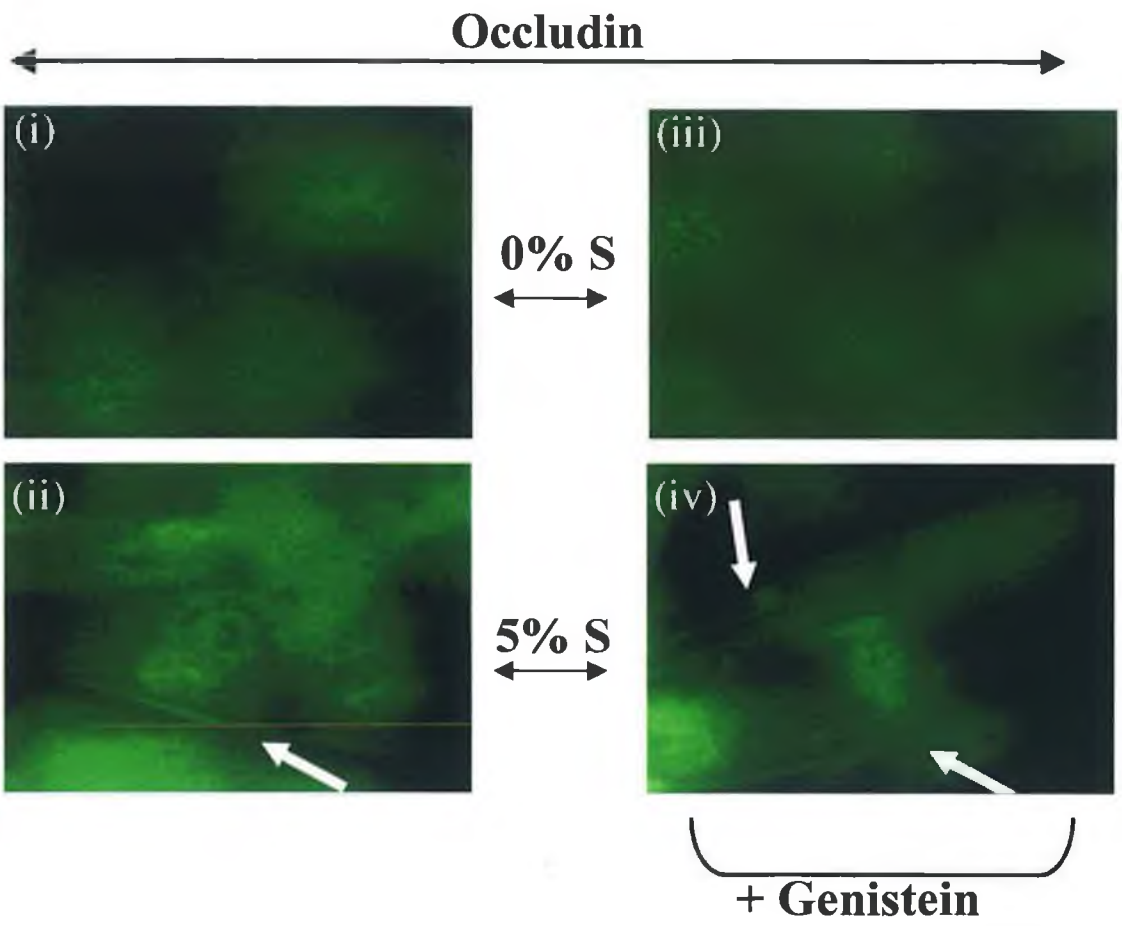

Fig. 5.3. Effect of Genistein on cyclic strain-induced occludin subcellular localisation in BAECs. Following exposure of BAECs to cyclic strain (5%, 24 h), localisation of occludin was monitored by immunocytochemistry. Occludin: (i) untreated control versus (ii) strain and (iii) Genistein-treated control versus (iv) strain. Occludin was monitored using standard fluorescence microscopy (1000x). White arrows indicate cell-cell border localisation. Images are representative of at least three individual sets of experiments.

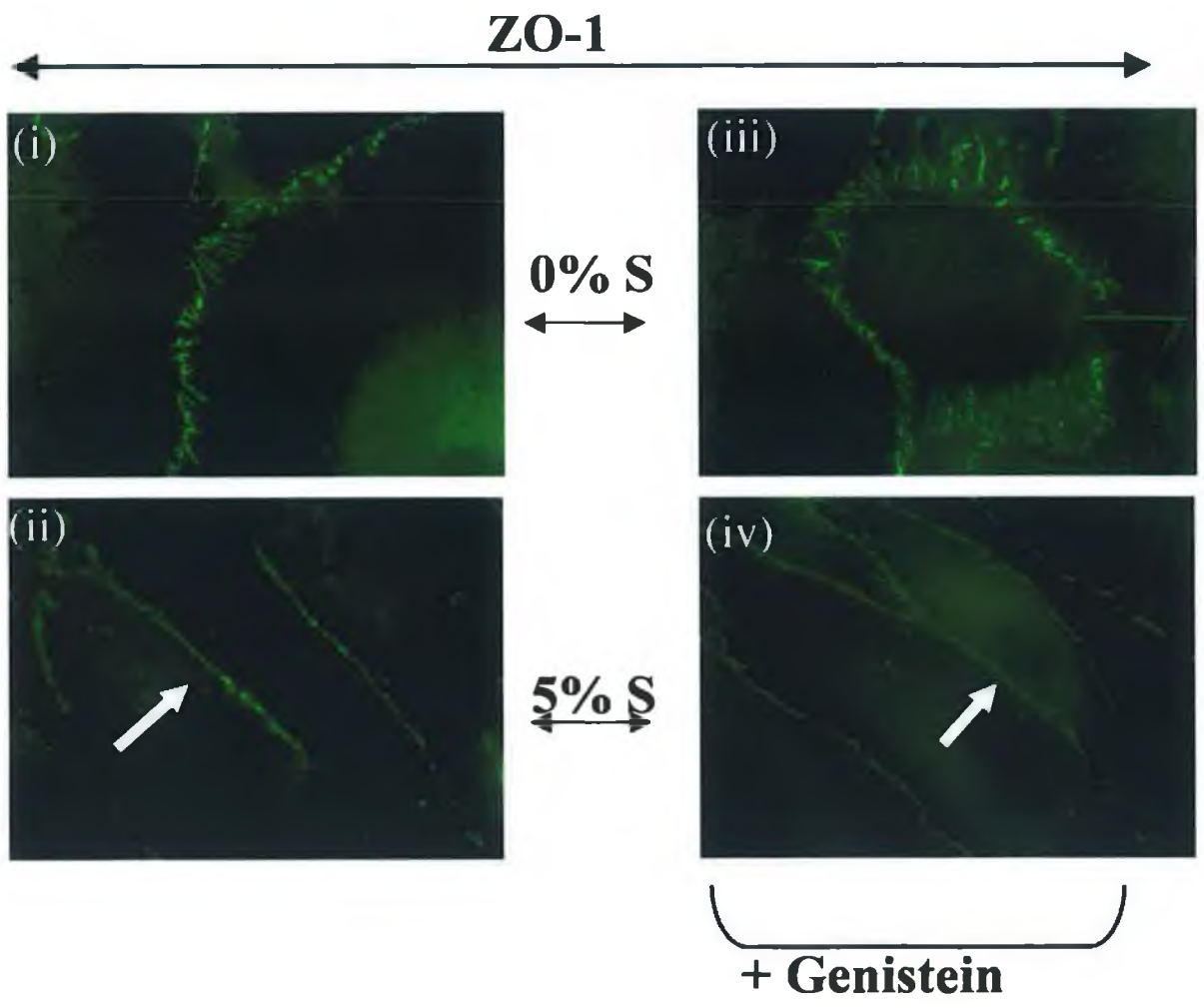

Fig. 5.4. Effect of Genistein on cyclic strain-induced ZO-1 subcellular localisation in BAECs. Following exposure of BAECs to cyclic strain (5%, 24 h), localisation of ZO-1 was monitored by immunocytochemistry. ZO-1: (i) untreated control versus (ii) strain and (iii) Genistein -treated control versus (iv) strain. ZO-1 was monitored using standard fluorescence microscopy (1000x). White arrows indicate cell-cell border localisation. Images are representative of at least three individual sets of experiments.

5.2.2 Effect of PTX on the cyclic strain regulation of occludin and ZO-1.

The regulatory effect of PTX (50 μM), a $G\alpha_i$ protein inhibitor, on cyclic strain-induced changes of occludin and ZO-1 protein expression in BAECs was determined by measuring total protein levels in cell lysates. Following exposure of BAECs to cyclic strain (5%, 24 h), occludin protein expression increased significantly by 2.1 ± 0.4 fold compared to unstrained control. However, addition of PTX reduced occludin protein expression to 1.2 ± 0.3 (Fig. 5.5A). Following exposure of BAECs to cyclic strain, ZO-1 protein expression increased significantly by 1.85 ± 0.1 fold. However addition of PTX reduced ZO-1 protein expression to 1.1 ± 0.1 (Fig. 5.5B) compared to unstrained control.

BAECs were exposed to cyclic strain (5%, 24 h) in the absence or presence of PTX and occludin/ZO-1 co-association monitored in total BAEC lysates by IP as described. Results indicated that the strain-induced increase in co-association of occludin/ZO-1 could be reduced from a 1.8 ± 0.1 fold increase following strain to a 1.2 ± 0.2 fold increase following treatment with PTX (Fig. 5.6).

Following BAEC exposure to cyclic strain (5%, 24 h) subcellular localisation of occludin and ZO-1 was monitored in the absence or presence of PTX. Results indicated that the localisation of occludin along the cell membrane observed in response to cyclic strain was completely ablated by PTX treatment (Fig. 5.7 i-iv). Furthermore, the continuous and well-defined organization of ZO-1 immunoreactivity initially observed

along the plasma membrane in response to cyclic strain was also completely ablated by PTX treatment, reverting to a discontinuous and jagged localisation pattern along the cell-cell border (Fig. 5.8 i-iv).

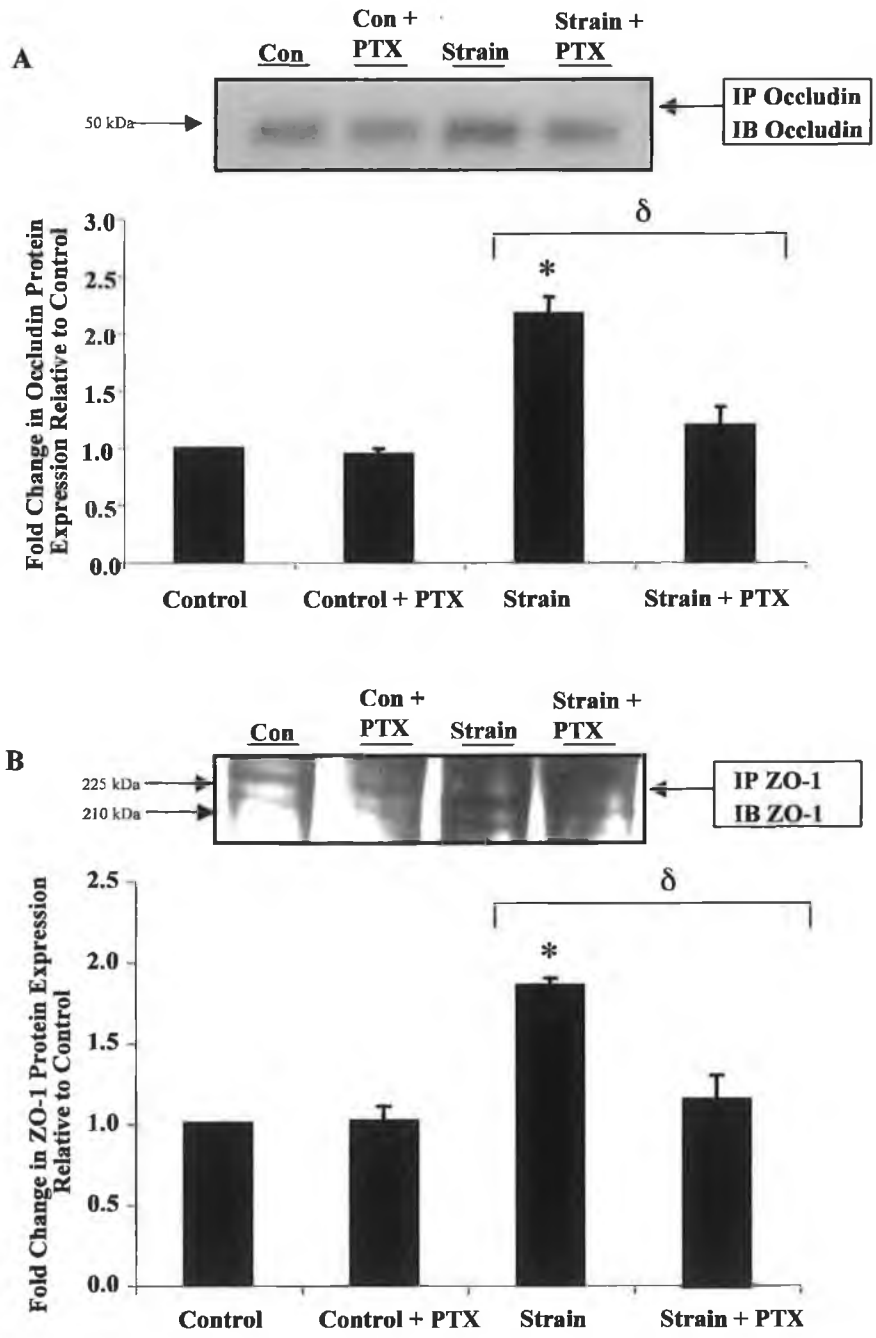

Fig. 5.5. The effect of PTX on occludin and ZO-1 protein expression following 5% strain for 24 h. BAECs were exposed to cyclic strain (5%, 24 h) ± PTX and monitored for (A) occludin or (B) ZO-1 protein expression by Western blotting. Representative blots are shown above each graph. Densitometric intensity of both bands has been combined. Histograms represent fold change in band intensity relative to unstrained controls and are averaged from three independent experiments ±SEM; $^{*}P \leq 0.05$ versus unstrained controls. $^{\delta}P \leq 0.05$ versus untreated strain.

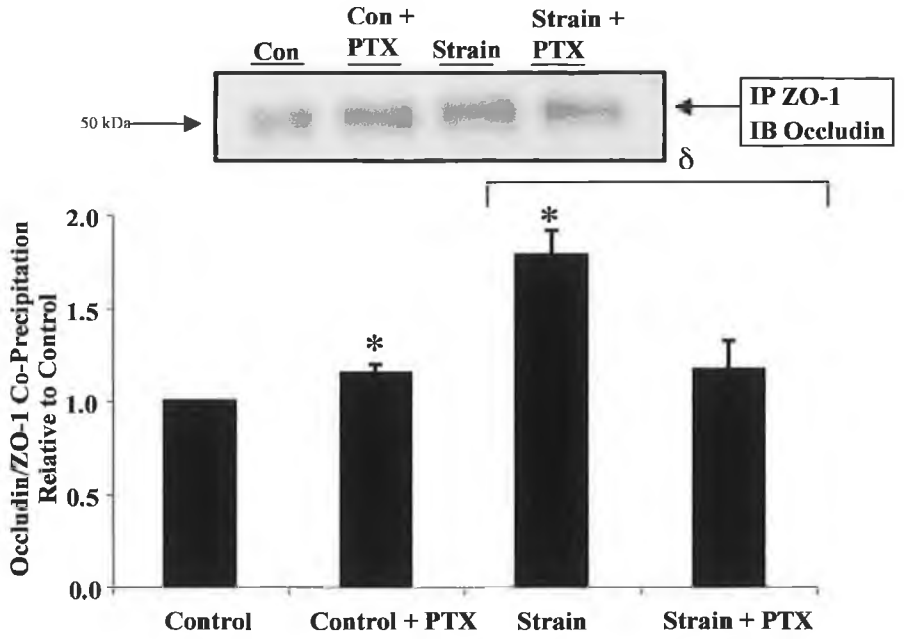

Fig. 5.6. Effect of 5% Strain + PTX on occludin and ZO-1 co-association in BAECs. Following exposure of BAECs to cyclic strain (5%, 24 h), co-association of occludin and ZO-1 were monitored by IP and Western blotting. Histogram represents fold change in band intensity relative to unstrained control and is averaged from three independent experiments ±SEM; $^{*}P \leq 0.05$ versus unstrained controls. $^{\delta}P \leq 0.05$ versus untreated strain.

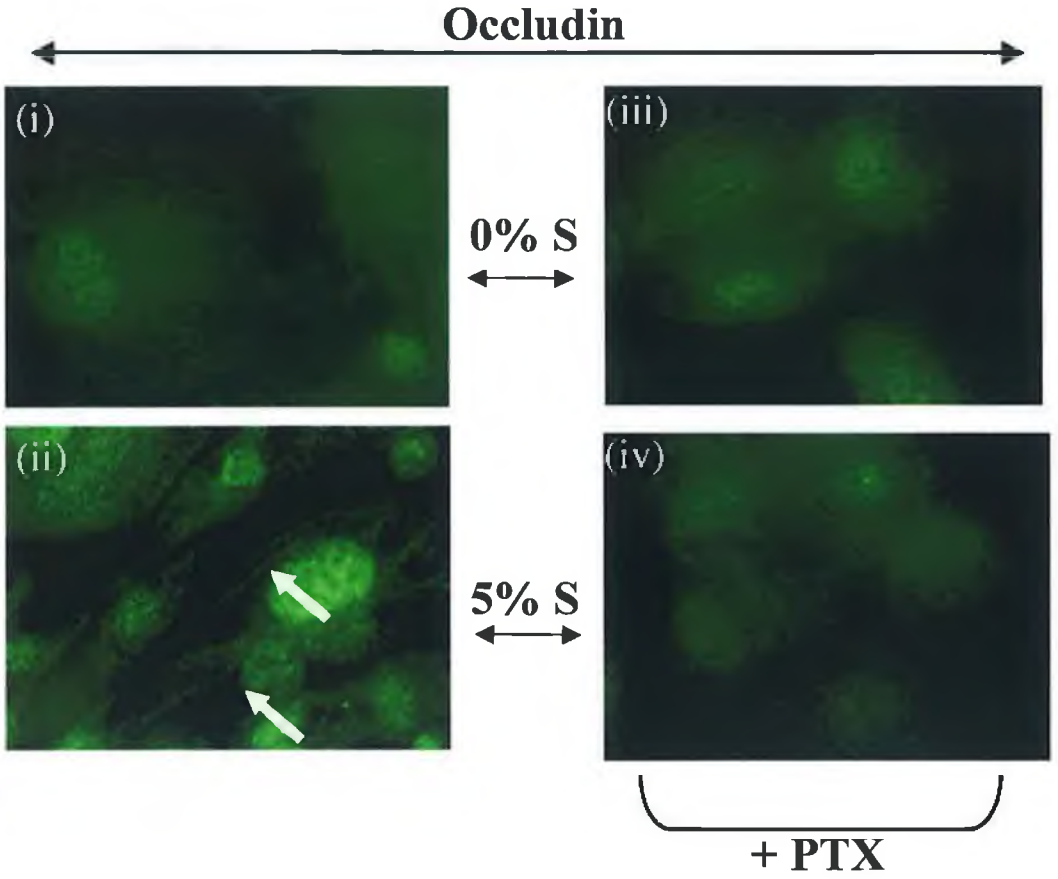

Fig. 5.7. Effect of PTX on cyclic strain-induced occludin subcellular localisation in BAECs. Following exposure of BAECs to cyclic strain (5%, 24 h), localisation of occludin was monitored by immunocytochemistry. Occludin: (i) untreated control versus (ii) strain and (iii) PTX -treated control versus (iv) strain. Occludin was monitored using standard fluorescence microscopy (1000x). White arrows indicate cell-cell border localisation. Images are representative of at least three individual sets of experiments.

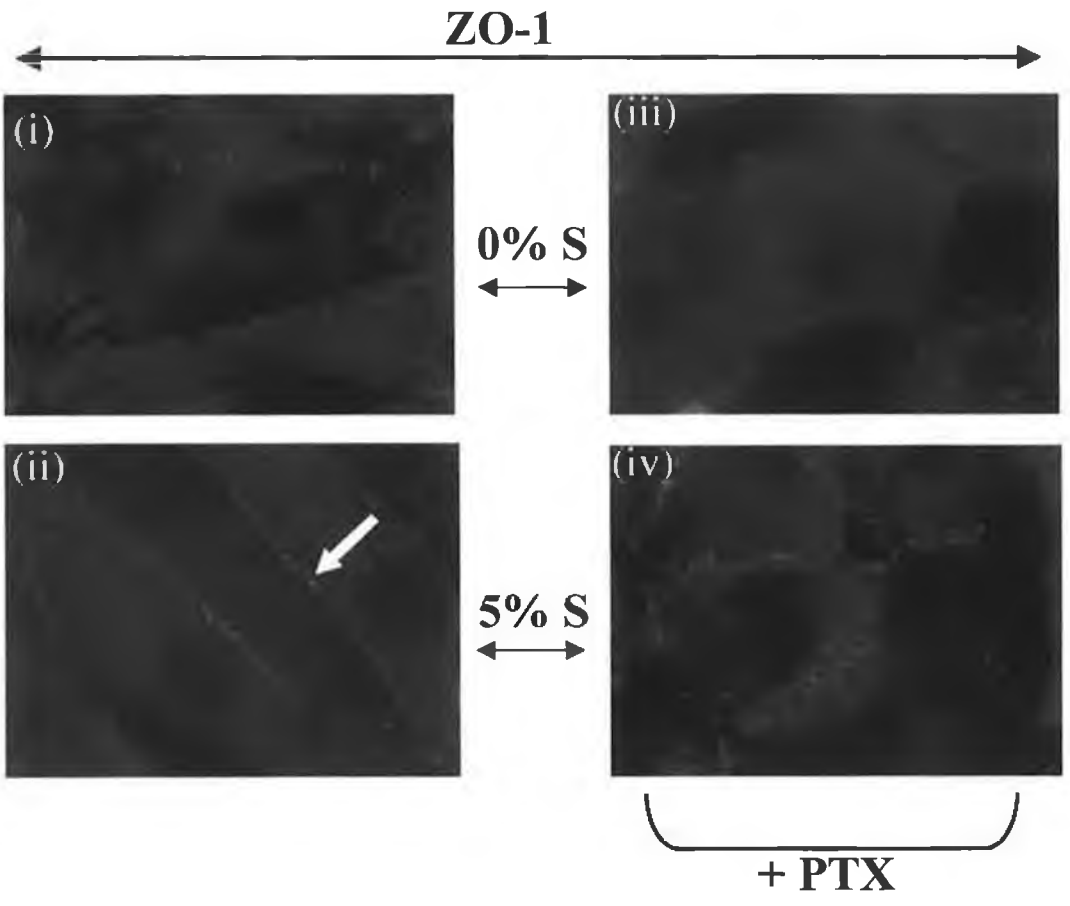

Fig. 5.8. Effect of PTX on cyclic strain-induced ZO-1 subcellular localisation in BAECs. Following exposure of BAECs to cyclic strain (5%, 24 h), localisation of ZO-1 was monitored by immunocytochemistry. ZO-1: (i) untreated control versus (ii) strain and (iii) PTX -treated control versus (iv) strain. ZO-1 was monitored using standard fluorescence microscopy (1000x). White arrows indicate cell-cell border localisation. Images are representative of at least three individual sets of experiments.

5.2.3. Effect of cyclic RGD on the cyclic strain regulation of occludin and ZO-1.

BAECs were exposed to cyclic strain (5%, 24 h) in the absence or presence of cRGD (0.1 μM), an integrin inhibitor, and occludin/ZO-1 co-association monitored (Fig. 5.9) in total BAEC lysates by IP as described. Results indicated that the strain-induced increase in co-association of occludin/ZO-1 was not significantly effected following treatment with cRGD, (1.9 ± 0.1 fold following strain and 2.1 ± 0.1 fold following strain and addition of cRGD).

Following BAEC exposure to cyclic strain (5%, 24 h) subcellular localisation of occludin and ZO-1 was monitored in the absence or presence of cRGD. Results indicated that cyclic strain-induced localisation of occludin to the cell-cell border was not effected by cRGD treatment (Fig. 5.10 i-iv). Furthermore, the cyclic strain-induced localisation of ZO-1 to the cell-cell border in a more continuous fashion was also not effected by cRGD treatment (Fig. 5.11 i-iv).

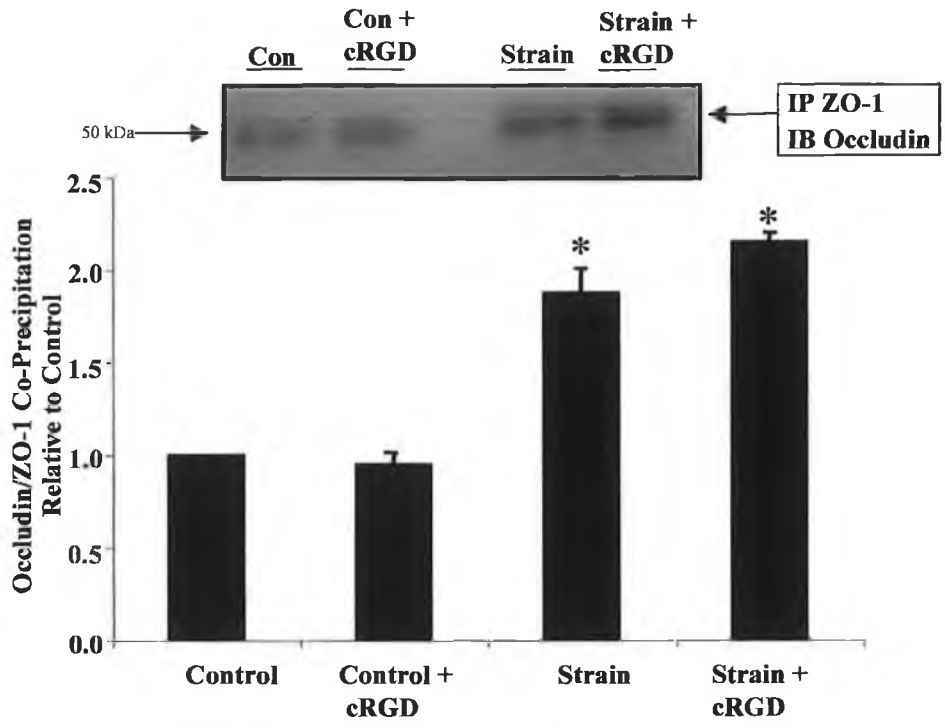

Fig. 5.9. Effect of 5% Strain + cRGD on occludin and ZO-1 co-association in BAECs. Following exposure of BAECs to cyclic strain (5%, 24 h), co-association of occludin and ZO-1 were monitored by IP and Western blotting. Histogram represents fold change in band intensity relative to unstrained control and is averaged from three independent experiments ±SEM; *$P \leq 0.05$ versus unstrained controls.

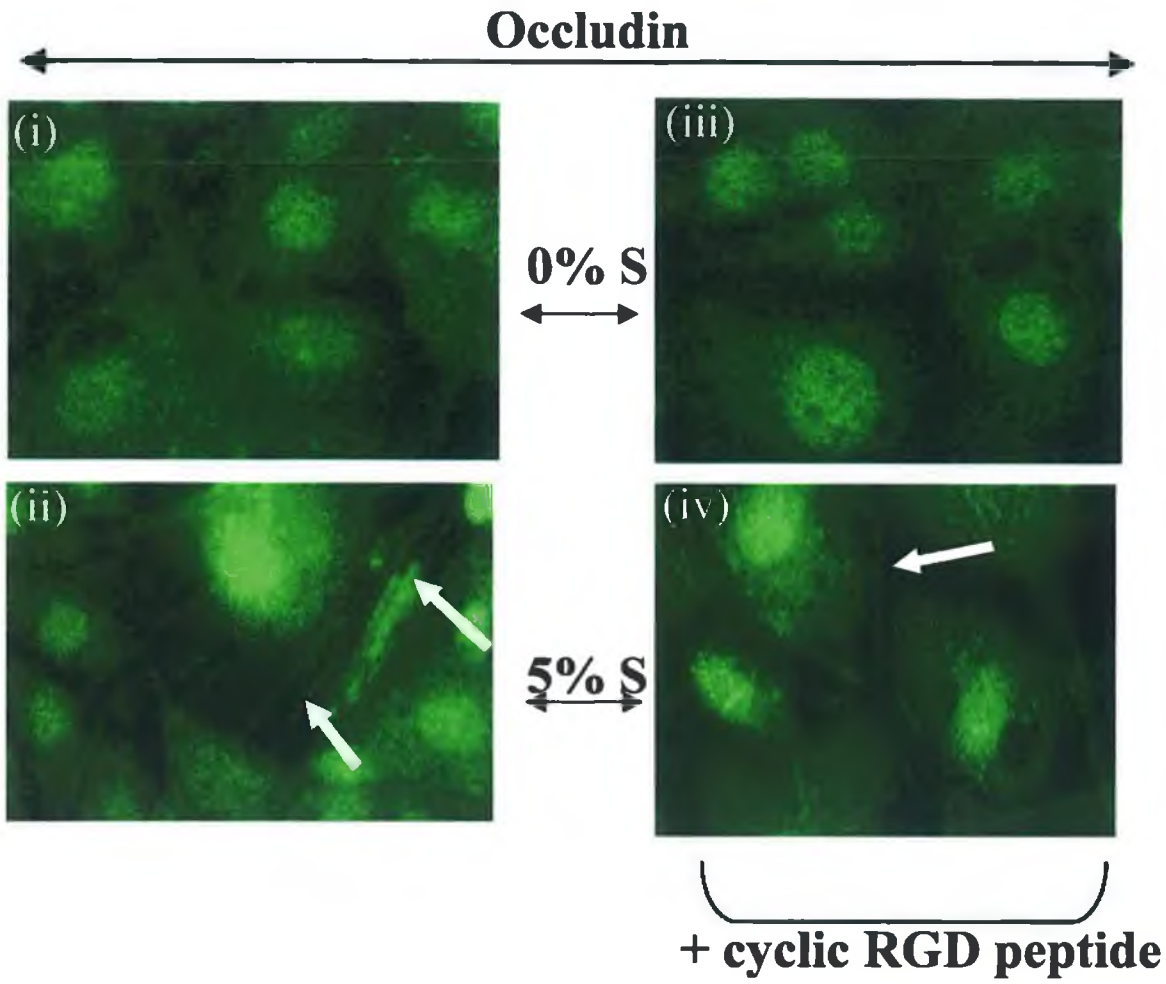

Fig. 5.10. Effect of cyclic RGD peptide on cyclic strain-induced occludin subcellular localisation in BAECs. Following exposure of BAEC to cyclic strain (5%, 24 h), localisation of Occludin was monitored by immunocytochemistry. Occludin : (i) untreated control versus (ii) strain and (iii) cyclic RGD peptide-treated control versus (iv) strain. occludin was monitored using standard fluorescence microscopy (1000x). White arrows indicate cell-cell border localisation. Images are representative of at least three individual sets of experiments.

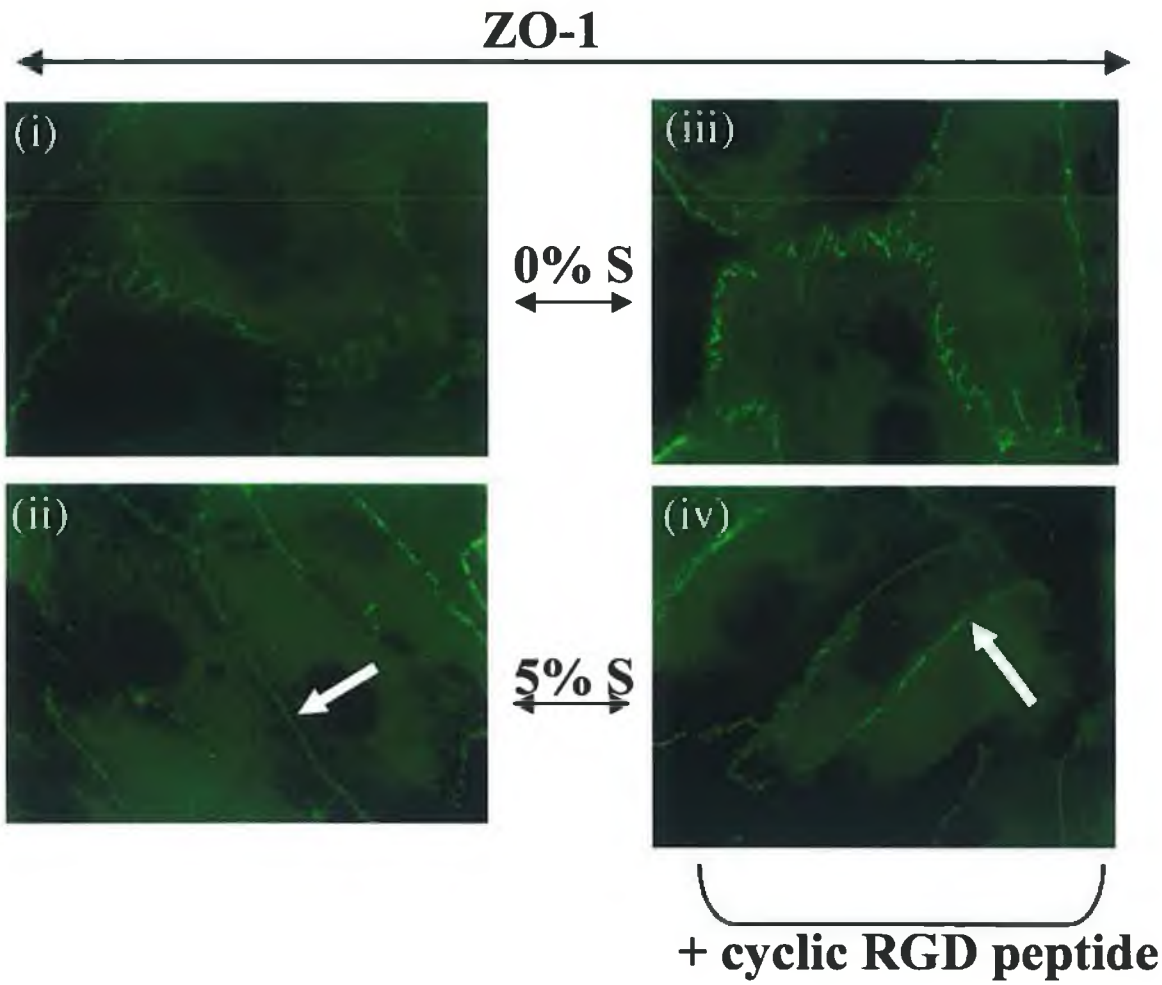

Fig. 5.11. Effect of cyclic RGD peptide on cyclic strain-induced ZO-1 subcellular localisation in BAECs. Following exposure of BAECs to cyclic strain (5%, 24 h), localisation of ZO-1 was monitored by immunocytochemistry. ZO-1: (i) untreated control versus (ii) strain and (iii) cyclic RGD peptide-treated control versus (iv) strain. ZO-1 was monitored using standard fluorescence microscopy (1000x). White arrows indicate cell-cell border localisation. Images are representative of at least three individual sets of experiments.

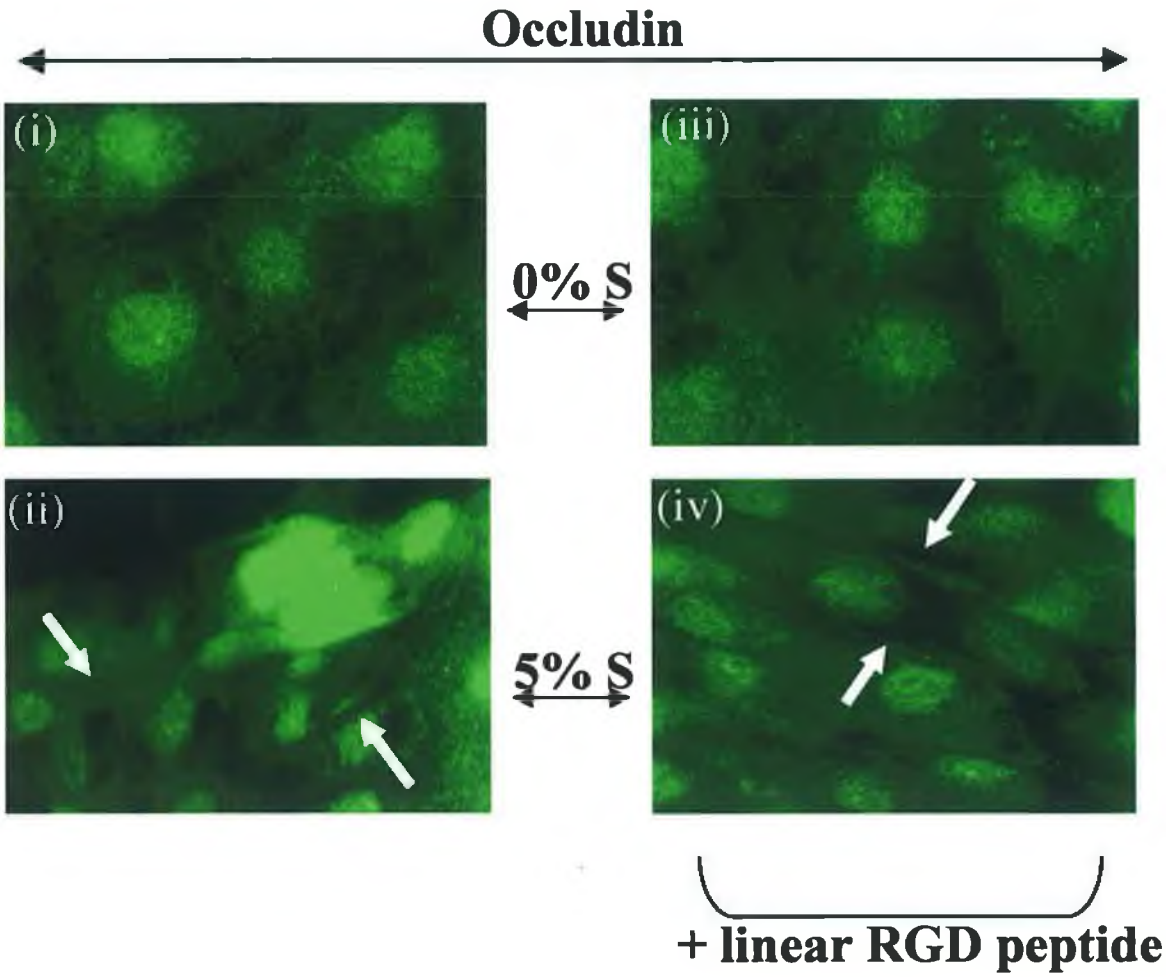

Fig. 5.12. Effect of linear RGD peptide on cyclic strain-induced occludin subcellular localisation in BAECs. Following exposure of BAECs to cyclic strain (5%, 24 h), localisation of occludin was monitored by immunocytochemistry. Occludin: (i) untreated control versus (ii) strain and (iii) linear RGD peptide -treated control versus (iv) strain. Occludin was monitored using standard fluorescence microscopy (1000x). White arrows indicate cell-cell border localisation. Images are representative of at least three individual sets of experiments.

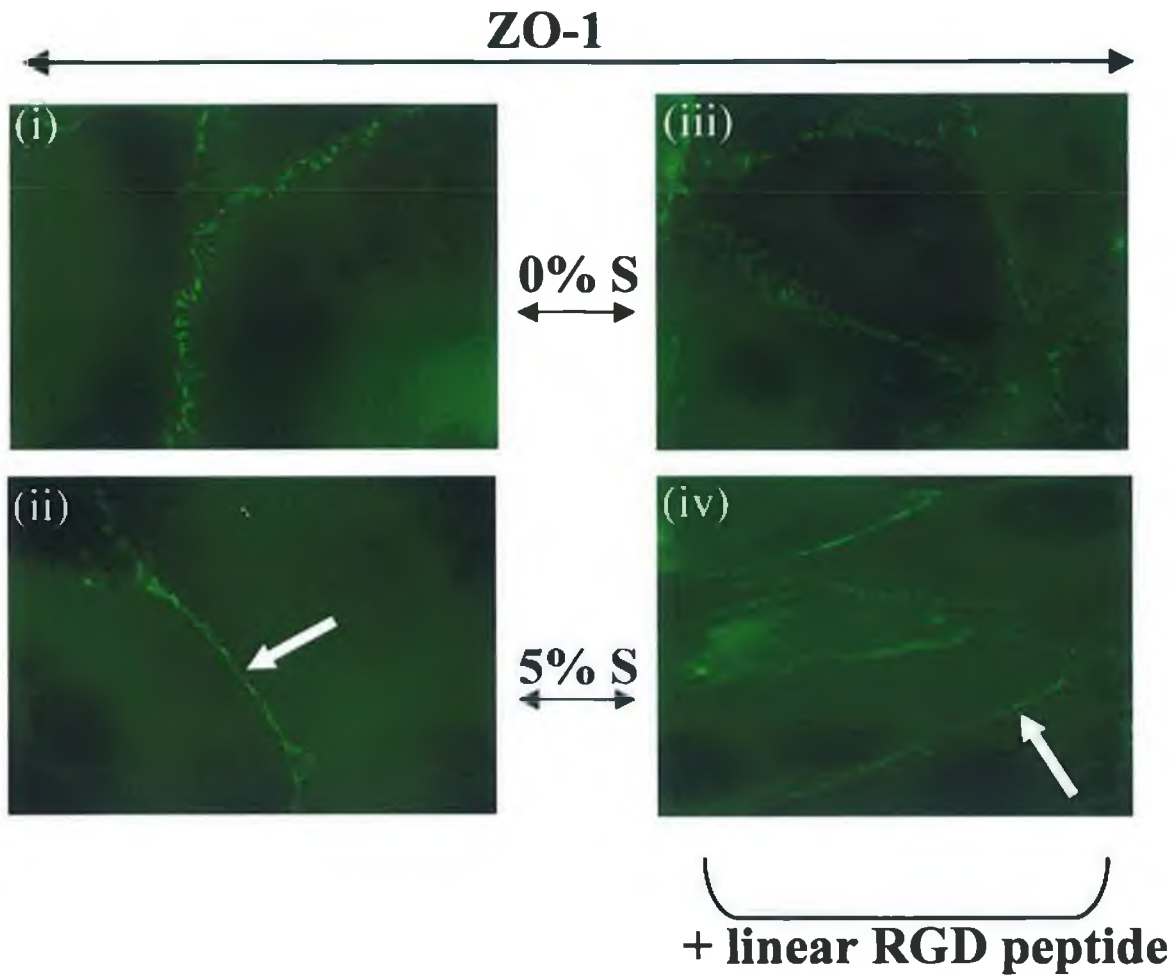

Fig. 5.13. Effect of linear RGD peptide on cyclic strain-induced ZO-1 subcellular localisation in BAECs. Following exposure of BAECs to cyclic strain (5%, 24 h), localisation of ZO-1 was monitored by immunocytochemistry. ZO-1: (i) untreated control versus (ii) strain and (iii) linear RGD peptide -treated control versus (iv) strain. ZO-1 was monitored using standard fluorescence microscopy (1000x). White arrows indicate cell-cell border localisation. Images are representative of at least three individual sets of experiments.

5.2.5. Effect of Rho Kinase inhibition on the cyclic strain regulation of occludin and ZO-1.

The regulatory effect of Y-27632 (20 μM), a Rho kinase inhibitor, on cyclic strain-induced changes of occludin and ZO-1 protein expression in BAECs was determined by measuring total protein levels in cell lysates. Following exposure of BAECs to cyclic strain, (5%, 24 h), occludin protein expression increased by 1.6 ± 0.2 fold compared with control. Following strain and addition of Y-27632, occludin protein expression increased significantly to 2.2 ± 0.2, compared to strain levels. (Fig. 5.14A). Following exposure of BAEC to cyclic strain, (5%, 24 h), ZO-1 protein expression increased by 1.7 ± 0.2 fold compared with control. Following strain and addition of Y-27632, ZO-1 protein expression increased to 2.3 ± 0.5 fold compared to strain levels (Fig. 5.14B).

BAECs were exposed to cyclic strain (5%, 24 h) in the absence or presence of Y-27632 and occludin/ZO-1 co-association monitored in total BAEC lysates by IP as described. Results indicated that strain-induced co-association of occludin/ZO-1 was increased significantly following treatment with Y-27632, (2.0 ± 0.1 fold following strain and 2.3 ± 0.1 fold following strain + Y-27632). (Fig. 5.15).

Following BAEC exposure to cyclic strain (5%, 24 h), in the absence or presence of Y-27632, subcellular localisation of occludin was monitored. Results indicated that cyclic strain-induced localisation of occludin to the cell-cell border was not affected by Y-27632 treatment (Fig. 5.16 i-iv).

Following BAEC exposure to cyclic strain (5%, 24 h) in the absence or presence of Y-27632, subcellular localisation of ZO-1 was also monitored by immunocytochemistry. Results indicated that cyclic strain-induced localisation of ZO-1 to the cell-cell border was not affected by Y-27632 treatment (Fig. 5.17 i-iv). Furthermore non-strained cells that were incubated with Y-27632 were observed to have a more continuous distribution of ZO-1 at the cell membrane (Fig. 5.17 iii).

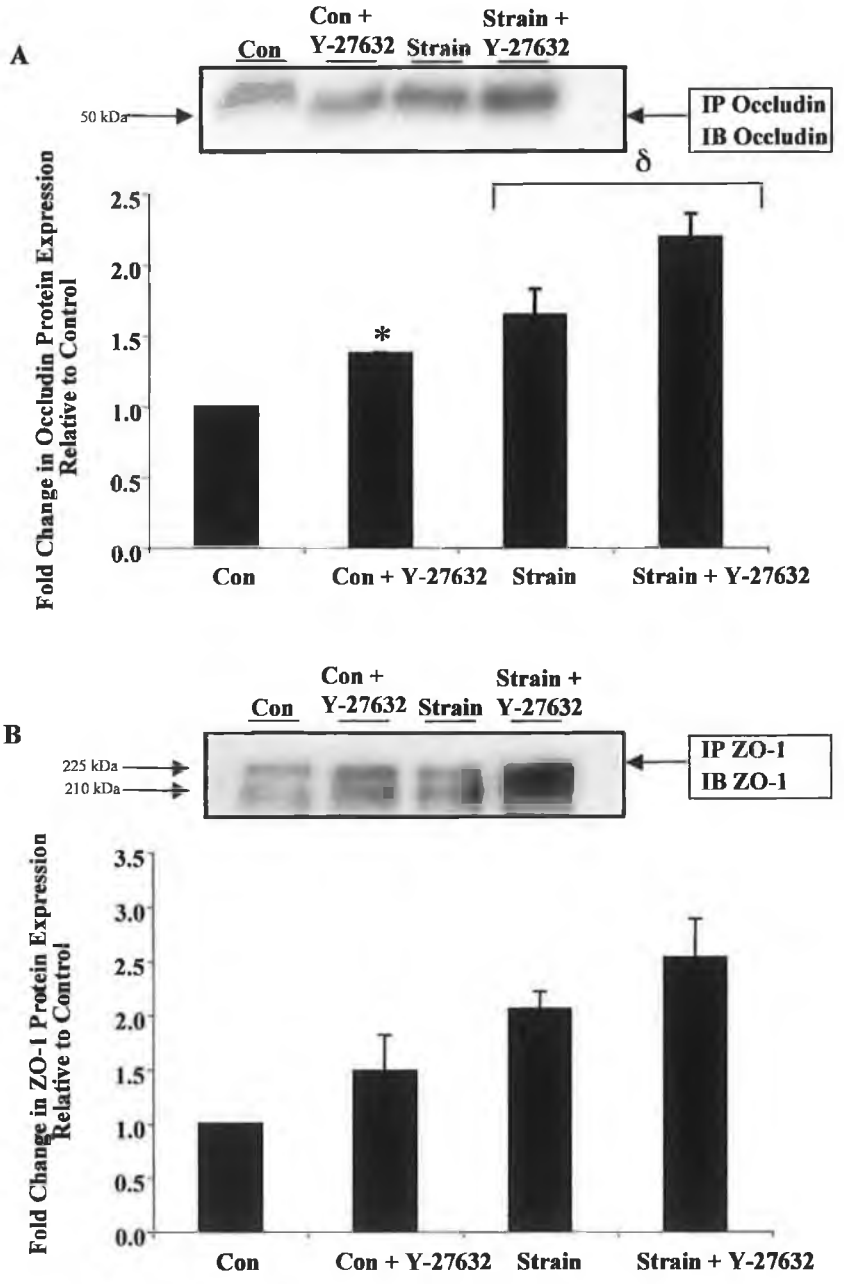

Fig. 5.14. The effect of Rho Kinase inhibition (Y-27632) on protein expression of occludin and ZO-1 following 5% Strain for 24 h. BAECs were exposed to cyclic strain (5%, 24 h) ± Y-27632 and monitored for (A) occludin or (B) ZO-1 protein expression by Western blotting. Representative blots are shown above each graph. Densitometric intensity of both bands has been combined. Histograms represent fold change in band intensity relative to unstrained controls and are averaged from three independent experiments ±SEM; $^{*}P \leq 0.05$ versus unstrained controls. $^{\delta}P \leq 0.05$ versus untreated strain.

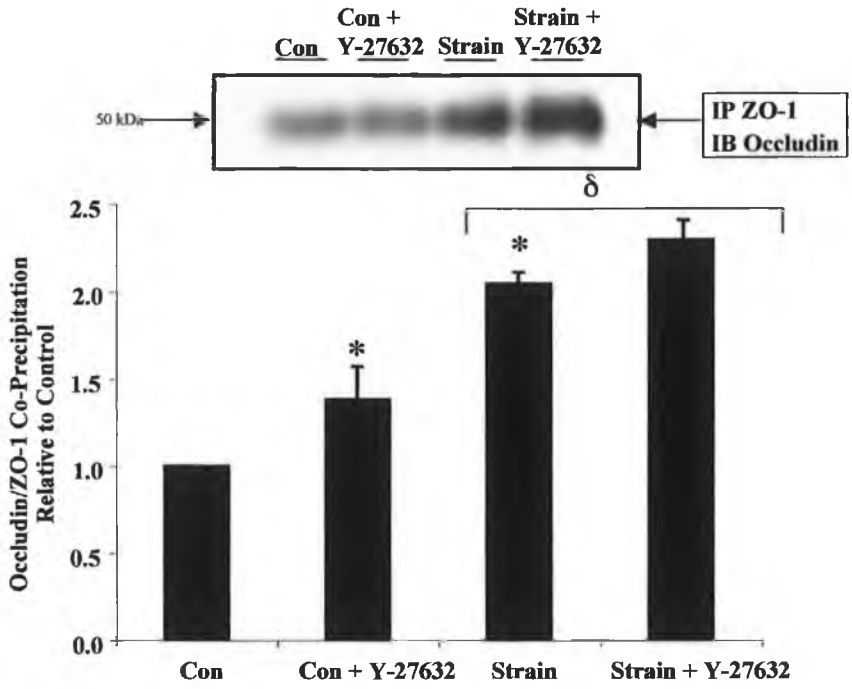

Fig. 5.15. Effect of 5% Strain + Rho Kinase inhibition (Y-27632) for 24hrs on occludin and ZO-1 co-association in BAECs. Following exposure of BAECs to cyclic strain (5%, 24 h), co-association of occludin and ZO-1 were monitored by IP and Western blotting. Representative blot is shown above graph. Histogram represents fold change in band intensity relative to unstrained control and is averaged from three independent experiments ±SEM; $^{*}P \leq 0.05$ versus unstrained controls. $^{\delta}P \leq 0.05$ versus untreated strain.

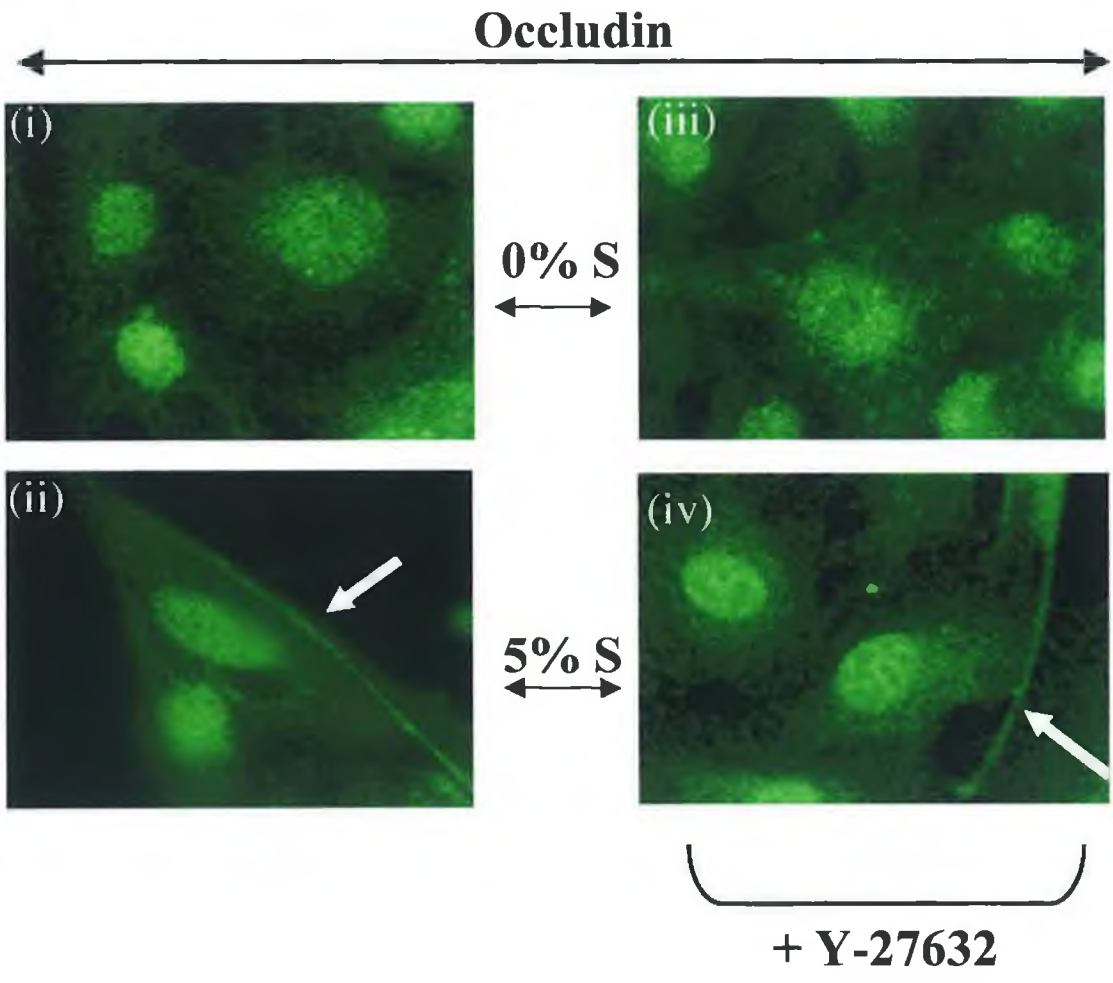

Fig. 5.16. Effect of Rho Kinase inhibition on cyclic strain-induced occludin subcellular localisation in BAECs. Following exposure of BAECs to cyclic strain (5%, 24 h), localisation of occludin was monitored by immunocytochemistry. Occludin: (i) untreated control versus (ii) strain and (iii) Y-27632-treated control versus (iv) strain. Occludin was monitored using standard fluorescence microscopy (1000x). White arrows indicate cell-cell border localisation. Images are representative of at least three individual sets of experiments.

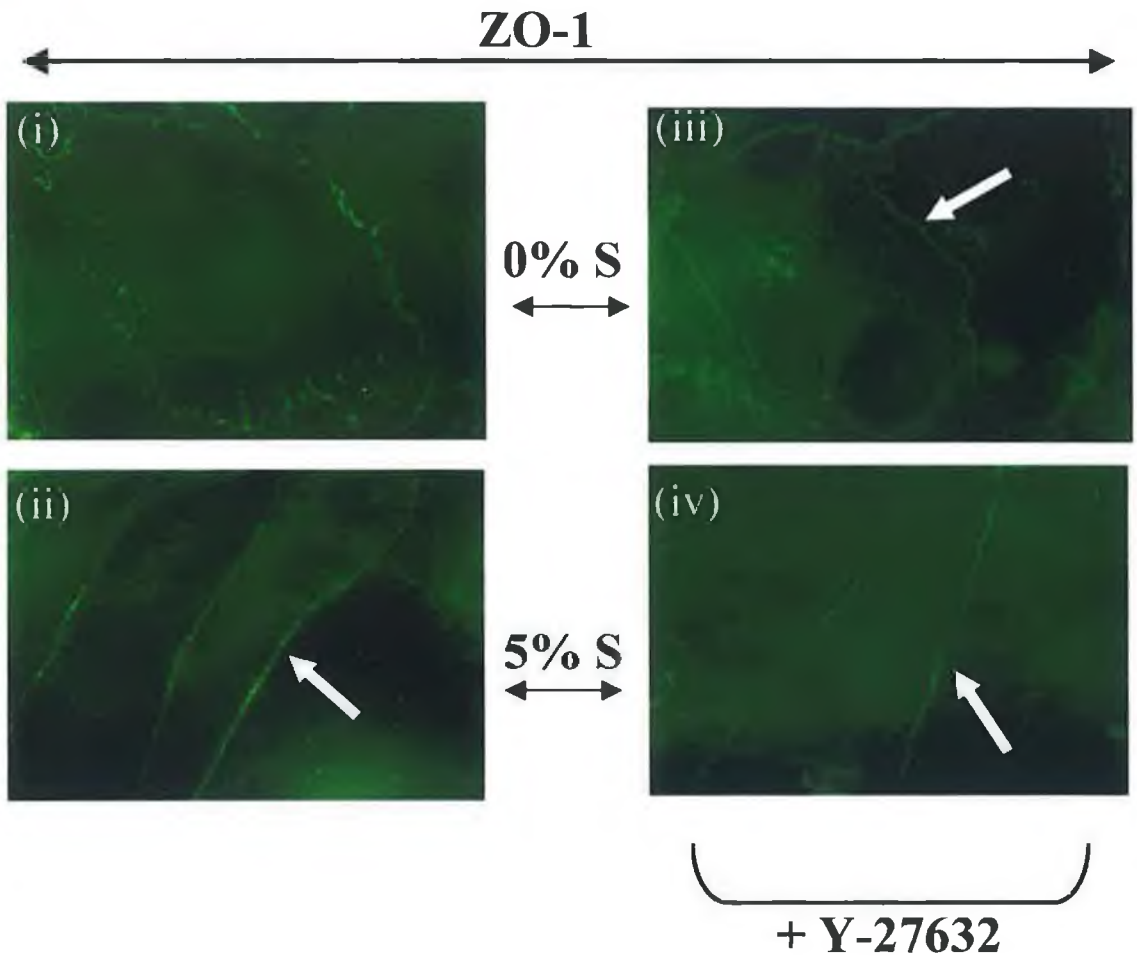

Fig. 5.17. Effect of Rho Kinase inhibition on cyclic strain-induced ZO-1 subcellular localisation in BAECs. Following exposure of BAECs to cyclic strain (5%, 24 h), localisation of ZO-1 was monitored by immunocytochemistry. ZO-1: (i) untreated control versus (ii) strain and (iii) Y-27632-treated control versus (iv) strain. ZO-1 was monitored using standard fluorescence microscopy (1000x). White arrows indicate cell-cell border localisation. Images are representative of at least three individual sets of experiments.

5.2.6. Effect of Rac-1 inhibition on the cyclic strain regulation of occludin and ZO-1.

BAEC were exposed to cyclic strain (5%, 24 h) in the absence or presence of NSC23766, (50 μM), a Rac-1 inhibitor, and occludin/ZO-1 co-association monitored in total BAEC lysates by IP as described. Results indicated that strain-induced co-association of occludin/ZO-1 could be significantly attenuated from an increase of 1.8 fold ± 0.2 fold following strain to 1.2 ± 0.2 fold following treatment with NSC23766 (Fig. 5.18).

Following BAEC exposure to cyclic strain (5%, 24 h) subcellular localisation of occludin was monitored in the absence or presence of NSC23766. Results indicated that the localisation of occludin along the cell membrane observed in response to cyclic strain was completely ablated by NSC23766 treatment (Fig. 5.19 i-iv).

Following BAEC exposure to cyclic strain (5%, 24 h) subcellular localisation of ZO-1 was also monitored in the absence or presence of NSC23766. Results indicated that the continuous and well-defined organization of ZO-1 immunoreactivity initially observed along the plasma membrane in response to cyclic strain was completely ablated by NSC23766 treatment, reverting to a discontinuous and jagged localisation pattern along the cell-cell border (Fig. 5.20 i-iv).

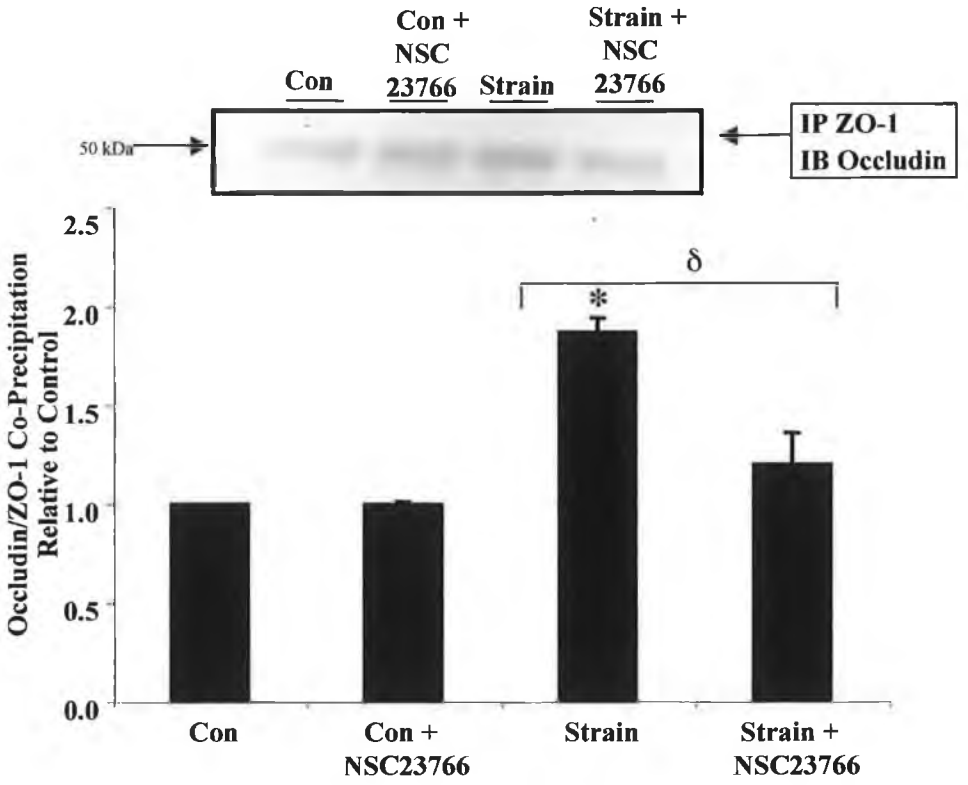

Fig. 5.18. Effect of 5% Strain + NSC23766 for 24 h on occludin and ZO-1 co-association in BAECs. Following exposure of BAECs to cyclic strain (5%, 24 h), co-association of occludin and ZO-1 was monitored by IP and Western blotting. Representative blot is shown above graph. Histogram represents fold change in band intensity relative to unstrained control and is averaged from three independent experiments ±SEM; $^{*}P \leq 0.05$ versus unstrained controls. $^{\delta}P \leq 0.05$ versus untreated strain.

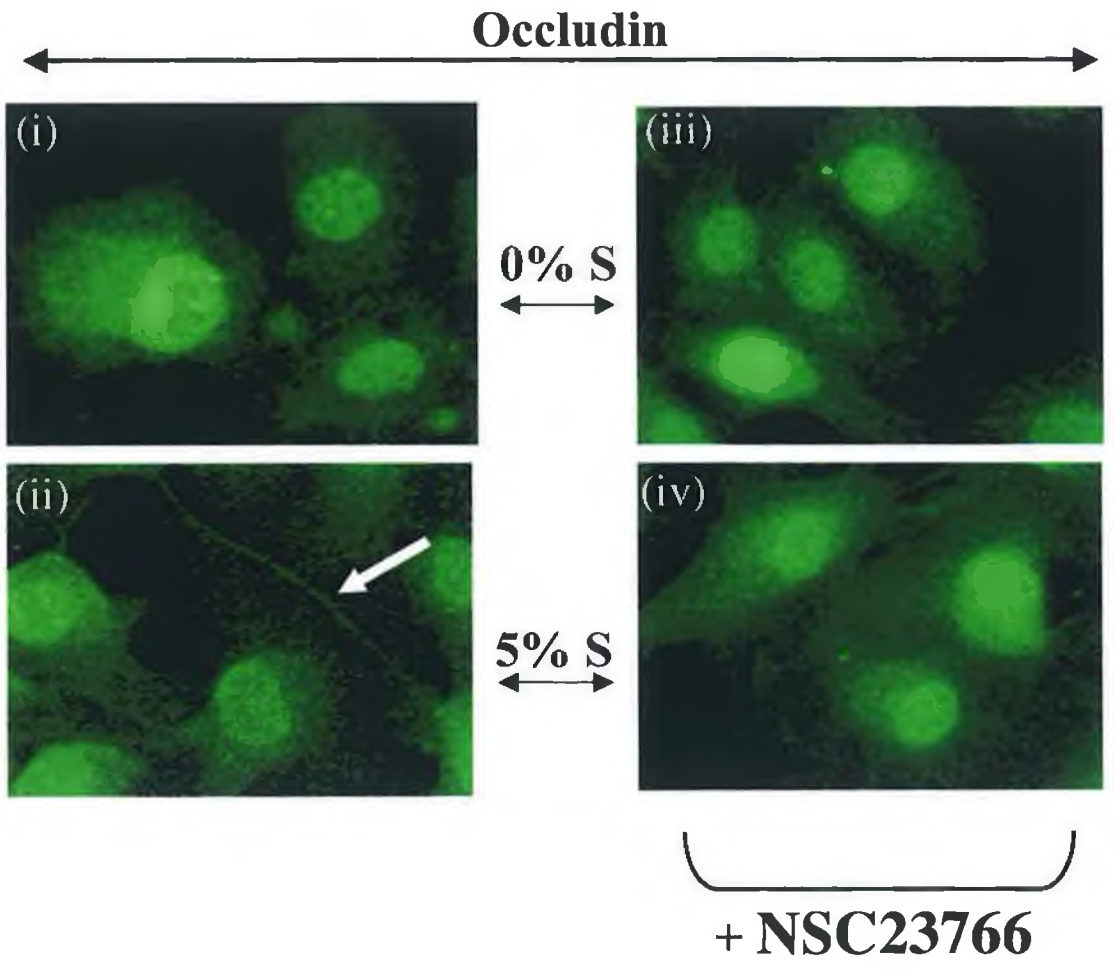

Fig. 5.19. Effect of NSC23766 on cyclic strain-induced occludin subcellular localisation in BAECs. Following exposure of BAECs to cyclic strain (5%, 24 h), localisation of occludin was monitored by immunocytochemistry. Occludin: (i) untreated control versus (ii) strain and (iii) NSC23766-treated control versus (iv) strain. Occludin was monitored using standard fluorescence microscopy (1000x). White arrows indicate cell-cell border localisation. Images are representative of at least three individual sets of experiments.

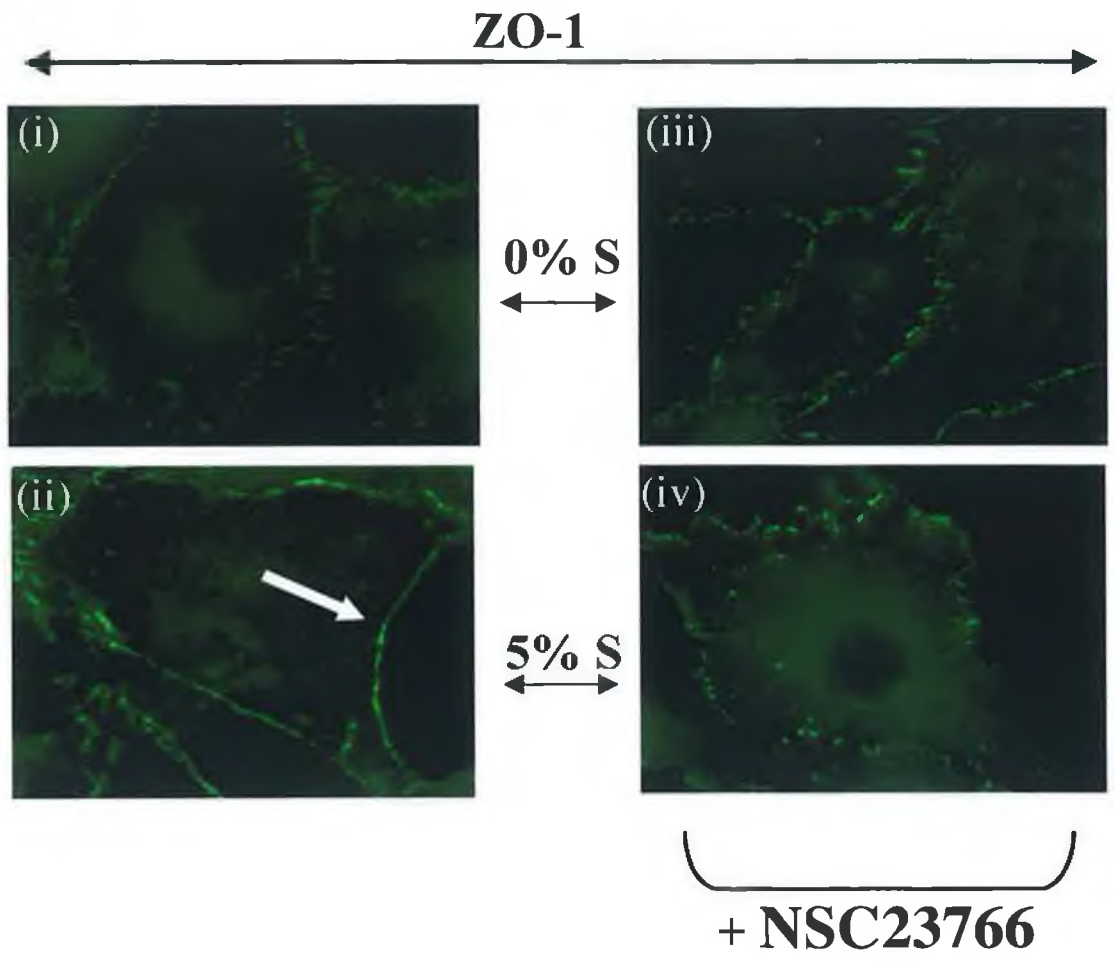

Fig. 5.20. Effect of NSC23766 on cyclic strain-induced ZO-1 subcellular localisation in BAECs. Following exposure of BAECs to cyclic strain (5%, 24 h), localisation of ZO-1 was monitored by immunocytochemistry. ZO-1: (i) untreated control versus (ii) strain and (iii) NSC23766-treated control versus (iv) strain. ZO-1 was monitored using standard fluorescence microscopy (1000x). White arrows indicate cell-cell border localisation. Images are representative of at least three individual sets of experiments.

5.2.7. Effect of p38 on the cyclic strain regulation of occludin and ZO-1 localisation.

Following BAEC exposure to cyclic strain (5%, 24 h) subcellular localisation of occludin was monitored in the absence or presence of PD169316 (10 μM), a p38 pharmacological inhibitor. Results indicated that the localisation of occludin along the cell membrane observed in response to cyclic strain was completely ablated by PD169316 treatment (Fig. 5.21 i-iv).

Following BAEC exposure to cyclic strain (5%, 24 h) subcellular localisation of ZO-1 was also monitored in the absence or presence of PD169316. Results indicated that the continuous and well-defined organization of ZO-1 immunoreactivity initially observed along the plasma membrane in response to cyclic strain was completely ablated by PD169316 treatment, reverting to a discontinuous and jagged localisation pattern along the cell-cell border (Fig. 5.22 i-iv).

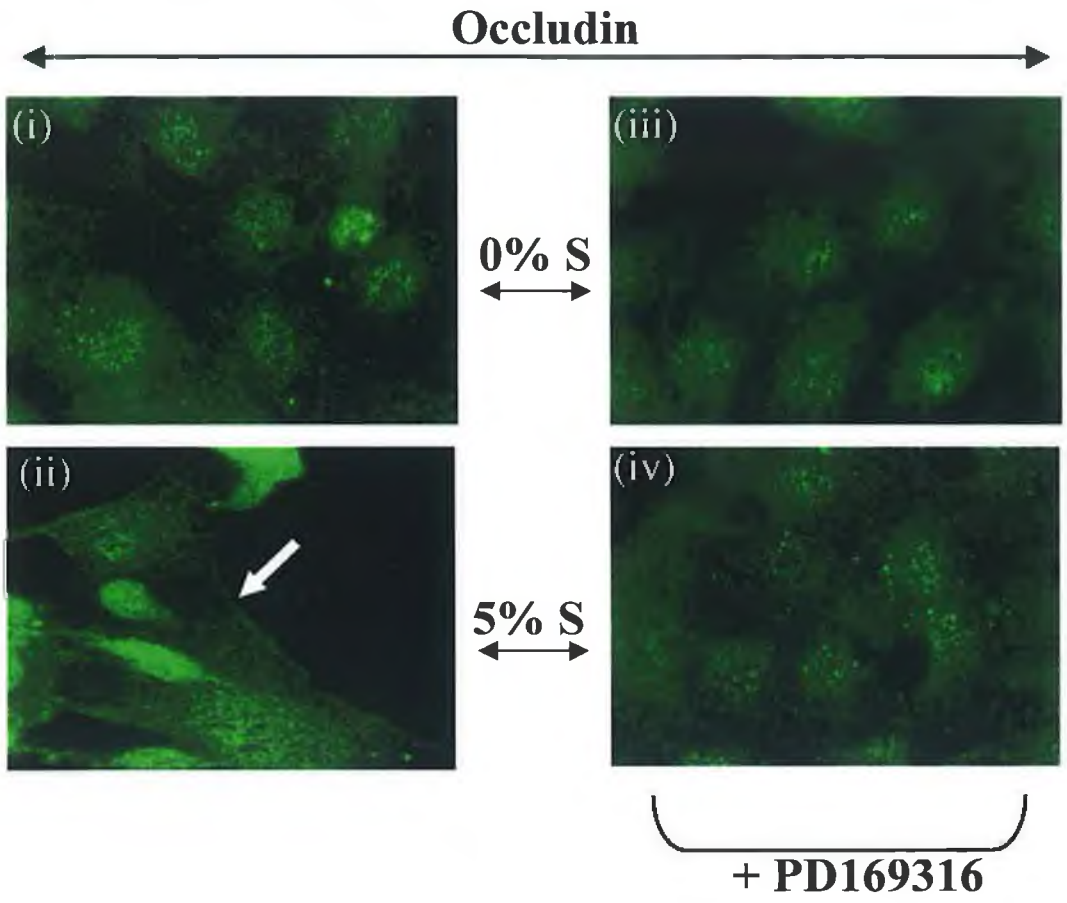

Fig. 5.21. Effect of PD169316 on cyclic strain-induced occludin subcellular localisation in BAECs. Following exposure of BAECs to cyclic strain (5%, 24 h), localisation of occludin was monitored by immunocytochemistry. Occludin: (i) untreated control versus (ii) strain and (iii) PD169316-treated control versus (iv) strain. Occludin was monitored using standard fluorescence microscopy (1000x). White arrows indicate cell-cell border localisation. Images are representative of at least three individual sets of experiments.

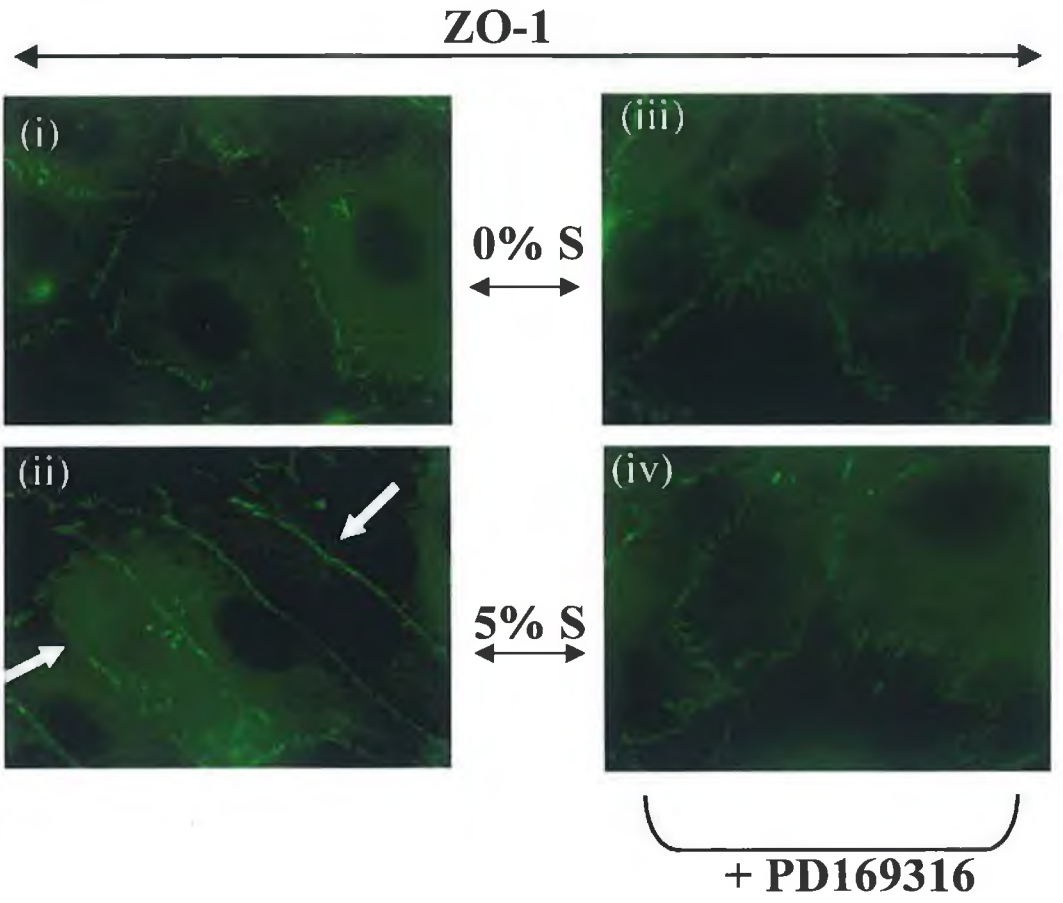

Fig. 5.22. Effect of PD169316 on cyclic strain-induced ZO-1 subcellular localisation in BAECs. Following exposure of BAECs to cyclic strain (5%, 24 h), localisation of ZO-1 was monitored by immunocytochemistry. ZO-1: (i) untreated control versus (ii) strain and (iii) PD169316-treated control versus (iv) strain. ZO-1 was monitored using standard fluorescence microscopy (1000x). White arrows indicate cell-cell border localisation. Images are representative of at least three individual sets of experiments.

5.2.8. Effect of MEK on the cyclic strain regulation of occludin and ZO-1 localisation.

Following BAEC exposure to cyclic strain (5%, 24 h) subcellular localisation of occludin was monitored in the absence or presence of PD98059 (10 μM), a MEK pharmacological inhibitor. Results indicated that cyclic strain-induced localisation of occludin to the cell-cell border was not effected by PD98059 treatment (Fig. 5.23 i-iv).

Following BAEC exposure to cyclic strain (5%, 24 h) in the absence or presence of PD98059, subcellular localisation of ZO-1 was also monitored by immunocytochemistry. Results indicated that cyclic strain-induced localisation of ZO-1 to the cell-cell border in a more continuous fashion was not effected by PD98059 treatment (Fig. 5.24 i-iv).

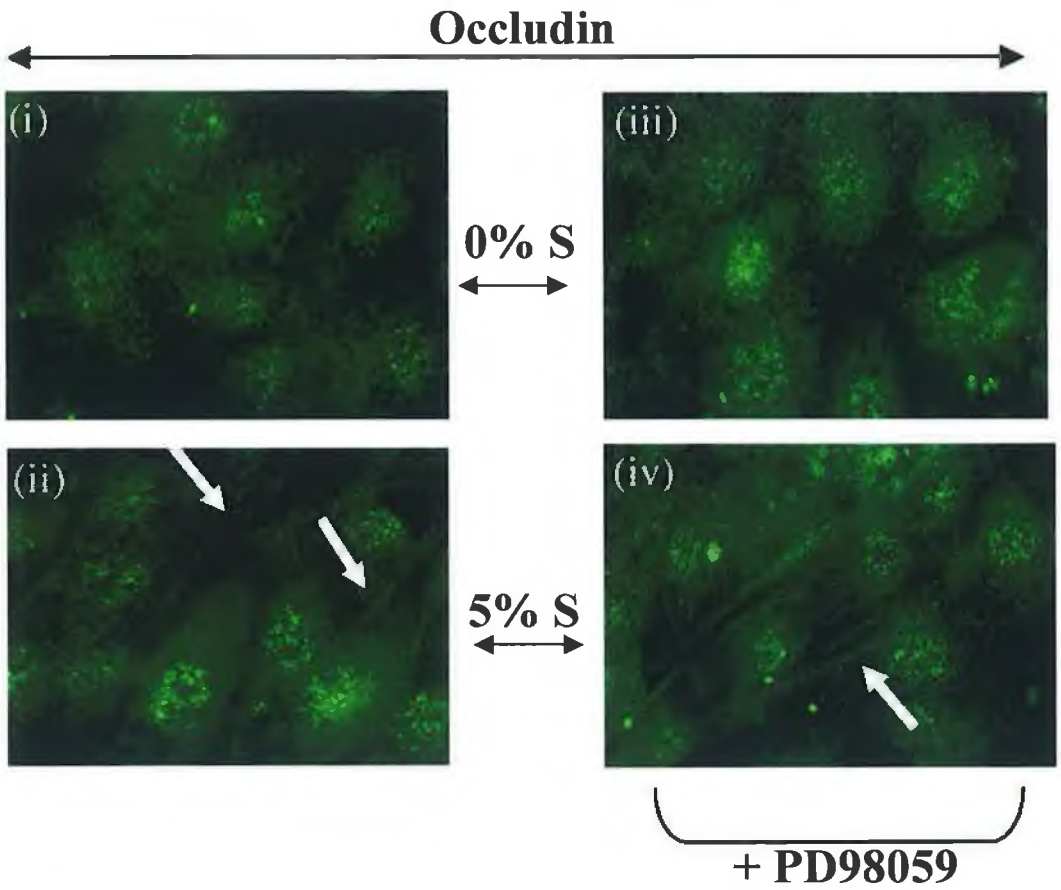

Fig. 5.23. Effect of PD98059 on cyclic strain-induced occludin subcellular localisation in BAECs. Following exposure of BAECs to cyclic strain (5%, 24 h), localisation of occludin was monitored by immunocytochemistry. Occludin: (i) untreated control versus (ii) strain and (iii) PD98059-treated control versus (iv) strain. Occludin was monitored using standard fluorescence microscopy (1000x). White arrows indicate cell-cell border localisation. Images are representative of at least three individual sets of experiments.

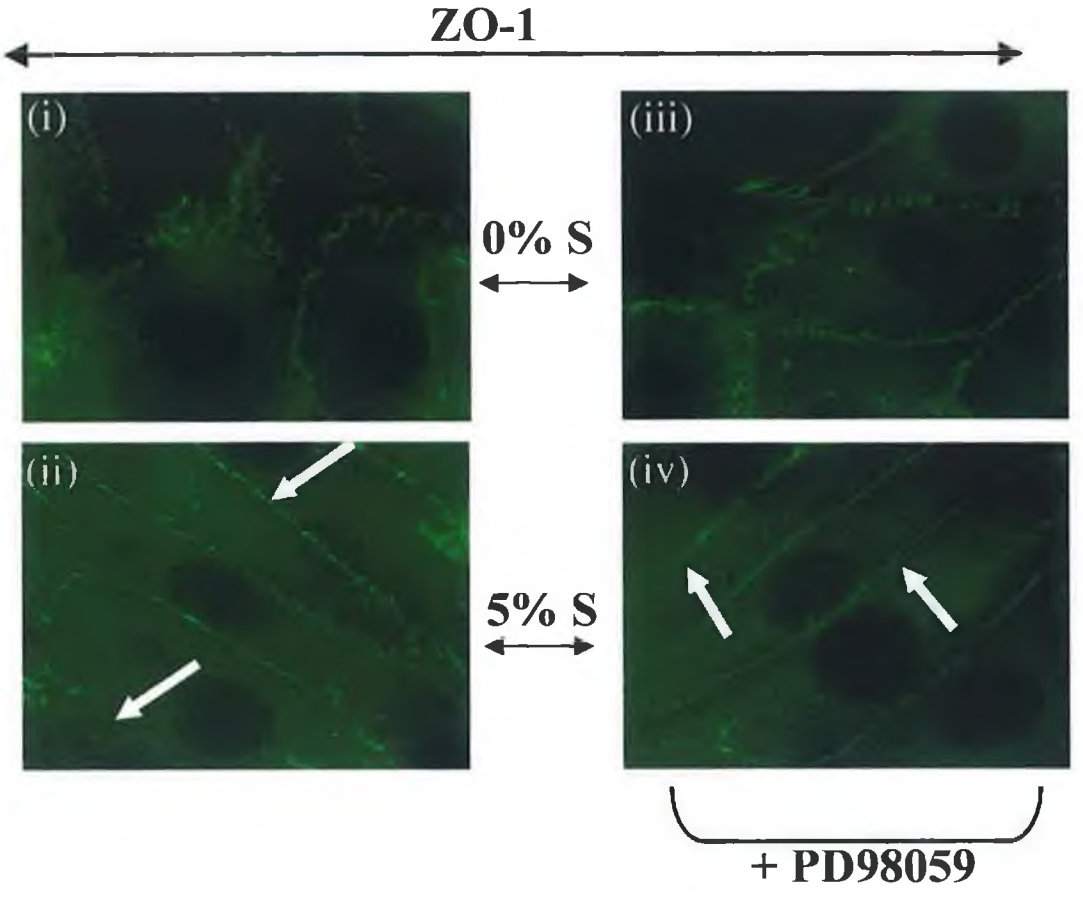

Fig. 5.24. Effect of PD98059 on cyclic strain-induced ZO-1 subcellular localisation in BAECs. Following exposure of BAECs to cyclic strain (5%, 24 h), localisation of ZO-1 was monitored by immunocytochemistry. ZO-1: (i) untreated control versus (ii) strain and (iii) PD98059-treated control versus (iv) strain. ZO-1 was monitored using standard fluorescence microscopy (1000x). White arrows indicate cell-cell border localisation. Images are representative of at least three individual sets of experiments.

5.3 Discussion

Structural adaptation of the vasculature occurs in response to both physiological and pathological changes in blood pressure and flow [Schwartz *et al.,* 1995; Libby *et al.,* 2003]. The effect of cyclic strain on the expression, localisation, co-association and phosphorylation of occludin and ZO-1 has been discussed in great detail in the previous two chapters. We have clearly demonstrated that physiological levels of cyclic strain increase endothelial cell tight junction function. In this chapter we have investigated PTKs, G-proteins, integrins, small GTPase (RhoA and Rac-1), p38 and MEK as possible components of the mechanical signaling cascade involved in the mechano-regulation of occludin and ZO-1 in BAECs.

Tyrosine kinases have been implicated in hemodynamic force-induced changes in EC function [Labrador *et al.,* 2003]. Therefore we examined the effects of inhibiting PTK using the pharmacological inhibitor Genistein, on tight junction assembly. Genistein had no effect on the strain-induced increase of occludin and ZO-1 protein expression nor any effect on the strain-induced co-association of both proteins. Moreover localisation of the proteins to the cell membrane following strain was not ablated following Genistein addition. Collectively, these results indicate that PTKs do not mediate biochemical changes to tight junction proteins in response to cyclic strain.

However, to fully elucidate the role to PTKs in TJ regulation, it would be interesting and necessary to repeat these experiments with a variety of inhibitors capable of inhibiting non-receptor TKs also. This would be especially relevant as the activity of

Src kinases and c-Yes appear to play a role in both assembly and disassembly of tight junctions [Kale *et al.*, 2003]. In fact, c-Yes is tightly linked to tight junction function due to its ability to bind to and phosphorylate occludin [Kale *et al.*, 2003].

We next turned our attention to G-proteins as their cellular localisation and rapid activation strongly implicate them as a primary sensor of hemodynamic forces [Gudi *et al.*, 1996]. In the current study, we inhibited G-protein signaling with the pharmacological inhibitor PTX. This inhibitor is known to block $G\alpha_i$ signaling [Gudi *et al.*, 1996]. Results indicated that PTX significantly inhibits the increase in occludin and ZO-1 protein expression observed following strain, suggesting that the changes are transduced via a $G\alpha_i$ protein pathway. Cyclic strain-induced increases in occludin and ZO-1 co-association were also significantly attenuated following addition of PTX and localisation of both proteins to the cell membrane following strain was also attenuated following inhibition of $G\alpha_i$ proteins. Thus, we have implicated $G\alpha_i$ signaling in transducing cyclic strain-induced changes to tight junction protein turnover and assembly. G-proteins have previously been reported to play a role a tight junction protein regulation. Saha *et al.* observed that treatment of MDCK cells with cholera toxin, a $G\alpha_s$ activator, accelerated TJ assembly as measured by transepithelial resistance [Saha *et al.*, 2001]. They also reported that $G\alpha_s$ was predominantly localised in the lateral membrane, but a fraction co-localises with ZO-1 in the TJ. Studies by Denker *et al.* also alluded to G-protein involvement in TJ biogenesis, indicating that G-proteins may participate in the maintenance and/or regulation of TJ assembly in MDCK cells [Denker *et al.* 1998]. In another study by Balda *et al.* in 1991 pertussis toxin increased

TEER in epithelial cells, indicating that junction formation may be controlled by a network of reactions including G-protein activation [Balda *et al.,* 1991]. Although this finding differs from ours, it suggests that G-proteins are be involved in TJ regulation

Integrins have also been linked to changes observed following mechanical stimulation [Shyy *et al.,* 2002] and therefore were examined in this study to ascertain their role in tight junction protein regulation following strain. To this end, synthetic cyclic RGD and synthetic linear RGD were applied to BAECs prior to and for the duration of strain. Results indicate that cRGD had no significant effect on strain-induced co-association of occludin and ZO-1 or on localisation of the proteins to the cell membrane following strain. The latter was also observed when the cells were treated with lRGD. Taken together these results indicate that integrins are not involved in tight junction regulation in response to strain.

With G-proteins implicated as the mechanotransducers of cyclic-strain-mediated changes in occludin and ZO-1, we then set out to examine the possible downstream signaling molecules that may be recruited in further transducing the mechanical signal. An obvious choice were the small GTPases RhoA and Rac-1 as they are known to play a major role in inflammation, permeability and cytoskeletal regulation [Dudek *et al.*, 2001]. Rho GTPases have now been implicated in signaling by many barrier-modifying substances including thrombin, vascular endothelial growth factor (VEGF) and histamine [Wojciak-Stothard *et al.*, 2002]. In fact, RhoA and Rac-1 have emerged as key permeability regulators acting antagonistically to regulate barrier function.

Results indicate that RhoA, investigated in the form of Rho Kinase, acts to weaken and destabilise the tight junction. We have ascertained this because when we inhibit Rho Kinase the junction becomes tighter, as indicated by the indices of permeability; co- association and subcellular localisation of TJ proteins. Rho Kinase blockade leads to an increase in occludin and ZO-1 protein levels following strain, in parallel with a significant increase in their co-association. Moreover, from our immunocytochemical evidence we can see that inhibiting Rho Kinase does not effect cyclic-strain induced localisation of occludin or ZO-1. Interestingly, under static, non-strained conditions, Rho Kinase blockade actually induced localisation of ZO-1 suggesting strengthening of the tight junction. This supports our hypothesis that RhoA and thus Rho Kinase, is inhibited during strain and thus further inhibition does not block strain-induced upregulation of barrier function. Moreover, inhibition of Rho Kinase would also be expected to increase barrier function under static non-strain conditions as seen here. Future studies involving a Rho Kinase activity kit would further strengthen this idea. Interestingly RhoA inhibition has been found to lead to Rac-1 activation [Rottner and Small, 1999], and this could also be responsible for the increase in barrier function observed in the static non-strained cells. In a study by Carbajal and Schaeffer, inhibition of RhoA with C3 transferase or expression of dominant negative RhoA as well as inhibition of Rho kinase with Y-27632 in endothelial cells prevents thrombin-induced permeability [Carbajal and Schaeffer, 1999]. It is plausible that Rho Kinase inhibition prevents the F-actin bundling caused by RhoA activation and hence precludes disruption of the tight junction. In fact, actomyosin contractility induced by RhoA is usually required for increased endothelial permeability [Wojciak-Stothard *et al.*, 2002]. A similar finding was reported in brain endothelial cells, with observations that ICAM 1

cross-linking induces Rho-dependent cytoskeletal rearrangements, which may facilitate transmigration and barrier disruption [Etienne *et al.*, 1998; Adamson *et al.*, 1999].

We then set about determining the role of Rac-1, another small GTPase that is known to work in tandem with RhoA. When we inhibit Rac-1, with the pharmacological inhibitor NSC23766, the increase in occludin/ZO-1 co-association is attenuated. This indicates that Rac-1 activation is involved in the increased binding of the two proteins following strain and therefore also involved in promoting a tight, functioning junction. In support of this, Rac-1 inhibition was also seen to inhibit the strain-induced localisation of occludin and ZO-1. Therefore Rac-1 is also responsible for the subcellular localisation of tight junction proteins, conceivably due to its effect on actin localisation. As previously mentioned, Rac-1 is required for the assembly and maturation of epithelial and endothelial junctions and its activity increases during junction formation [Noren *et al.*, 2001]. Moreover, in the absence of vasoactive stimuli, dominant negative Rac increases endothelial permeability and affects adherens and tight junctions, consistent with Rac being important for maintaining cell–cell junctions [Wojciak-Stothard *et al.*, 2001]. In fact, *Yersinia* bacteria decreases endothelial barrier function by injecting effector proteins into the cell cytoplasm. One of these proteins, YopE, is a GAP that has been shown to inhibit Rac-1 responses in cells and thereby induce breakdown in intercellular junctions in HUVECs [von Pawel-Rammingen *et al.*, 2000]

Collectively therefore, these findings suggest a synergistic regulation of the tight junction by RhoA/Rho kinase and Rac-1. RhoA and Rac-1 generally have opposing

effects on junctional integrity and activation of one GTPase coincides with inactivation of the other [Sander *et al.*, 1999; Noren *et al.*, 2001]. Thus, in a simplified model, improvement of endothelial barrier function may be achieved by increasing the Rac-1/RhoA activity ratio either by inactivation of RhoA or activation of Rac-1. Conversely, increased permeability may be achieved by activating RhoA and/or inactivating Rac-1. However, the results of many experiments suggest that junctional stability requires a very fine balance between Rac-1 and RhoA activity [Wojciak-Stothard *et al.*, 2002].

Several studies have linked MAPK pathways to tight junction protein regulation [Basuroy *et al.,* 2006: Pedram *et al.*, 2002] and therefore, finally, we looked at the signaling molecules p38 and MEK. MEK activity has been found to be required for claudin-mediated formation of tight junctions in human intestinal epithelial cells [Kinugasa et al., 2000]. However, in another study by Chen *et al.* after treatment of Ras-transformed MDCK cells with the mitogen-activated protein kinase inhibitor PD98059, occludin, claudin-1, and ZO-1 were recruited to the cell membrane, tight junctions were assembled, and E-cadherin protein expression was induced [Chen *et al.,* 2000]. In our study, MEK, a MAP kinase inhibitor had no effect on occludin or ZO-1 localisation following strain and therefore is not involved in the cyclic strain-induced modification of TJ proteins. As previously discussed, P38 has been implicated in tight junction regulation. Inhibition of p38 during oxidant challenge in HUVECs was found to attenuate the increase in permeability following addition of hydrogen peroxide [Kelvil *et al.*, 2001], indicating that the increase in TJ permeability is mediated by p38 in this instance. In our study, inhibition of p38, leads to an attenuation of the subcellular localisation of both proteins to the cell membrane, indicating that the decrease in TJ

permeability, following strain is mediated by p38. The conflicting findings in these studies may be explained by the fact that different cell types and stimuli are involved. However, it is likely that p38 plays a signaling role in transducing the cyclic strain stimuli to the tight junction proteins in this instance, although its sequence in these events would require further investigation.

5.4 Conclusion

In conclusion, our data demonstrates that cyclic strain-mediated changes in tight junction function are transduced in a G-protein-dependent but integrin- and PTK-independent fashion. Inhibition of the small GTPase Rac-1 attenuates the strain-induced changes in occludin and ZO-1 and therefore also plays a pivotal role in cyclic strain signal transduction to ECs. Moreover, the MAPK, p38 is also involved in transducing the cyclic-strain mediated response to TJ proteins.

Chapter 6

Final Summary

Hemodynamic forces, such as shear stress and cyclic strain, are potent mediators of endothelial cell remodelling, angiogenesis and maintenance of vascular tone. Common hemodynamic factors, such as low or oscillatory shear stress, can be found at areas of compromised permeability in the vascular tree, implying that there may be a relationship between EC permeability and physical forces. Also, as disruption of vascular endothelial barrier integrity is a central feature of many homeostatic and pathophysiological processes (e.g. atherosclerosis, sepsis etc.), and frequently correlates with a perturbation in vessel hemodynamic loading and remodelling [Harhaj *et al.,* 2004; Van Nieuw-Armerongen *et al.,* 2002; Tinsley *et al.,* 2004; Koschinsky *et al.,* 2004], we endeavoured to determine the link between physiological levels of hemodynamic force and tight junction protein regulation. The focus of this study was the hemodynamic force cyclic strain.

Endothelial permeability is controlled by a series of junctional complexes. One of the chief contributors to barrier function is the tight junction, which forms between adjacent endothelial cells. It is comprised of several proteins, two of which; occludin and ZO-1, formed the basis of this study. Occludin is a membrane-bound protein whose expression can be directly linked to barrier function. ZO-1 is a transmembrane protein, best known for its role in organising the proteins of the tight junction due to its many binding sites.

It was our hypothesis that there exists a dynamic regulatory co-association between vascular endothelial permeability and hemodynamic stimuli. We also sought to

determine the mechanotransduction pathway by which this modulation took place (See Fig. 6.1 for diagrammatic representation of experimental approach).

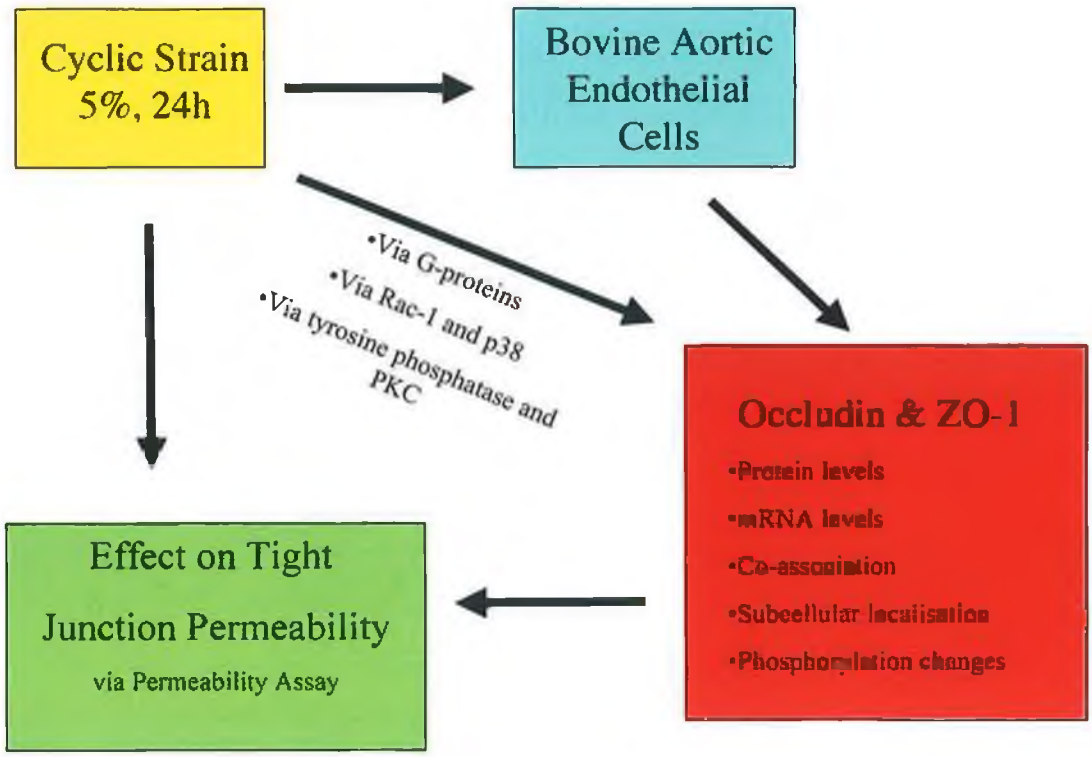

Fig. 6.1 Diagrammatic representation of experimental approach. The regulation of occludin and ZO-1 in response to cyclic strain was investigated along with the identification of signaling pathways involved in the cyclic strain-induced increase in TJ barrier function.

Investigations clearly demonstrate that chronic cyclic strain (5%, 24h) of BAECs up-regulates protein expression of occludin and ZO-1, in parallel with an increase in mRNA for occludin (but not ZO-1). It is plausible that TJ proteins are increased following strain to allow a tighter junction to form between ECs. The increase in ZO-1 protein, but not mRNA, can be reconciled by the fact there is possibly increased protein translation and/or decreased protein turnover of ZO-1. The integrity of the TJ is

regulated by junctional protein interactions. As such, one would expect changes in subcellular localisation of occludin and ZO-1 at the cell-cell border where tight junctions form, coupled with parallel changes in occludin/ZO-1 co-association, to accompany a change in endothelial barrier function, as previously evidenced by numerous studies [Furuse *et al.*, 1994; Rao *et al.*, 2002; Wang *et al.,* 2002; Lee *et al.*, 2004; Fischer *et al.,* 2004; Sheth *et al.*, 2003]. With this in mind, we decided to broaden our early investigations by examining occludin/ZO-1 co-association and subcellular localisation (i.e. measurable indices of tight junction assembly) in our cyclic strain model. Our results clearly indicate a significant strain-dependent increase in occludin and ZO-1 co-association by way of immunoprecipitation. As mentioned previously, ZO-1 can bind specifically to the COOH-terminal cytoplasmic tail of occludin. Therefore, we can deduce that the two proteins are bound together at the tight junction, thus contributing to the functional barrier. Also, following strain we observe that the proteins have re-localised within the cell, as seen by immunocytochemistry. Occludin protein moves from the cytosol to the cell membrane and ZO-1 staining becomes more linear and continuous at the cell-cell junction. This is also indicative of a tighter junction. We can also observe a change in F-actin staining, as the actin cytoskeleton is also subject to modification by cyclic strain. Actin staining becomes more cortical and intimately linked to the proteins of the tight junction as it is directly bound to ZO-1 [Fanning *et al.*, 1998]. In parallel with these strain-induced changes, we observe a decreased permeability of the endothelial monolayer to small macromolecules such as FD40, thus indicating that cyclic strain leads to an increase in the barrier function of ECs. When viewed collectively, these data lead us to conclude that cyclic strain up-regulates

endothelial occludin/ZO-1 expression and tight junction assembly, putatively leading to increased barrier integrity. Consistent with this conclusion as previously mentioned, a study by Shin *et al.* demonstrated reduced transendothelial permeability to albumin following exposure of HUVECs to chronic pulse pressure (cyclic pressure) [Shin *et al.*, 2003].

Having ascertained that cyclic strain causes an increase in barrier function in BAECs, we set about examining the pathway by which this increase took place. As protein levels of both occludin and ZO-1 were increased following strain, we wanted to determine the contribution of this new protein synthesis to the changes in barrier function indices observed. To this end we utilised cycloheximide, which stops new protein production, and observed that the new protein produced did not lead to the changes in localisation of either protein. However, inclusion of cycloheximide was seen to reduce the increase in co-association observed following strain, suggesting that approximately 40% of the co-association is a direct consequence of new protein synthesis. Therefore, the majority of strain-induced co-association of occludin and ZO-1, along with the localisation of both proteins to the cell membrane is not dependent on new protein synthesis but due to some other modification of TJ proteins following strain.

It was subsequently hypothesised that strain-induced phosphorylation changes in occludin and ZO-1 were responsible for the up-regulation in tight junction function observed. In this regard, tyrosine dephosphorylation of occludin, via tyrosine phosphatase and serine/threonine phosphorylation of ZO-1, via PKC, were correlated to the strain-induced localisation and co-association of both proteins in this study.

Treatment of BAECs with either dephostatin or rottlerin was also found to reverse the strain-mediated reduction in transendothelial permeability as measured by the flow of FD40 across the monolayer. This suggests a causal link between changes in occludin/ZO-1 biochemical properties and endothelial barrier function in response to cyclic strain. The specific mechanistic roles of these phosphorylation events in tight junction assembly however remain unclear. Perturbation of the interaction between proximal membrane occludin domains leading to activation of intracellular signalling cascades and subsequent alteration in the phosphorylation state of tight junction proteins is an attractive and highly probable model of barrier regulation by extracellular stimuli [Alexander *et al.*, 2002; Balda *et al.,* 1998]. Consistent with the current study, previous papers have clearly demonstrated that reduction in endothelial barrier integrity is usually accompanied by increased tyrosine phosphorylation of occludin leading to loss of junctional protein co-association and membrane localisation [Rao *et al.,* 2002; Sheth *et al.,* 2003; Kale *et al.,* 2003]. Published evidence also indicates that binding of ZO-1 (and ZO-2/3) to the COOH-terminal cytoplasmic tail of occludin is important for targeting of the latter to the tight junction as well as for it's cytoskeletal tethering [Mitic *et al.,* 1999], a process which is almost certainly sensitive to the phosphorylation state of both junctional and cytoskeletal components involved [Balda *et al.,* 1998; Sakakibara *et al.,* 1997; Rao *et al.,* 2002; Kale *et al.,* 2003]. Also consistent with the current study, papers by Lohmann *et al.* and Wachtel *et al.* have demonstrated that pharmacological blockade of tyrosine phosphatase significantly reduces vascular endothelial barrier integrity in blood brain barrier and peripheral vasculature, respectively [Lohmann *et al.,* 2004; Wachtel *et al.,* 1999]. Stuart *et al.* observed that activation of PKC is directly linked to tight junction assembly [Stuart *et al.*, 1995]. Overall however, whilst clearly central to

tight junction regulation, further investigation will be necessary to clarify the precise mechanistic roles of these phosphorylation events in this process.

The composition and integrity of tight junctions can vary immensely depending on environmental and humoral factors. Tight junction assembly is a highly regulated process that involves a complex network of signalling pathways that include G-proteins, integrins, tyrosine phosphatase, protein kinase C, phospholipase C and calmodulin [Rosson *et al.,* 1997; Balda *et al.*, 1991; Denker *et al.*, 1996]. Having demonstrated that cyclic strain upregulates tight junction function in ECs, we next tried to ascertain if the phenomenon was mediated by protein tyrosine kinases, G-proteins and/or integrins. Pharmacological inhibitors were employed to determine the mechanotransduction pathway(s) involved. Results indicated that protein tyrosine kinase and integrin inhibition did not block strain-induced upregulation of the barrier. However, inhibition of G-protein signaling attenuated the strain-induced modifications. More specifically, addition of PTX resulted in attenuation of the increase in occludin and ZO-1 protein levels observed following strain. PTX also resulted in attenuation of the increase in occludin/ZO-1 co-association and membrane localisation. To our knowledge this is the first time G-protein mechanotransduction has been implicated in hemodynamic regulation of TJ protein assembly. G-proteins have however been linked to TJ function in several other studies. For example, LPA binds to a seven transmembrane domain G protein-coupled receptor on endothelial cells, leading to compromised junctional permeability [Schulze *et al.*, 1997], which can be partially attributed to dissociation of actin from its binding proteins [Meerschaert *et al.*, 1998]. Several other lines of evidence suggest that regulation of TJ assembly may be mediated through the actions of multiple

Gα-subunits. Confocal studies demonstrate several Gα subunits ($G\alpha_{i2}$, $G\alpha_{o}$ and $G\alpha_{12}$) and multiple isoforms of PKC to be present in the TJ [de Almeida *et al.*, 1994; Denker *et al.*, 1996; Dodane *et al.*, 1996; Hamilton *et al.*, 1997; Rosson *et al.*, 1997]. In addition, modulators of G-protein activity such as, cholera toxin and pertussis toxin have been shown to affect TJ biogenesis [Saha *et al.*, 1998; Balda *et al.*, 1991].

We hypothesised that the intermediate signaling molecules recruited following G-protein activation would be the small GTPases RhoA and Rac-1 as they are known to play a major role in inflammation, permeability and cytoskeletal regulation [Dudek *et al.*, 2001]. RhoA destabilises the tight junction via the activation of its effector ROCK. It leads to phosphorylation of myosin light chain and causes F-actin bundling, thereby leading to stress fiber formation [Mackay *et al.*, 1998]. This is a key event in various models of barrier dysfunction [Dudek *et al.*, 2001; Wojciak-Stothard *et al.*, 2002]. Therefore inhibiting RhoA, via Rho Kinase, should have no negative effect on barrier function. In fact, strain-induced changes in occludin and ZO-1 protein expression, co-association and localisation were not attenuated by Rho Kinase inhibition. Moreover, under static, non-strained conditions, Rho Kinase blockade actually induced localisation of ZO-1 to the cell-cell border, suggesting strengthening of the tight junction. In addition to this, inhibiting Rho Kinase under static, non-strained conditions led to a significant increase in occludin protein and occludin/ZO-1 co-association in parallel with an increase in ZO-1 protein expression. This supports our hypothesis that RhoA, and thus Rho Kinase is inhibited during strain. Conversely, Rac-1 activation leads to an increase in barrier function. It leads to formation of cortical F-actin, which is usually associated with stabilisation of intercellular endothelial junctions [Garcia *et al.*, 2001]. Therefore

we would expect Rac-1 inhibition to lead to an increase in TJ permeability. Our results indicate that Rac-1 inhibition lead to attenuation of the strain-induced increases in occludin/ZO-1 co-association and localisation. This indicates that Rac activation is involved in the increased binding of the two proteins following strain and their subcellular localisation, and therefore also involved in promoting a tight, functioning junction (See Fig. 6.2).

Future work should include looking at the distribution of F-actin in BAECs by immunocytochemistry following addition of Rac-1 and RhoA inhibition to visualise the effect on F-actin reorganisation observed following strain. Also of interest would be to ascertain the functional consequences of RhoA and Rac-1 inhibition on TJ permeability via a permeability assay.

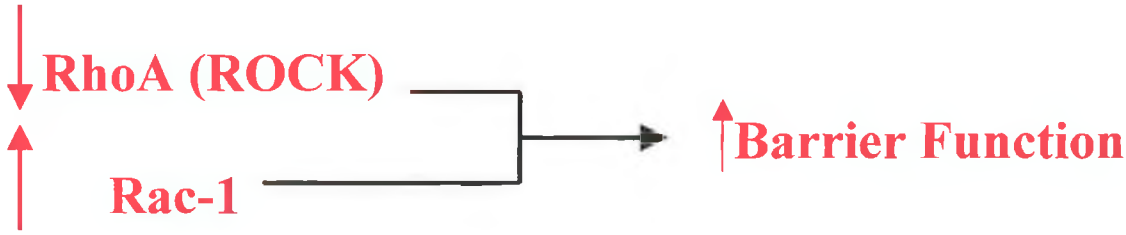

Fig. 6.2 Tight junction barrier function is regulated by the small GTPases RhoA and Rac-1.

As previously mentioned, inhibition of integrins, using the synthetic inhibitors cRGD and lRGD, had no effect on TJ junction protein co-association and localisation following strain. However we did observe a relationship between cyclic strain and the small GTPases, RhoA and Rac-1. It is well documented in the literature that there exists an intimate relationship between integrin stimulation and small GTPase activation [Shyy

et al., 2002]. In fact, integrins can lead directly to activation of RhoA, and Rac-1 and therefore it was believed they would play a major role in transducing the cyclic strain signal to the proteins of the TJ [Grande-García *et al.*, 2005]. How can we reconcile the findings in our study, with regards to integrins and small GTPases? It is possible that the integrin synthetic inhibitors used only inhibited a sub-class of integrins present on the EC, RGD-dependent and not RGD-independent integrins. Therefore it is plausible that the cyclic strain-induced integrin signal was not fully inhibited. Further studies are needed using alternative integrin inhibitors and dominant negative mutants of Shc, which is known to be recruited by integrins and to cause activation/inhibition of the small GTPases RhoA and Rac-1 [Shyy *et al.,* 2002], to fully illicit the role of integrin signalling in TJ protein regulation.

We also investigated the role of p38 and MEK in the cyclic strain-mediated regulation of occludin and ZO-1 as several studies have linked MAPK pathways to tight junction protein regulation [Basuroy *et al.,* 2006; Pedram *et al.*, 2002]. Our data indicates the p38, but not MEK, also plays a role in cyclic strain-signal transduction in BAECs. P38 has previously been implicated in tight junction regulation. Kelvil *et al.* reported a contrasting role of p38 in TJ permeability. They observed that inhibition of p38 MAP kinase during oxidant challenge in HUVECs significantly attenuated actin stress fiber formation and prevented gap formation, thus attenuating the increase in permeability following addition of hydrogen peroxide [Kelvil *et al.* 2001]. Another study using Sertoli cells has shown that TJ dynamics are regulated, at least in part, by TGF-beta3 via the p38 mitogen activated protein (MAP) kinase pathway. This in turn

regulates the production of occludin, by Sertoli cells and a specific p38 MAP kinase inhibitor could block occludin loss from the blood testis barrier [Lui *et al.*, 2003]. The differences in the role of p38 reported in these studies is likely be related to different experimental paradigms and different cell types.

G-proteins have been implicated as the mechanotransducers of cyclic strain-mediated alterations of tight junction proteins in BAECs. Rac-1 and p38-mediation of occludin and ZO-1 has also been reported. A direct association between G-protein activation and Rac-1 activation has previously been shown [Mehta *et al.*, 2005]. In this study it was reported that Sphingosine 1-phosphate ligation of endothelial differentiation gene-1 receptor coupled to a heterotrimeric Gi-protein, promotes endothelial barrier strengthening via rac-dependent assembly of adherens junctions. In another study it was reported that LPA, working through a GPCR mechanism in addition to stimulating cell proliferation, also induced cytoskeletal changes and promoted cell migration in a RhoA- and Rac-1-dependent manner [van Leeuwen *et al.*, 2003]. Significantly in this study LPA-induced Rac-1 activation is inhibited by pertussis toxin also indicating a clear association between PTX-sensitive G-proteins and Rac-1 activation. Therefore it is probable that G-protein activation by cyclic strain leads to Rac-1 activation in BAECs. As previously discussed, Rac-1 acts to stabilise the TJ and promote a healthy, functioning barrier.

In our study, p38 inhibition ablated the stain-induced sub-cellular localisation of occludin and ZO-1, indicating that p38 is involved in the mechanotransduction process. It is possible that p38 is activated by G-proteins and/or Rac-1. G-protein-mediated

activation of P38 has previously been reported in a study by Li *et al.* In this study, rat SMCs exposed to cyclic strain, showed activation of p38. Inhibition of Gi-proteins with PTX significantly inhibited p38 phosphorylation, suggesting that the p38 signal pathway is at least partially dependent on Gi-protein in response to mechanical stress in SMCs. Rac-1 also played a role in the strain-induced activation of p38, as dominant-negative Rac-1 cells attenuated p38 phosphorylation following strain [Li *et al.*, 2000]. Activation of p38 can lead to phosphorylation of p38 at both Ser/Thr and Tyr sites, which can then lead to protein synthesis and gene transcription [Cowen *et al.*, 2003]. It is therefore possible that p38 is involved in new occludin and ZO-1 protein synthesis and gene transcription, stimulated by either G-proteins and/or Rac-1, to increase barrier function in ECs following strain.

As previously discussed, strain-induced phosphorylation changes in occludin and ZO-1 were responsible for the up-regulation in tight junction function observed. Furthermore, these phosphorylation/dephosphorylation events were mediated by PKC and tyrosine phosphatase respectively. Cyclic strain-induced activation of PKC has been previously reported in ECs [Cheng *et al.* 2001]. Moreover, a study by Dodane *et al.* suggests that the formation and permeability of tight junctions are actively regulated by second-messenger-generating systems involving G proteins and protein kinase C in MDCK and Caco-2 epithelial cells [Dodane *et al.*, 1996]. Another study by Denker *et al.* suggests that TJ biogenesis appears to be regulated, in part, by classic signal transduction pathways involving heterotrimeric G proteins and activation of protein kinase C [Denker *et al.,* 1998]. Signals that stimulate members of the large families of G protein-coupled receptors, tyrosine kinase receptors, or non-receptor tyrosine kinases

can cause diacylglycerol production and PKC activation [Newton, 1995]. Therefore PKC is necessary for cyclic strain-induced modification of TJs, possibly directly stimulated by cyclic strain or in a G-protein dependent manner. Furthermore, evidence exists for G-protein dependent modulation of tyrosine phosphatase. Pan and co-workers demonstrated that protein tyrosine phosphatase activation by either guanyl-5'-yl imidodiphosphate or somatostatin can be blocked by pertussis toxin in mouse fibroblast cells [Pan *et al.,* 1992]. However, the number and identity of protein tyrosine phosphatases regulated, directly or indirectly, by G proteins is unknown. Fig. 6.3 depicts probable signaling pathways involved in cyclic strain regulation of the tight junctions proteins occludin and ZO-1.

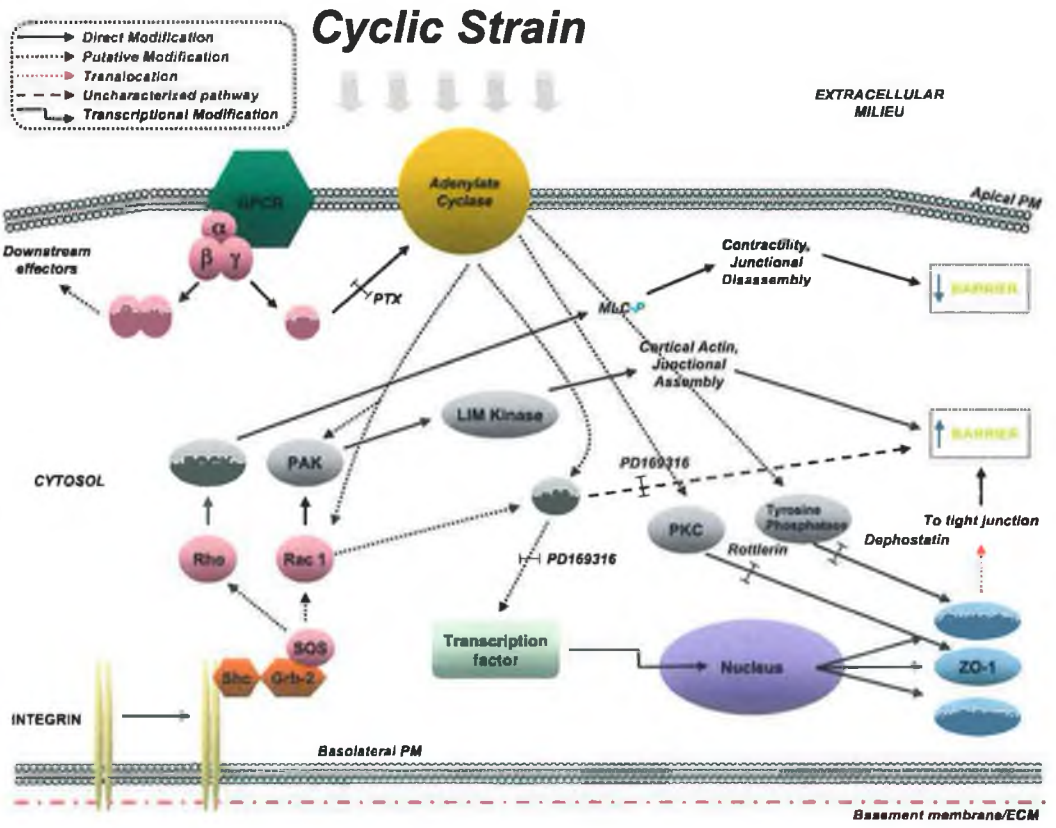

Fig. 6.3 Model for signalling pathways downstream of hemodynamic stimuli in endothelial cells. Figure offers a simplified view of probable signalling pathways involved in cyclic strain regulation of the tight junctions proteins occludin and ZO-1.

It should be noted that all investigations were carried out using pharmacological inhibitors, however, future investigations will use dominant-negative phosphorylation mutants of occludin/ZO-1, in conjunction with small interfering RNA strategies to selectively knock down certain pathways, to definitively reconfirm these findings.

This is the first conclusive study to look at the mechanotransduction signaling pathway in relation to tight junction permeability. To the best of our knowledge, no other study has looked in-depth at the effect of cyclic strain on EC tight junction permeability from initiation of the stimulus to recording of the downstream effects on tight junction proteins. Thus, the data enhances our overall understanding of how hemodynamic forces regulate vascular endothelial functions and behavior.

Increased knowledge of how tight junctions are regulated could have important therapeutic benefits. These would focus on developing methods to increase or decrease tight junction permeability in specific areas of the vasculature. For example most small drugs molecules are transported by way of the transcellular pathway by passive and active transport, easily entering the systemic circulation and distributing to various tissues in the bodies [Ohtake *et al.*, 2003]. However there is no easy way to deliver large hydrophilic molecules such as bioactive peptides, hormones, vaccines, and genes by way of the transmucosal routes into the systemic circulations. Therefore the ability to produce a transient reduction in barrier function of TJ to deliver large amounts of these compounds without disruption to epithelial cellular sheets would be convenient [Ohtake *et al.*, 2003]. Recently it has been noted that polycationic materials such as poly-l-lysine, ploy-l-arginine [Mc Ewan *et al.*, 1993], protamine [Hammes *et al.,* 1999], and chitosan

[Illum *et al.,* 1994], have the potential to promote transmucosal delivery of macromolecules due in part to alteration of the TJ proteins occludin and ZO-1 and adherens junction protein E-cadherin. These finding could be useful in developing a transnasal delivery system for macromolecules.

Conversely in disease states such as atherosclerosis and diabetic retinopathy we would strive to tighten the barrier between endothelial cells. In this way we would hope to halt the movement of molecules such as low-density lipoprotein, and as such, prevent disease progression because it known that the accumulation of atherogenic substances such LDL, growth factors, and fibrinogen in the intima of arteries is one of the initiating factors of atherosclerosis [Fry *et al.*, 1987; Curmi *et al.*, 1990; Nielson *et al.*, 1992]. In fact, occludin expression had been increased experimentally in transfected cultured epithelial cells, and this coincided with improved barrier function [McCarthy *et al.*, 1996]. This is the exact opposite situation that has been chronicled in so many diverse disease states, where both occludin levels fall and TJ permeability rises eg. inflammatory bowel disease, diabetes and cancer. Indeed in our study, decreased levels of occludin protein were found in non-strained BAECs that also had increased endothelial cell permeability. It is possible therefore, that approaches to prevent or reverse the downregulation of occludin may provide a useful therapeutic strategy in the future. Probiotics such as *Lactobacillus acidophilus* when incubated with epithelial cell cultures have been shown to increase transepithelial electrical resistance while enhancing occludin protein phosphorylation [Resta-Lenert *et al.*, 2003]. Hydrocortisone has likewise been observed to increase occludin content at the cell border while also decreasing paracellular leakage in the blood–brain barrier [Antonetti *et al.*, 2002]. These

and other possible therapeutic strategies aimed at occludin may in future provide beneficial results in a variety of diseases.

Moreover, our results indicated that the small GTPases RhoA and Rac-1 played an important role in endothelial cell permeability and as such may also be useful targets for therapeutical intervention in disease states involving impaired endothelial barrier function. In fact, the Rho kinase inhibitor, Y-27632, has been used in several animal models mainly because it is a small cell-permeable molecule. For example, Y-27632 administered as an aerosol in guinea pigs in vivo partially reduced airway microvascular leakage caused by leukotriene D4 and inhibited the lung resistance induced by histamine and leukotriene D4 [Tokuyama *et al.*, 2002].

In conclusion, our data suggests that cyclic strain plays a key role in tight junction regulation in BAECs, an important component of normal vascular function. Moreover, it suggests that physiological levels of cyclic strain may be athro-protective and lead to an intact, fully functioning endothelial barrier. Identification of the mechanisms by which cyclic strain regulates occludin and ZO-1 may lead to possible new drug targets that can promote/inhibit tight junctions *in vivo*. Similarly a more complete understanding of how tight junctions interact in response to hemodynamic forces may lead to a better understanding of pathological conditions such as atherosclerosis.

Chapter 7

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