

# The role of AMP-activated protein kinase in endothelial VEGF signalling

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Submitted in fulfilment of the requirements for the  
Degree of Doctor of Philosophy



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September 2009

## Abstract

The endothelium acts to maintain vascular homeostasis, including the regulation of vascular tone, blood fluidity and coagulation. Endothelial dysfunction, a condition largely characterised by reduced NO bioavailability, is an important feature associated with the aetiology of several pathophysiological disorders including type 2 diabetes and cardiovascular disease.

AMPK is the downstream component of a protein kinase cascade important in the regulation of cellular and whole body metabolism. AMPK has been demonstrated to mediate a number of physiological responses in the endothelium, including the stimulation of eNOS phosphorylation and NO synthesis; and as such AMPK represents a therapeutic target in the dysfunctional endothelium.

VEGF has been established as the prime angiogenic molecule during development, adult physiology and pathology. VEGF stimulates NO production, proposed to be a result of phosphorylation of Ser-1177 on eNOS, a residue also phosphorylated upon AMPK activation in cultured endothelial cells. The present study, utilising HAEC as a model, provides the first demonstration that AMPK is activated by physiological concentrations of VEGF; and furthermore, partially mediates VEGF-stimulated phosphorylation of eNOS on Ser-1177 and subsequent NO production. In addition, the present investigation demonstrates that the upstream AMPK kinase CaMKK is responsible for these VEGF-mediated effects.

VEGF is known to increase intracellular calcium levels in endothelial cells via the generation of DAG and IP<sub>3</sub>. DAG increases Ca<sup>2+</sup> influx through a family of non-selective cation channels, whereas IP<sub>3</sub> promotes the release of Ca<sup>2+</sup> from intracellular stores. High potassium-induced depolarisation, which reduces the driving force for Ca<sup>2+</sup> entry through non-selective cation channels in endothelial cells, abolished VEGF-mediated AMPK activation, whereas the IP<sub>3</sub> receptor blocker 2-APB was without effect. Exposure of HAEC to a DAG mimetic (OAG) also stimulated AMPK, an effect which was sensitive to the CaMKK inhibitor STO-609 and high potassium induced depolarization.

The functional effects of VEGF-stimulated AMPK were also assessed in HAEC. Ablation of AMPK abrogated VEGF-stimulated HAEC migration and proliferation, two key features of the angiogenic process. While AMPK was necessary for VEGF-stimulated

endothelial cell proliferation direct activation of the kinase was insufficient to induce this process. AICAR-stimulated AMPK activation has been demonstrated to stimulate fatty acid oxidation in endothelial cells. However, exposure of HAEC to VEGF did not alter fatty acid oxidation in the present study.

Together, the current investigation suggests that a VEGF-Ca<sup>2+</sup>-CaMKK-AMPK-eNOS-NO pathway is present in HAEC, and furthermore, that AMPK is required, albeit insufficient, for the VEGF-stimulated angiogenic response.

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## **Acknowledgements**

I would like to thank all of those who have provided valuable assistance during the course of my Ph.D. studies, in particular Dr. Ian Salt who has been of enormous help throughout. I would also like to thank everyone in the Salt group, past and present, and indeed everyone in lab 241 who have provided much needed assistance with many aspects of this project.

I would also like to extend my appreciation to any other members of the department for their help and discussion pertaining to this study, in particular the members of my Ph.D. review panel Professor Gwyn Gould, Dr Chris McInerny and Dr Anna Amtmann.

Funding was provided by the Biotechnology and Biological Science Research Council.

## **Author's Declaration**

I hereby declare that the thesis that follows is my own composition, that it is a record of the work done myself, except where otherwise acknowledged, and that it has not been presented in any previous application for a Higher degree.

James Anthony Reihill

## Abbreviations

<b>2-APB</b>	2-Aminoethoxydiphenyl borate
<b>ACC</b>	Acetyl-CoA carboxylase
<b>ADP</b>	Adenosine diphosphate
<b>AICAR</b>	5-aminoimidazole-4- carboxamide-1- $\beta$ -riboside
<b>AMP</b>	Adenosine monophosphate
<b>AMPK</b>	AMP-activated protein kinase
<b>AMPKK</b>	AMP-activated protein kinase kinase
<b>APS</b>	Ammonium persulphate
<b>ATP</b>	Adenosine trisphosphate
<b>BAD</b>	B-cell lymphoma 2-associated death promoter homologue
<b>BAEC</b>	Bovine aortic endothelial cells
<b>Bcl2</b>	B-cell CLL/lymphoma 2
<b>bFGF</b>	Basic fibroblast growth factor
<b>BH<sub>4</sub></b>	Tetrahydrobiopterin
<b>BK<sub>ca</sub></b>	Large-conductance calcium-activated potassium channels
<b>CaM</b>	Calmodulin
<b>CaMK</b>	Ca <sup>2+</sup> /calmodulin-dependent protein kinase
<b>CaMKII</b>	Ca <sup>2+</sup> /calmodulin-dependent protein kinase II

<b>CaMKK</b>	Ca <sup>2+</sup> /calmodulin-dependent protein kinase kinase
<b>cAMP</b>	Cyclic adenosine monophosphate
<b>cGMP</b>	Cyclic guanosine monophosphate
<b>CDC42</b>	Cell division cycle 42, GTP binding protein
<b>CDK</b>	Cyclin-dependent kinase
<b>CDKI</b>	Cyclin-dependent kinase inhibitor
<b>cGMP</b>	Cyclic guanosine monophosphate
<b>CPT1</b>	Carnitine palmitoyltransferase-1
<b>CYP</b>	Cytochrome P450
<b>DAG</b>	Diacylglycerol
<b>DMEM</b>	Dulbecco's modified Eagle medium
<b>DSCR1</b>	Down syndrome critical region-1
<b>DTT</b>	Dithiothreitol
<b>DYRK</b>	Dual specificity tyrosine phosphorylated and regulated kinase
<b>ECM</b>	Extracellular matrix
<b>EDTA</b>	Ethylenediaminetetraacetic acid
<b>EETs</b>	Epoxyeicosatrienoic acids
<b>EGTA</b>	Ethylene glycol tetraacetic acid
<b>eNOS</b>	Endothelial nitric oxide synthase



<b>EPCs</b>	Endothelial progenitor cells
<b>ERK1/2</b>	Extracellular signal regulated kinases 1 and 2
<b>FAD</b>	Flavin adenine dinucleotide
<b>FAK</b>	Focal adhesion kinase
<b>FAS</b>	Fatty acid synthase
<b>FCS</b>	Foetal calf serum
<b>FGF</b>	Fibroblast growth factor
<b>FMN</b>	Flavin mononucleotide
<b>GBD</b>	Glycogen binding domain
<b>Gd<sup>3+</sup></b>	Gadolinium
<b>GFP</b>	Green fluorescent protein
<b>GPCRs</b>	G-protein coupled receptors
<b>GTP</b>	Guanosine-5'-triphosphate
<b>HAEC</b>	Human aortic endothelial cells
<b>HepG2</b>	Human hepatocellular liver carcinoma cell line
<b>HIF-1</b>	Hypoxic-inducible factor-1
<b>HIPK2</b>	Homeodomain-interacting protein kinase 2
<b>HMDEC</b>	Human microdermal endothelial cells
<b>HMEC</b>	Human microvascular endothelial cells

<b>HMGR</b>	3-hydroxy-3-methyl-glutaryl-CoA reductase
<b>HRP</b>	Horse radish peroxidase
<b>HSP90</b>	Heat shock protein 90
<b>HUVEC</b>	Human umbilical vein endothelial cells
<b>Ig</b>	Immunglobulin
<b>IGF</b>	Insulin-like growth factor
<b>IL</b>	Interleukin
<b>IP<sub>3</sub></b>	Inositol 1,4,5,-trisphosphate
<b>IP</b>	Immunoprecipitation
<b>IPTG</b>	Isopropyl $\beta$ -D-1-thiogalactopyranoside
<b>IQGAP1</b>	IQ motif containing GTPase activating protein 1
<b>JAK3</b>	Janus kinase 3
<b>KGF</b>	Keratinocyte growth factor
<b>KRH</b>	Krebs-Ringer-Hepes buffer
<b>La<sup>3+</sup></b>	Lanthanium
<b>MAPK</b>	Mitogen activated protein kinase
<b>MCP-1</b>	Monocyte chemotactic protein 1
<b>MEFs</b>	Mouse embryo fibroblasts
<b>MEK-1</b>	Mitogen activated protein kinase kinase 1

<b>M-MLV-RT</b>	Moloney murine leukemia virus reverse transcriptase
<b>MMP-9</b>	Matrix metalloproteinase-9
<b>MNK-1</b>	Nicotinamide adenine dinucleotide phosphate
<b>MP</b>	Milk powder
<b>mTOR</b>	Mammalian target of rapamycin
<b>NADPH</b>	Nicotinamide adenine dinucleotide phosphate
<b>NFAT</b>	Nuclear factor of activated T-cells
<b>NF-<math>\kappa</math>B</b>	Nuclear factor- $\kappa$ B
<b>NO</b>	Nitric oxide
<b>NRP</b>	Neuropilin
<b>L-NAME</b>	N (G)-nitro-L-arginine methyl ester
<b>MAPK</b>	Mitogen-activated protein kinase
<b>MCP-1</b>	Monocyte chemoattractant protein-1
<b>OAG</b>	1-oleoyl 2-acetyl-sn glycerol
<b>OCT</b>	Organic cation transporter
<b>PBS</b>	Phosphate buffer solution
<b>PCR</b>	Polymerase chain reaction
<b>PDGF</b>	Platelet-derived growth factor
<b>PDK-1</b>	Phosphoinositide dependent kinase-1

<b>PFK-2</b>	Phosphofructokinase-2
<b>PGC1A</b>	Peroxisome proliferator-activated receptor $\gamma$ coactivator-1a
<b>PI3K</b>	Phosphoinoside-3 kinase
<b>PIGF</b>	Placenta growth factor
<b>PIP<sub>3</sub></b>	Phosphatidylinositol 3,4,5 trisphosphate
<b>PLC</b>	Phospholipase C
<b>PKA</b>	Protein kinase A
<b>PKB</b>	Protein kinase B
<b>PKC</b>	Protein kinase C
<b>PKG</b>	Protein kinase G
<b>PMA</b>	Phorbol 12-myristate 13-acetate
<b>PMSF</b>	Phenylmethanesulphonylfluoride
<b>PPAR<math>\gamma</math></b>	Peroxisome proliferator-activated receptor
<b>PP</b>	Protein phosphatase
<b>ROS</b>	Reactive oxygen species
<b>RPAEC</b>	Rat pulmonary artery endothelial cells
<b>RTKs</b>	Receptor tyrosine kinases
<b>RT-PCR</b>	Reverse transcriptase polymerase chain reaction
<b>SBTI</b>	Soy bean trypsin inhibitor

<b>SDS</b>	Sodium dodecyl sulphate
<b>SDS-PAGE</b>	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
<b>SERCA</b>	Sarco/endoplasmic reticulum Ca <sup>2+</sup> ATPase
<b>SNF-1</b>	Sucrose non-fermenting-1
<b>SOC</b>	Store operated Ca <sup>2+</sup> entry
<b>S.O.C medium</b>	Super optimal broth (with catabolite repression)
<b>SREBP</b>	Sterol regulatory element binding protein
<b>Src</b>	v-src sarcoma viral oncogene
<b>Syk</b>	Spleen tyrosine kinase
<b>TAK1</b>	Transforming growth factor- $\beta$ -activated kinase 1
<b>TBST</b>	Tris-buffered saline
<b>TCA</b>	Trichloroacetic acid
<b>TCR</b>	T-cell receptor
<b>Temed</b>	Tetramethylethylenediamine
<b>TGF</b>	Transforming growth factor
<b>TNF</b>	Tumour necrosis factor
<b>TORC2</b>	Transducer of regulated CREB-2
<b>TRPC</b>	Canonical transient receptor potential channel
<b>TZDs</b>	Thiazolidinediones

<b>VCAM</b>	Vascular cell adhesion molecule
<b>VEGF</b>	Vascular endothelial growth factor
<b>VEGF-R</b>	VEGF receptor
<b>Yes</b>	Yamaguchi sarcoma viral oncogene homologue
<b>ZMP</b>	AICAR monophosphate

# **1 INTRODUCTION**

## 1.1 Cellular signalling

Organisms exist in a continually changing environment where they are constantly bombarded and challenged with stimuli to which they must respond in order to survive. Cell signalling is a complex system of communication that enables cells to perceive and correctly respond to their microenvironment. In practice a cell detects a signal, such as a hormone or potentially damaging free radical, and activates a series of enzymes in a signalling cascade that ultimately determines the appropriate biological outcome.

Signal transduction networks are formed, in large part, by interacting protein kinases and phosphatases, which catalyse protein phosphorylation and dephosphorylation respectively. Phosphorylation of proteins or kinases (or their dephosphorylation by phosphatases) can provide docking sites for interacting partners or may trigger conformational changes that alter the enzymatic activity of a protein and/or its interaction with other proteins or DNA (Salazar and Hofer, 2009). These altered enzymatic or interacting properties may transmit signals in various ways.

A simple signalling cascade is a linear one where an enzyme regulates the activity of another which subsequently activates another and so on. If there is an increase in the number of activatable enzymes at each step, signal amplification occurs, thus generating a robust response. Consequently a single activation event may lead to the activation of numerous downstream enzymes. In addition to phosphorylation several post translational modifications are involved in signal transduction greatly enhancing the complexity of signalling networks where crosstalk is also a common theme.

### 1.1.1 Phosphorylation

Phosphorylation, (catalysed by protein kinases; discussed in section 1.1.2), is a process consisting of the esterification of an amino acid side chain with the terminal  $\gamma$ -phosphate group from ATP. A key feature of protein phosphorylation is that it is a readily reversible process whereby protein phosphatases catalyse the reverse reaction, hydrolysing the ester bond with a resultant release of inorganic phosphate (dephosphorylation). Within a protein, phosphorylation can occur on several amino acids. Most kinases act on both serine and threonine (about 98 % of the phosphate found on proteins is covalently linked to the hydroxyl moiety of these residues), others act on tyrosine, and a number (dual-specificity kinases) act on all three (Olsen et al., 2006).



The addition of a negatively charged phosphate group to an amino acid residue turns a non-polar serine, threonine or tyrosine residue into a hydrophilic molecule. This results in a major conformational change in the protein which may dramatically alter its function.

Phosphorylation (or dephosphorylation) is not merely a mechanism to switch the activity of a protein on or off, but can have many additional roles including the modulation of its intrinsic biological activity, subcellular location, half-life and docking with other proteins (Cohen, 2000). Multisite phosphorylation can enable several such effects to operate in the same protein and can determine the extent and duration of a response and is the key to signal integration allowing different agonists to work synergistically or antagonistically as required (Cohen, 2000). It is now known that reversible protein phosphorylation regulates almost all aspects of cell life (Manning et al., 2002a; Manning et al., 2002b).

## **1.1.2 Protein kinases**

### **1.1.2.1 An historical perspective**

Protein kinase activity was first observed in 1954 with the discovery of a liver enzyme that catalysed the phosphorylation of casein (Burnett and Kennedy, 1954). Two forms of glycogen phosphorylase (a and b), the enzyme that catalyses the rate limiting step of glycogenolysis, had been identified in the late 1930's. Work by Fischer and Krebs (Fischer and Krebs, 1955; Krebs and Fischer, 1956), as well as Wosilait and Sutherland (Sutherland, Jr. and Wosilait, 1955) demonstrated that the interconversion of phosphorylase b to phosphorylase a involved a phosphorylation/dephosphorylation mechanism. Fischer and Krebs further demonstrated that the b form could be converted to the a form in the presence of MgATP and an enzyme they termed phosphorylase kinase (Fischer and Krebs, 1955; Krebs and Fischer, 1956). In the following decades the significance of protein kinases in other metabolic pathways and organelles (in addition to glycogen metabolism) began to emerge; for review see (Cohen, 2002a). PKA, a kinase widely distributed in animal tissues and other organisms (Kuo and Greengard, 1969), was shown to activate hormone sensitive lipase (Corbin et al., 1970). This was of particular interest as the widespread distribution of PKA in animal tissues and other organisms suggested a wider range of functions.

### 1.1.2.2 The discovery of AMPK

With hindsight, the AMPK system was first observed concordantly by two groups more than 3 decades ago. Both Beg and co-workers and Carlson and Kim found that soluble protein factors, in the presence of ATP, inactivated two enzymes involved in liver fat metabolism, HMGR and ACC respectively (Carlson and Kim, 1973; Beg et al., 1973). Although these factors were correctly surmised to be protein kinases, and subsequent studies revealed the kinases responsible for the action on ACC and HMGR were both stimulated by AMP (Yeh et al., 1980; Ferrer et al., 1985), it was not realised that these two functions were mediated by the same protein kinase for more than a decade (Carling et al., 1987). It soon became apparent that this was a true multisubstrate protein kinase; and thus it was renamed AMP-activated protein kinase after its allosteric activator, AMP, in accordance with the precedent set by PKA (cyclic AMP-dependent protein kinase).

### 1.1.2.3 The importance of protein kinases

It is now known that there are in excess of 500 protein kinase genes in the human genome, forming the third most populous gene family (constituting of about 2% of all human genes) (Manning et al., 2002b; Caenepeel et al., 2004; Zhu et al., 2000). Furthermore it is thought that up to 30 % of all human proteins may be modified by kinase activity (Hubbard and Cohen, 1993). Protein kinases regulate the majority of cellular pathways, especially those involved in signal transduction (Manning et al., 2002b; Manning et al., 2002a). Defects in protein kinase function result in a variety of diseases thus kinases are major targets for drug design (Cohen, 2002b).

### 1.1.2.4 Mechanisms of substrate specificity

It has been estimated that the majority of human proteins might be phosphorylated at multiple sites (giving more than 100 000 potential phosphorylation sites) (Zhang et al., 2002; Blom et al., 2004) Therefore, to ensure signalling fidelity, kinases must be sufficiently specific and act only on a defined subset of cellular targets. This precision of specificity is essential for the integrity of signal transduction.

In general protein kinases exhibit specificities that are often primarily determined by the amino acids around the phosphorylation site (Kemp and Pearson, 1991). Early studies on PKA and phosphorylase kinase revealed that the principal substrate specificity determinants for these kinases were 'recognition motifs', located in short segments of the primary sequence around the phosphorylation sites (Kemp et al., 1975; Zetterqvist et al.,

1976). The finding that protein kinases phosphorylate short peptide sequences based on known phosphorylation sites in physiological substrates led to the consensus sequence model where the kinase recognizes residues directly flanking the site of phosphorylation (Pearson and Kemp, 1991). In most cases, the active site of the kinase interacts with four amino acids on either side of the phosphorylation site. Sequences that are further away from the phosphorylation site can interact with portions of the protein kinase that are just outside the active site (Knighton et al., 1991). AMPK substrate specificity based on the consensus sequence model is discussed further in section 1.3.1.

In addition to consensus motifs there are several other control mechanisms, outlined as follows, that ensure substrate specificity; reviewed in (Ubersax and Ferrell, Jr., 2007). 1) The nature of the active site, such as its depth, charge or hydrophobicity, often enhances its binding preference for substrates with complementary characteristics. 2) Interactions between docking motifs on the substrate; often spatially separate from the kinase active site and the substrate phosphorylation site, with interaction domains on the kinase (Biondi and Nebreda, 2003; Holland and Cooper, 1999), may also act as determinants of substrate specificity. 3) Some Ser/Thr kinases use binding partners containing docking domains which target the kinase to specific substrates. 4) In addition to direct interactions between protein kinases and their substrates two proteins may interact through intermediate adaptors or scaffolds, which recruit both the kinase and the substrate to the same complex (Bhattacharyya et al., 2006). 5) Localization of protein kinases to distinct subcellular compartments or structures can promote specificity by limiting the number of substrates to which a kinase has access.

## 1.2 AMPK

### 1.2.1 A general overview

The AMPK system is now known to act as an intracellular energy sensor that is conserved throughout all eukaryotes (Towler and Hardie, 2007). AMPK is activated in response to an increase in the cellular AMP:ATP ratio caused by any metabolic stress that either increases ATP consumption (eg, muscle contraction) or compromises its production (e.g., deprivation of oxygen or glucose) (Towler and Hardie, 2007). The AMPK complex is comprised of a catalytic  $\alpha$  subunit and regulatory  $\beta$  and  $\gamma$  subunits (Oakhill et al., 2009). The  $\beta$  subunit contains a GBD (McBride et al., 2009; Hudson et al., 2003; Polekhina et al., 2003), whereas the  $\gamma$  subunit is responsible for AMP/ATP sensing (Cheung et al., 2000). Activation of AMPK involves phosphorylation on Thr-172 catalysed by an upstream kinase. In addition to LKB1 (Hawley et al., 2003), it has been recently demonstrated that in a number of tissues AMPK may be activated by an alternate  $\text{Ca}^{2+}$ -dependent, AMP independent upstream kinase, CaMKK (Hawley et al., 2005). Once activated, AMPK phosphorylates several downstream substrates resulting in the stimulation of ATP-generating (catabolic) pathways including glucose transport, glycolysis, and fatty acid oxidation whilst also inhibiting ATP consuming (anabolic) pathways non-essential to short term survival such as fatty acid and cholesterol synthesis (Carling, 2004; Hardie, 2008). In addition to the acute effects of AMPK on energy metabolism, activation of AMPK has longer term effects, altering both gene (Foretz et al., 1998; Leclerc et al., 1998) and protein expression (Winder et al., 2000). Although it may have evolved to respond to metabolic stress at the cellular level, AMPK is also regulated by physiological stimuli, independent of the energy charge of the cell, including hormones and nutrients. The effect of hormones on AMPK, coupled with the role of AMPK in response to muscle contraction, provide direct evidence that the kinase plays a part in determining whole body energy metabolism (Carling, 2004).

### 1.2.2 AMPK subunit structure

AMPK is a heterotrimeric protein composed of an  $\alpha$  subunit, which contains the catalytic domain, and non-catalytic regulatory  $\beta$  and  $\gamma$  subunits. The AMPK subunit structure is summarised in Figure 1.1. Each AMPK subunit is encoded by two or three distinct genes ( $\alpha 1$ ,  $\alpha 2$ ,  $\beta 1$ ,  $\beta 2$ ,  $\gamma 1$ ,  $\gamma 2$ ,  $\gamma 3$ ), giving rise to 12 possible heterotrimeric combinations, with splice variants further increasing the potential for diversity (Hardie, 2008). Genes encoding

the subunits of the AMPK complex have been identified in all eukaryotes for which the genome sequences have been completed including vertebrates, invertebrates, plants, fungi and protozoa (Hardie et al., 2003). Isoforms of AMPK differ in tissue and subcellular localisation and may direct different functions (Hardie, 2007).

### 1.2.2.1 Alpha subunit

The  $\alpha 2$  catalytic subunit isoform is the subunit of AMPK predominantly found within skeletal and cardiac muscle, whereas, approximately equal distribution of both the  $\alpha 1$  and  $\alpha 2$  isoforms are present in hepatic AMPK. Within pancreatic islet  $\beta$ -cells the  $\alpha 1$  isoform predominates. In the endothelium both  $\alpha 1$  and  $\alpha 2$  subunits are expressed (Davis et al., 2006), with  $\alpha 1$  being the dominant isoform (Davis et al., 2006; Zou et al., 2004). The  $\alpha$  subunit ( $\alpha 1$ : 550 residues) contains an N-terminal catalytic core (1-312) followed by an autoinhibitory sequence (Pang et al., 2007) and a C-terminal sequence (313-548) responsible for autoregulation and binding the  $\beta\gamma$  subunits (Iseli et al., 2005; Crute et al., 1998). The  $\alpha 1$  and  $\alpha 2$  subunit sequence are highly conserved within the catalytic core ( $\alpha 1$ :1-314/ $\alpha 2$ : 1-312, 89 % identity) but diverge in their C-terminal sequences ( $\alpha 1$ :315-550/ $\alpha 2$ : 313-552, 60 % identity) (Stapleton et al., 1996). Both  $\alpha$  subunits appear to have similar substrate specificities (Woods et al., 1996), but the  $\alpha 2$  isoform is enriched in the nucleus of several cell types, including pancreatic  $\beta$  cells (Salt et al., 1998), neurones (Turnley et al., 1999) and skeletal muscle (Ai et al., 2002), whereas  $\alpha 1$  is predominantly cytoplasmic. The  $\alpha 1$  isoform has been shown to localise with the apical membrane of airway (Hallows et al., 2003) and nasal epithelial cells (Kongsuphol et al., 2009), and the plasma membrane in carotid body type 1 cells (Evans et al., 2005). Differences in the subcellular localisation of mammalian  $\alpha$  subunit isoforms may well depend on the associated  $\beta$  and  $\gamma$  isoforms.

### 1.2.2.2 Beta subunit

The  $\beta$  subunit acts as a scaffold for the binding of the  $\alpha$  and  $\gamma$  subunits (Woods et al 1996), and furthermore regulates subcellular localisation of AMPK by targeting the kinase to membranes via an N-terminal myristoyl group (Warden 2001). Indeed 3  $\beta$  subunits in *Saccharomyces cerevisiae* (Sip1, Sip2 and Gal83) direct Snf1 to the vacuole, cytoplasm or nucleus respectively (Vincent et al., 2001). Mammalian  $\beta 1$  is a 270 residue protein which contains 2 conserved regions, located in the central and C-terminal regions (Jiang and Carlson, 1997). The C-terminal region is necessary for a functional  $\alpha\beta\gamma$  complex, and is sufficient on its own to form a complex with  $\beta$  and  $\gamma$  (Hudson et al., 2003), whereas the

central conserved region is recognised to be a glycogen binding domain (GBD) (Hudson et al., 2003; Polekhina et al., 2003) which is conserved in the  $\beta$  subunits of all eukaryotic species.

The GBD is classified as an N-isoamylase domain, which is a non-catalytic domain also found in enzymes that synthesize or degrade the  $\alpha$ 1-6 branches in  $\alpha$ 1-4 linked glucans, such as glycogen or starch. Overexpression of  $\alpha\beta\gamma$  complexes in mammalian cells results in the formation of cytoplasmic inclusions containing AMPK associated with glycogen, an effect which is not observed when a truncated  $\beta$  subunit lacking the GBD is used (Hudson et al 2003). High levels of muscle glycogen have been shown to inhibit AMPK activation during exercise in human muscle (Wojtaszewski et al 2003). Furthermore, a recent report has demonstrated that glycogen inhibits purified AMPK in cell-free assays, an effect that is dependent on binding to the GBD and varies according to the branching content of the glycogen (McBride et al., 2009). Oligosaccharides with single  $\alpha$ 1 $\rightarrow$ 6 branch points are allosteric inhibitors of AMPK that also inhibit phosphorylation and activation by upstream kinases (McBride et al., 2009). Interestingly the GBD may be a regulatory domain that allows AMPK to act as a glycogen sensor *in vivo* thus providing another level whereby AMPK regulates overall energy metabolism (McBride and Hardie, 2009).

### 1.2.2.3 Gamma subunit

The three  $\gamma$  subunit isoforms each contain four tandem repeats of a sequence termed a CBS motif (Bateman, 1997), named after the corresponding sequences in cystathionine  $\beta$ -synthase. CBS motifs occurring in tandem pairs form a structure termed a “Bateman domain” which is responsible for AMP binding. The importance of the  $\gamma$  subunit for AMP binding is highlighted by the observation that the AMP dependence of a particular AMPK complex varies with the  $\gamma$  isoform present (Cheung et al., 2000). Labeling experiments using 8-azido- $^{32}\text{P}$  AMP have also demonstrated that the  $\gamma$  subunit participates directly in the binding of AMP to the AMPK complex (Cheung et al., 2000). Interestingly 3 naturally occurring mutations within the CBS domains have been shown to decrease AMPK activity by dramatically reducing the AMP activation of the kinase (Daniel and Carling, 2002). The Bateman domains antagonistically bind ATP with a lower affinity than AMP (Scott et al., 2004), consistent with the observation that high concentrations of ATP oppose activation of the AMPK complex by AMP (Corton et al., 1995). Thus, AMPK activity is affected by the AMP:ATP ratio and not merely AMP levels alone.

The crystal structure of the regulatory fragment of mammalian AMPK $\gamma$ 1 in complexes with AMP and ATP has recently been reported (Xiao et al., 2007). From this work it has emerged that there are two sites on the  $\gamma$  domain that readily exchange AMP/ATP, whereas a third site contains a tightly bound AMP that is non-exchangeable (Xiao et al., 2007). The presence of two exchangeable sites is consistent with binding studies that detected two moles of AMP or ATP binding per  $\gamma$  subunit (Scott et al., 2004). The results reported by (Xiao et al., 2007) suggest that, at least for mammalian enzyme, AMP binding at this non-exchangeable site is not involved in AMP/ATP sensing. Binding of ATP to the exchangeable sites was found to elicit little structural rearrangement. Mg.ATP or Mg<sup>2+</sup>-free ATP were found to bind with similar affinity consistent with earlier studies which also found the affinity of the protein for ATP was independent of Mg<sup>2+</sup> (Scott et al., 2004). Currently it remains uncertain as to how the occupancy state of the AMP/ATP exchangeable sites affects allosteric activation of the catalytic domain.

Interestingly, mutations in the CBS domains of the  $\gamma$ 2 subunit (gene symbol PRKAG2) are associated with Wolff-Parkinson-White syndrome (a syndrome of pre-excitation of the ventricles of the heart) and familial hypertrophic cardiomyopathy (Blair et al., 2001; Gollob et al., 2001b; Gollob et al., 2001a). An additional inherited disorder associated with mutations in the PRKAG2 gene is a severe cardiac condition called lethal congenital glycogen storage disease of the heart (Arad et al., 2002). This disorder is caused by a single amino acid substitution of glutamate for arginine at position 531 (R531Q) (Burwinkel et al., 2005).

### 1.2.3 Regulation of the AMPK complex by AMP

AMPK senses changes in the ratio of AMP:ATP by binding both species in a competitive manner (Hardie et al., 1998). If the reaction catalysed by adenylate cyclase ( $2\text{ADP} \leftrightarrow \text{ATP} + \text{AMP}$ ) is assumed to be at or near equilibrium, the AMP:ATP ratio will vary approximately as the square of the ADP:ATP ratio, making the former ratio a much more sensitive indicator of reduced cellular energy status (Hardie et al., 1998). Indeed, only a 6-fold increase in nucleotide concentration is required for maximal activity to progress from 10 % to 90 % (Hardie et al., 1999). AMP binding to the  $\gamma$  subunit results in allosteric activation of AMPK, and furthermore inhibits the action of protein phosphatases, thus regulates the enzyme by two distinct mechanisms (Sanders et al., 2007b).

The degree of allosteric activation by AMP depends on the particular  $\alpha$  and  $\gamma$  subunit isoforms present in the complex (Hardie et al., 1999). Complexes containing the  $\alpha 2$ - and  $\gamma 2$ -isoforms exhibit the greatest degree of activation (almost 5-fold), whereas complexes containing the  $\gamma 3$  isoform are only activated <2-fold by AMP (Cheung et al., 2000). As AMP stimulation of the kinase is only observed if the intact  $\alpha\beta\gamma$  complex, and not the isolated kinase domain (which lacks the AMP binding site) is used as the substrate, it is clear the nucleotides interact with AMPK rather than with the upstream kinase (Hawley et al., 2003). Work conducted by Xiao and colleagues would suggest AMPK mainly exists in its inactive form in complex with the much more abundant MgATP with only a small proportion binding AMP (Xiao et al., 2007). A model proposed by the authors of this study suggests that a 2-3 fold increase in the AMP concentration (low  $\mu\text{M}$  range) results in a similar fold increase in the proportion of AMP bound enzyme in the presence of a much higher (mM range) concentration of MgATP (Xiao et al., 2007).

#### 1.2.4 Phosphorylation of AMPK on Thr-172

AMPK is activated by phosphorylation at a specific threonine residue (Thr-172) on the  $\alpha$  subunit, catalysed by an upstream kinase (Hawley et al., 1996). Although AMPK was so named as a result of its allosteric activation by AMP, this effect is largely insignificant (up to 5-fold) (Carling et al., 1987) in comparison with the effect of Thr-172 phosphorylation (>100-fold) (Suter et al., 2006). Phosphorylation of Thr-172 appears to be essential for AMPK activity as site directed mutagenesis of Thr-172 to alanine completely abolishes kinase activity (Crute et al., 1998; Stein et al., 2000).

Mass spectrometry studies have shown the  $\alpha$  subunit of AMPK is phosphorylated on at least 2 other residues, Thr-258 and Ser-485 (Ser491 in  $\alpha 2$ ); however phosphorylation at these sites has no effect on AMPK activity (Woods et al., 2003a). Mutagenesis of Thr-258, and Ser-485 to acidic residues to mimic phosphorylation in the recombinant proteins indicates that Thr-258 and Ser-485 are not involved in AMPK activation (Woods et al., 2003b). Transfection of the non-phosphorylatable S485A and T258A mutants in CCL13 cells subjected to stresses known to activate AMPK further indicate these sites are not involved in AMPK activation (Woods et al., 2003b). It may be the case these phosphorylation sites may play a less prominent role in the regulation of AMPK, e.g. in subcellular localization or substrate recognition.



## 1.2.5 Regulation of AMPK by upstream kinases

Although it had been apparent that the mechanism of AMPK activation by metabolic stress involved the action of an AMPKK, for many years the molecular identity remained elusive, despite partial purification of the kinase from rat liver (Hawley et al 1996), and many attempts to uncover it by conventional protein purification. This led a number of groups to focus on the yeast *Saccharomyces cerevisiae* system. In this system genetic studies show that the AMPK homologue (the Snf1 complex) is required for the response to glucose starvation (Hardie et al 1998), while in the moss *Physcomitrella patens* it is required to allow the plant to survive periods of darkness, the equivalent of carbon source starvation for a photosynthetic plant (Thelander et al 2004). Two groups subsequently reported the identification of three protein kinases encoded in the yeast *Saccharomyces cerevisiae* genome i.e. Elm1, Sak1 (originally called Pak1) and Tos3, that phosphorylated the yeast orthologue of the  $\alpha$  subunit of AMPK (Snf1) at the equivalent site (Thr-210), and provided genetic evidence that these kinases acted upstream of Snf1 in a redundant manner *in vivo* (Hong et al 2003; Sutherland et al 2003). The nearest relatives of Elm1, Sak1 and Tos3 in the human genome are CaMKK and the tumour suppressor kinase LKB1.

### 1.2.5.1 LKB1

Historically, interest in LKB1 was first aroused in 1998 after it was found that germline inactivating mutations in this tumour suppressing kinase causes the rare Peutz-Jeghers syndrome (Mehenni et al., 1998). Peutz-Jeghers is a cancer predisposition syndrome characterized by the development of gastrointestinal polyps and mucocutaneous pigmentation abnormalities.

#### 1.2.5.1.1 Identification of LKB1 as an AMPKK

Considering its intimate association with cancer development, it was of surprise when LKB1 was identified as an AMPK kinase (Hawley et al., 2003; Woods et al., 2003a; Shaw et al., 2004). LKB1 exists as a heterotrimeric complex requiring two accessory subunits, STRAD and MO25, for activity of the kinase (Boudeau et al., 2003). Recombinant LKB1 expressed in COS7 cells was found to activate AMPK by phosphorylation of Thr-172 (Woods et al., 2003a). Homozygous LKB1 knockout mice die during embryonic development, but immortalised MEFs can be derived from the embryos. An experiment

using MEFs demonstrated that LKB1 was necessary and sufficient for activation of AMPK by the drugs, AICAR and phenformin (Shaw et al., 2004; Hawley et al., 2003).

#### 1.2.5.1.2 Importance of LKB1

It is not clear whether the tumour suppressor effects of LKB1 occur via AMPK, as LKB1 acts upstream of at least 13 distinct AMPK-related protein kinases (Lizcano et al., 2004). It is apparent however that LKB1-mediated activation of AMPK is an important regulatory pathway. Deletion of LKB1 in the liver significantly reduces total AMPK activity suggesting LKB1 is the predominant upstream kinase for both AMPK  $\alpha 1$  and  $\alpha 2$  in this tissue. Loss of LKB1 activity in adult mouse liver leads to near complete loss of AMPK activity and is associated with hyperglycemia. The hyperglycemia is, in part, due to an increase in the transcription of gluconeogenic genes. Of particular significance is the increased expression of PGC-1 $\alpha$  which drives gluconeogenesis. Reduction in PGC-1 $\alpha$  activity results in normalized blood glucose levels in LKB1-deficient mice. Deficiency of LKB1 in skeletal muscle prevents AMPK $\alpha 2$  activation and glucose uptake during contraction or in response to AICAR or phenformin (Sakamoto et al., 2005). The activity of  $\alpha 1$ -containing complexes is reduced but not abolished (Sakamoto et al., 2005). Similarly, in heart lacking LKB1, the basal activity of AMPK $\alpha 2$  is vastly reduced and not increased by ischemia or anoxia, whereas the activity of AMPK $\alpha 1$  is reduced by a small degree (Sakamoto et al., 2006). Interestingly, these studies indicate that an alternative upstream kinase can activate AMPK $\alpha 1$  in cardiac and skeletal muscle.

#### 1.2.5.2 The CaMK cascade

An increase in the intracellular Ca<sup>2+</sup> concentration, often acting through the Ca<sup>2+</sup> receptor protein calmodulin (CaM), is a ubiquitous signal that regulates diverse cellular responses. The CaMK cascade consists of three related members where CaMKK is the activating kinase for two multifunctional CaM kinases, CaMKI and CaMKIV (Soderling, 1999). CaMKK phosphorylates a Thr in the T-loop of the catalytic domain of CaMKI and CaMKIV, resulting in a large increase in the catalytic efficiency of each CaMK (Soderling, 1999).

CaMKI is broadly distributed in mammal and is mainly cytosolic (Picciotto et al., 1995) whereas the two splice variants of CaMKIV are well expressed in neural tissue, T cells and testis (Soderling, 1996). CaMKIV is predominantly restricted to the nucleus but also is detectable in cytoplasm (Jensen et al., 1991). Distinct genes encode the two isoforms of

CaMKK ( $\alpha$  and  $\beta$ ), which exhibit cytoplasmic and nuclear localisation respectively. High levels are found in brain, testis and spleen, whereas other tissues contain much lower levels of CaMKK (Tokumitsu et al., 1995; Kitani et al., 1997; Anderson et al., 1998). There are two isoforms of CaMKK,  $\alpha$  and  $\beta$ . The binding of  $\text{Ca}^{2+}/\text{CaM}$  to CaMKK $\alpha$  is absolutely required for activation and phosphorylation of target protein kinases (Tokumitsu and Soderling, 1996; Selbert et al., 1995; Haribabu et al., 1995), while CaMKK $\beta$  activity is enhanced but not completely dependent on  $\text{Ca}^{2+}/\text{CaM}$ , *in vitro* and in transfected cells (Anderson et al., 1998).

#### 1.2.5.2.1 CaMKK structure and regulation

CaMKK, as well as CaMKI and CaMKIV, contains an amino-terminal catalytic domain and a regulatory domain (autoinhibitory and CaM-binding segments) at its carboxyl terminus (Tokumitsu and Soderling, 1996; Tokumitsu et al., 1997; Matsushita and Nairn, 1998). An interaction between the autoinhibitory domain and the catalytic domain maintains the kinase in an inactive conformation by preventing binding of protein substrate as well as MgATP (Soderling, 1996). Binding of  $\text{Ca}^{2+}/\text{CaM}$  to the CaM binding domain alters the conformation of the overlapping autoinhibitory domain such that it no longer interferes with substrate binding resulting in activation of the kinase.

#### 1.2.5.2.2 Downstream of the CaMK cascade

In the cytosol members of the CaMK cascade regulate cell survival through the activation of PKB (Yano et al., 1998), and transcription through indirect activation of MAPK (Soderling, 1999). CaMKIV can also phosphorylate and inactivate type I adenylate kinase, thereby decreasing cAMP levels (Wayman et al., 1996). Elevated levels of cAMP can exert negative feedback by activating PKA, which phosphorylates and inactivates CaMKK (Wayman et al., 1997). There is extensive cross-talk between CaMKK, CaMKIV and other signalling cascades, including those that involve the PKA, MAPK and PKB (Soderling, 1999). CaMKK and CaMKIV are also present in the nucleus where they stimulate transcription through the phosphorylation of transcription factors (Jensen et al., 1991; Soderling, 1999).

#### 1.2.5.2.3 Identification of CaMKK as an AMPK kinase

Although LKB1 had been identified as a major AMPKK a number of observations indicated other kinases could activate AMPK in a number of tissues. LKB1<sup>-/-</sup> fibroblasts and the naturally LKB1-deficient HeLa cells still possessed AMPK activity and Thr-172

phosphorylation, albeit with reduced responses to known AMPK activators (Hawley et al., 2005; Shaw et al., 2004).

CaMKK shows more sequence similarity to Pak1, Tos3 and Elm1 than does LKB1, and in 1995 it was reported that CaMKK could phosphorylate and activate the AMPK complex in cell-free assays (Hawley et al., 1995). However, the major upstream kinase that had been purified from rat liver was not calmodulin dependent and as a result CaMKK was ruled out as an AMPKK. In hindsight this can be easily explained as in those initial studies AMPK kinase was purified from a tissue where LKB1 is the major AMPKK. Three independent reports subsequently identified that CaMKK is an upstream kinase for AMPK, that acts in a  $\text{Ca}^{2+}$ -dependent, AMP-independent manner (Hurley et al., 2005; Hawley et al., 2005; Woods et al., 2005).

### 1.2.5.3 The mechanism of CaMKK/LKB1-mediated AMPK activation

In LKB1-deficient HeLa cells activation of AMPK is abolished in response to AICAR or other stimuli that increase the AMP:ATP ratio (Hawley et al., 2003). However, whilst these observations demonstrate that LKB1 mediates AMPK activity in response to an increased AMP:ATP ratio, evidence suggests that AMP does not promote phosphorylation of AMPK by recombinant LKB1, nor does it alter LKB1 activity (Woods et al., 2003a; Sakamoto et al., 2004). Rather AMP inhibits the dephosphorylation of AMPK by protein phosphatases (Figure 1.2) (Sanders et al., 2007a; Suter et al., 2006), and as LKB1 appears to be constitutively active (Lizcano et al., 2004; Sakamoto et al., 2004) this results in phosphorylation of AMPK at Thr-172.

Activation of AMPK in response to increased intracellular  $\text{Ca}^{2+}$  occurs through increased CaMKK activity and is independent of adenine nucleotide levels. Once  $\text{Ca}^{2+}$  levels fall AMPK is rapidly dephosphorylated (Figure 1.2).

Recent studies using cultured endothelial cells have suggested that LKB1-mediated activation of AMPK occurs in response to peroxynitrite (Xie et al., 2006), whereas CaMKK has been reported to mediate the activation of AMPK in response to thrombin (Stahmann et al., 2006), suggesting both the AMP-dependent and  $\text{Ca}^{2+}$ -dependent mechanisms are present in cultured endothelial cells.

#### 1.2.5.4 Additional AMPKK's

Considering that there are 3 upstream kinases for Snf1 in yeast it seems feasible that there may be at least three kinases in mammalian cells. Tak1, a member of the MAPK kinase family, has recently been proposed as a possible AMPKK in mammals (Momcilovic et al., 2006). The physiological significance of Tak1 and its role *in vivo* however is uncertain at present. Further work is necessary to further elucidate the role Tak1, and possibly other kinases, in relation to the regulation of AMPK.

### 1.2.6 Pharmacological activators of AMPK

#### 1.2.6.1 Metformin and phenformin

Metformin and phenformin are derivatives of guanidine, the active ingredient of French lilac, used to treat diabetes in medieval Europe (Witters, 2001). Currently metformin is widely used in both Europe and the US as a treatment for type 2 diabetes (prescribed to more than 120 million people worldwide). Phenformin was withdrawn as a treatment as it can cause lactic acidosis (Kwong and Brubacher, 1998). These agents were introduced in Europe as oral anti-hyperglycemic agents during the late 1950's, even though their mechanism of action was uncertain. Thus, it was of great interest when it was demonstrated that metformin activates AMPK in intact cells *in vivo* (Zhou et al., 2001). It is likely the therapeutic actions of metformin are primarily on the liver, where it has been shown to decrease glucose production and increase fatty acid oxidation in hepatocytes (Zhou et al., 2001). OCT-1, which is not expressed in many cultured cells but well expressed in liver, facilitates the entry of metformin into tissues (Wang et al., 2002). Indeed a liver-specific LKB1 deletion in mouse liver results prevents the anti-hyperglycemic effects of metformin (Shaw et al., 2005). Zhou and co-workers proposed that metformin might act by stimulating phosphorylation of AMPK by upstream kinase(s) or by inhibiting dephosphorylation by protein phosphatases (Zhou et al., 2001). However it has been reported that metformin does not activate AMPK in cell-free assays by either of the two upstream kinases found to phosphorylate Thr-172 (LKB1 and CaMKK), either in the presence or absence of AMP (Hawley et al., 2002). Similarly, metformin was found to have no effect on inactivation of AMPK by PP2C (Zhou et al., 2001; Hawley et al., 2002).

Metformin, phenformin and the TZDs, another group of anti-diabetic agents which are discussed in section 1.2.7.2, can act as an inhibitor of complex 1 of the respiratory chain

(Owen et al., 2000; Brunmair et al., 2004). Any metabolic poison that inhibits mitochondrial ATP synthesis will raise the AMP:ATP ratio triggering phosphorylation and activation of AMPK. It was therefore proposed that these drugs may act as weak poisons resulting in a cellular stress which activates AMPK via an increase in the AMP:ATP ratio. The cellular AMP:ATP ratio is significantly altered after treatment of cultured cells with phenformin (Hawley et al., 2005), and decreases in ATP have been reported in rodent hepatocytes treated with metformin (Guigas et al., 2006). Notably, as intestinal epithelial cells also express OCT transporters (Koepsell et al., 2007), it seems possible that inhibition of the respiratory chain is responsible for the adverse gastrointestinal side effects of biguanides, as well as the lactic acidosis that led to the withdrawal of phenformin. Indeed it has been reported that the lactic acid produced in animals treated with biguanides is derived from gut (Bailey et al., 1992).

#### 1.2.6.2 TZDs

The TZDs (thiazolidinediones), a class of anti-diabetic drugs including rosiglitazone, troglitazone and pioglitazone, improve endothelial function, as assessed by endothelium-dependent vasodilatation in patients with type 2 diabetes or insulin resistance (Pistrosch et al., 2004; Wang et al., 2004; Natali et al., 2004; Satoh et al., 2003). While the hypoglycemic effects of TZDs are mediated PPAR $\gamma$ -activated genes (Semple et al., 2006), evidence suggests that the improvement of endothelial function by TZDs is independent of the effect on glycerin (Pistrosch et al., 2004; Satoh et al., 2003). Interestingly, AMPK has been identified as an alternative mechanism also modulated upon exposure to TZDs (Fryer et al., 2002). A recent report from our laboratory has demonstrated that rosiglitazone stimulates NO production in human aortic endothelial cells in an AMPK-dependent manner (Boyle et al., 2008).

Similar to metformin and phenformin TZDs have been demonstrated act as an inhibitor of complex 1 of the respiratory chain (Owen et al., 2000; Brunmair et al., 2004), thus an increase in the AMP:ATP ratio may be the mechanism for AMPK activation in response to these agents. Notably, TZDs also increase plasma adiponectin and decrease plasma resistin levels, which may indirectly contribute to the effects on AMPK activity (Yamauchi et al., 2002; Wu et al., 2003; Banerjee and Lazar, 2003; Muse et al., 2004).

### 1.2.6.3 AICAR

AICAR was the first drug demonstrated to activate AMPK in intact cells (Sullivan et al., 1994; Corton et al., 1995; Henin et al., 1995). AICAR is a pro-drug taken up into cells by adenosine transporters (Gadalla et al., 2004) then converted to its active component (ZMP, an AMP analogue) inside the cell, which acts as a mimetic of AMP thus activating AMPK (Corton et al., 1995). When administered to rodent models of obesity and insulin resistance such as the ob/ob mouse, the fa/fa rat, and high fat-fed rat, AICAR has been demonstrated to reverse many of the adverse abnormalities (Song et al., 2002; Bergeron et al., 2001; Iglesias et al., 2002; Buhl et al., 2002). For a number of reasons AICAR is not used clinically including its tendency to cause bradycardia in perfused hearts and hypoglycemia when administered intravenously (Young et al., 2005).

### 1.2.6.4 A-769662

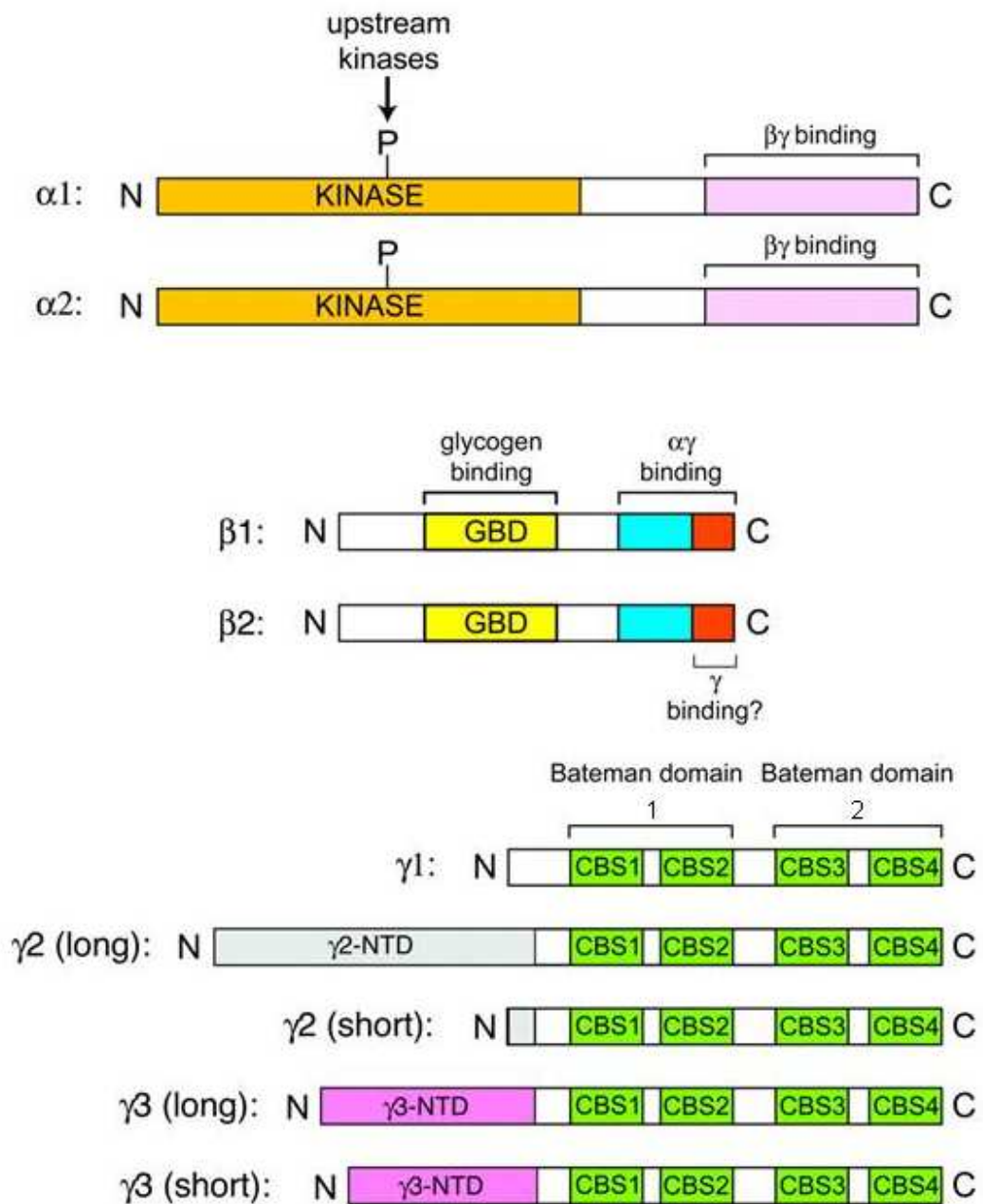
A-769662, a newly identified small molecule from the thienopyridone family, activates AMPK both allosterically and by inhibiting dephosphorylation of the kinase at Thr-172 (Sanders et al., 2007a; Goransson et al., 2007; Cool et al., 2006). A-769662 has been shown to act independently of the upstream kinases LKB1 and CaMKK (Goransson et al., 2007), and in cell-free assays has been shown to have no direct effect on the ability of LKB1 or CaMKK to phosphorylate AMPK (Sanders et al., 2007a). Whilst activation of AMPK by A-769662 is similar to that caused by AMP (Sanders et al., 2007a; Goransson et al., 2007) the drug has not been demonstrated to have any effect on adenine nucleotide levels (Sanders et al., 2007a; Cool et al., 2006), and a number of observations suggest its mechanism of action is distinct from activation by AMP. Indeed A-769662, unlike AMP, retains the ability to activate AMPK containing a mutation in the  $\gamma$  subunit (Sanders et al., 2007a). Furthermore, a mutation in the  $\beta$ 1 subunit (Ser-108 to alanine) which prevents activation by A-769662 is only partially effective in reducing activation as a result of AMP (Sanders et al., 2007a).

Current evidence indicates that the  $\beta$ 1 subunit plays a key role in mediating the activation of AMPK by A-769662. Indeed it has been reported recently that A-769662 exclusively activates AMPK complexes containing the  $\beta$ 1 subunit isoform (Scott et al., 2008). The activation of AMPK by A-769662 seems to involve an interaction between the  $\beta$ 1 subunit GBD and residues from the  $\gamma$  subunit (that are not involved in AMP binding) (Scott et al., 2008). The mechanism of A-769662 action however does not seem to involve binding of the drug to the GBD nor the nucleotide binding sites of the  $\gamma$  subunit (Scott et al., 2008). At

present it is unclear as to why  $\beta$ 2-containing AMPK complexes are insensitive to A-769662 as there is little variation in the GBD of these two isoforms.

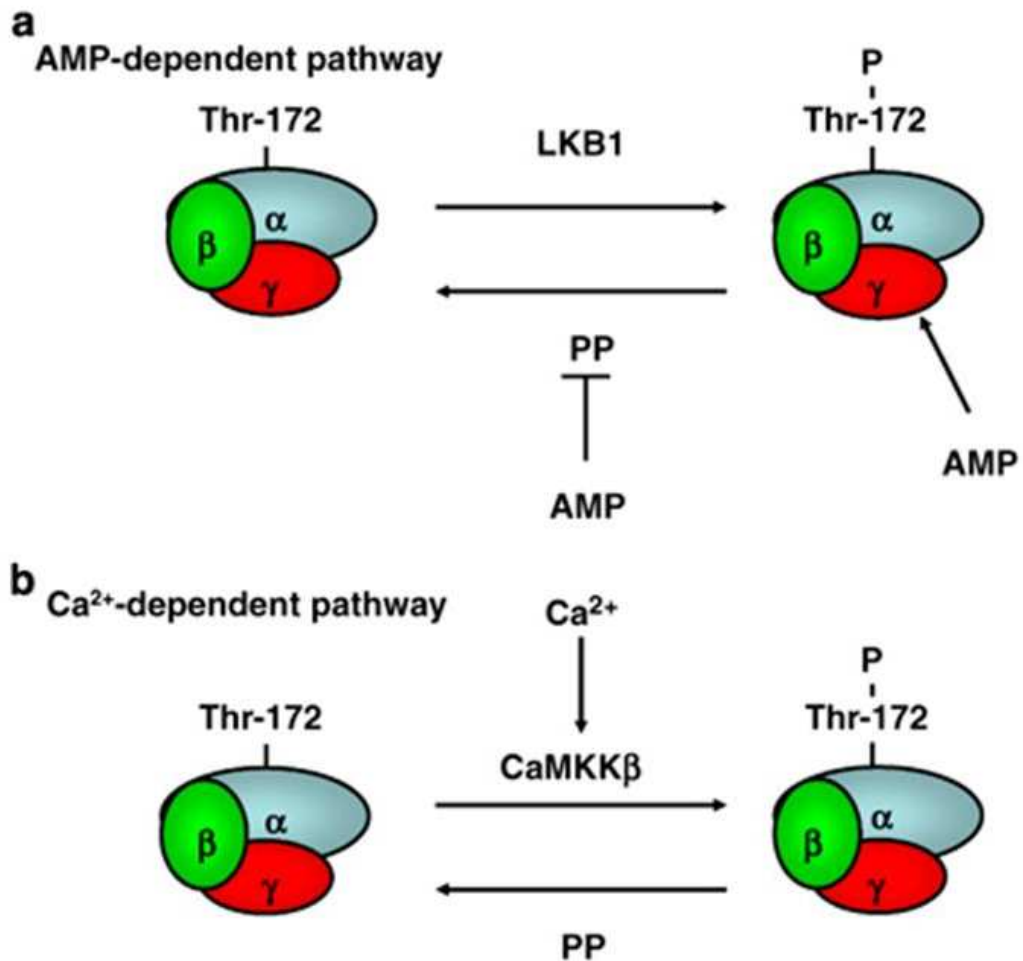
*In vivo* A-769662 has previously been reported to lower plasma glucose levels in obese mice, likely through a primary action on the liver (Cool et al., 2006). However, more recently Scott and co-workers have demonstrated that the glucose lowering effects of A-769662 occur independently of AMPK activation in hepatocytes generated from AMPK  $\beta$ 1-null mice (Scott et al., 2008). This observation and the finding that A-769662 can inhibit non-proteolytic components of the 26S proteasome (Moreno et al., 2008) highlight the potential for off-target effects of the drug.





**Figure 1-1 Domain structure of AMPK subunit isoforms and splice variants**

Regions shown in the same colour are related, and their functions, where known, are indicated. (Figure adapted from (Towler and Hardie, 2007)).



**Figure 1-2 Activation of AMPK by upstream kinases**

(A) As LKB1 appears to be constitutively active activation of AMPK in response to an increase in the AMP:ATP ratio is proposed to occur through inhibition of the dephosphorylation reaction, catalysed by protein phosphatases (PP). The net result is increased phosphorylation of Thr-172. Inactivation of AMPK would occur following a drop in the AMP:ATP ratio, and relief of inhibition of dephosphorylation. (B) Activation of AMPK in response to increased intracellular Ca<sup>2+</sup> occurs through increased CaMKKβ activity. In this model, AMPK would be rapidly dephosphorylated following a fall in Ca<sup>2+</sup> levels and this may account for the transient nature of AMPK activation observed after CaMKKβ activation. It should be noted that these pathways do not need to be mutually exclusive and activation of AMPK by both LKB1 and CaMKKβ could occur under conditions where AMP and Ca<sup>2+</sup> levels rise in concert. (Figure adapted from (Carling et al., 2008)).

## 1.3 Downstream of AMPK activation

The signalling cascades initiated by the activation of AMPK exert both short and long term effects through its action on a range of processes including glucose and lipid metabolism, gene expression and protein synthesis (for review see (Kahn et al., 2005)). In general (as summarised in Figure 1.3), AMPK switches on catabolic processes that generate ATP (e.g., glucose uptake, glycolysis, fatty acid oxidation and mitochondrial biogenesis), while switching off anabolic processes that consume ATP, (e.g., fatty acid, triglyceride, cholesterol, glucose (via gluconeogenesis) and glycogen synthesis (reviewed in (Kahn et al., 2005))).

### 1.3.1 AMPK substrate specificity

As of 2007 there were almost 20 direct physiological targets identified for AMPK (Towler and Hardie, 2007); though given the critical importance of maintaining energy balance at both the cellular and whole body levels, it is likely there are many more which remain, as of yet, undiscovered.

As discussed in section 1.1.2.3, the amino acids that are situated immediately N-terminal and C-terminal to the phosphorylation site (P-site) often contribute substantially to kinase-substrate recognition (consensus sequence model). Early studies on the substrate specificity of AMPK, using variant synthetic peptides, indicated basic and hydrophobic residues in the substrate are important specificity determinants (Weekes et al., 1993; Dale et al., 1995). The SAMS peptide has been most widely used, and the consensus recognition sequence around the phosphorylation site established from synthetic peptide studies is hydrophobic ( $\Phi$ ) at P-5 and P+4 and at least one basic ( $\beta$ ) between P-1 and P-4 - i.e. [ $\beta$ ,X]-X-X-S/T-X-X-X- $\Phi$  (Weekes et al., 1993; Dale et al., 1995). A number of subsequent studies have further confirmed this consensus phosphorylation motif for AMPK substrates (Scott et al., 2002; Towler and Hardie, 2007; Gwinn et al., 2008). Figure 1.4 is an alignment of 14 sequences containing sites phosphorylated by AMPK in cell-free assays, all of which are believed to be targets *in vivo*. Although a serine residue is phosphorylated in each instance, threonine can be phosphorylated by AMPK in synthetic peptide substrates (Weekes et al., 1993). In most cases, there are bulky hydrophobic residues (shown in bold) at positions P-5 and P+4, and at least 1 basic residue (underlined) at either P-4 or P-3 in accordance with the consensus sequence described for AMPK substrates.

Two major AMPK targets ACC and eNOS (Figure 1.4), are central to the present investigation. ACC, phosphorylated by AMPK on Ser-80 (human sequence), is regarded as perhaps the most efficient substrate for AMPK (Towler and Hardie, 2007). Knowledge of AMPK substrate recognition has been enhanced by modeling studies using the binding of the sequence around Ser-79 on rat ACC1 to a 3D model of the  $\alpha$ 1 kinase domain (Scott et al., 2002). Briefly this work, indicates a basic residue at P-6 and additional hydrophobic side chains on the N-terminal side occurring every third or fourth residue, i.e., at P-9, P-13, and P-16 may be an additional positive determinants of substrate specificity. This pattern of hydrophobic residues is also discernible in a few of the other substrates, including HMGR, eNOS, TSC2, and TORC2. Although Ser-1177 on eNOS is known to be the consensus phosphorylation site of AMPK (Chen et al., 1999), Figure 1.4 demonstrates that the basic-hydrophobic-basic motif that usually occurs at the P-6, P-5, and P-4 or P-3 positions is displaced by 1 residue, i.e., at P-5, P-4, and P-3 (similar to cardiac PFK2).

### 1.3.1.1 ACC

In animals the storage of body fat provides a crucial reserve to sustain energy metabolism during starvation, hibernation and migration, as well as providing thermal insulation and other functions (Brownsey et al., 2006). However, excess body fat, especially in certain adipose depots and other tissue sites, can be maladaptive in humans, substantially increasing the risk of diabetes and cardiovascular diseases (Gurevich-Panigrahi et al., 2009). ACC is a key regulator of lipid storage and overall energy metabolism through the synthesis of malonyl-CoA, an essential substrate for fatty acid synthesis and a potent inhibitor of fatty acid oxidation (McGarry, 2002). As mentioned previously ACC was one of the first substrates identified for AMPK (Carlson and Kim, 1973). AMPK is a negative regulator of ACC activities and as such the AMPK-ACC system is a major therapeutic target against insulin resistance and type 2 diabetes (Ruderman and Prentki, 2004).

### 1.3.1.2 ACC isoforms

ACC orthologues have been demonstrated in yeast, animals and higher plants, including two major mammalian isoforms; ACC1 (265 kDa) and ACC2 (280 kDa) (Lane et al., 1974; Cronan, Jr. and Waldrop, 2002; Barber et al., 2005). The predicted amino acid sequence of ACC2 contains an additional 136 amino acids relative to ACC1, 114 of which constitute the unique N terminal sequence of ACC2. The N-terminal sequence of ACC2 contains a potential mitochondrial targeting motif. Indeed the expressed N-terminal domain appears to co-localize with mitochondria in intact cells (Abu-Elheiga et al., 2000), an observation

that is supported by a number of genetic studies (Abu-Elheiga et al., 2003; Abu-Elheiga et al., 2005; Abu-Elheiga et al., 2001). Immunofluorescence microscopic analysis has demonstrated that ACC1 is a cytosolic protein (Abu-Elheiga et al., 2000).

### 1.3.1.3 ACC and the regulation of fatty acid synthesis and oxidation

ACC acts as key metabolic enzyme through the formation of malonyl-CoA. ACC1-generated malonyl CoA is utilised by fatty acid synthase (FAS) for the synthesis of fatty acids in the cytosol (Davies et al., 1990), whereas ACC2-generated malonyl-CoA functions as a potent inhibitor of CPT-1, a rate limiting step for the entry of long chain fatty acids into mitochondria for oxidation (McGarry, 2002). ACC2<sup>-/-</sup> mice have a normal lifespan, a higher fatty acid oxidation rate and accumulate less fat, thus making it a potential therapeutic target for obesity and its related disorders. Mutant mice lacking ACC1, unlike ACC2-null mice, are embryonically lethal (Abu-Elheiga et al., 2005) demonstrating the importance of *de novo* fatty acid synthesis in development, and further highlighting that ACC1 and ACC2 have distinct roles in fatty acid metabolism.

### 1.3.1.4 ACC as a fuel sensor

When metabolic fuel is low (glucose deprivation, increased energy expenditure) and ATP is required, both ACC1 and ACC2 are turned off by phosphorylation and the consequential reduction in malonyl-CoA leads to the generation of ATP through increased fatty acid oxidation and a decrease in ATP consumption for fatty acid synthesis (Ruderman et al., 2003). Conversely, in response to an increase in glucose availability, many cells inhibit the oxidation of fatty acids (Ruderman et al., 2003). Indeed, just as AMPK acts as a fuel sensor (in response to an increase in the AMP:ATP ratio), ACC may also act as a metabolic sensor, allosterically (via citrate and fatty acids) and through AMPK phosphorylation, directing fatty acid synthesis (fuel storage) and  $\beta$  oxidation (fuel supply) (Steinberg et al., 2006).

### 1.3.1.5 Regulation of ACC

ACC is tightly regulated through a variety of dietary, hormonal and metabolic responses. The gene expression of ACC is largely regulated by SREBP1c (Andreolas et al., 2002; Diraison et al., 2004), whereas acute changes are mediated by allosteric and covalent mechanisms (Brownsey et al., 2006).

### 1.3.1.5.1 Gene expression

ACC1 is highly expressed (10–50  $\mu\text{g/g}$  of wet weight) in white and brown fat, liver and lactating mammary gland, with expression being inhibited during starvation or insulin deficiency and restored by refeeding a low-fat diet or by insulin treatment (Iritani, 1992). The ACC1 gene is under the control of at least three promoters with distinct roles in constitutive or inducible expression in response to a variety of factors including glucose, insulin, thyroid hormone, catabolic hormones and leptin (Kim, 1997; Mao et al., 2003; Zhang et al., 2003).

ACC2 is expressed at low levels (1–2  $\mu\text{g/g}$  of wet weight) in heart and skeletal muscle (Thampy, 1989), whereas much higher concentrations of ACC2 are found in liver, where it represents approximately 20–25% of total hepatic ACC (Winz et al., 1994). The expression of ACC2 is increased during the differentiation of heart and skeletal-muscle cells, with a parallel decline in expression of ACC1 (Lopaschuk et al., 1994). In contrast, in liver cells, the expression of ACC2 appears to parallel that of ACC1, being decreased during starvation and restored on refeeding (Iverson et al., 1990).

Among the key transcription factors SREBP1c plays a major role in controlling genes for ACC1 and other lipogenic enzymes, in adipogenesis and in nutritional regulation (Shimano et al., 1999). Other transcription factors that contribute to the control of ACC expression include liver X receptor, retinoid X receptor, PPARs, FOXO and PGC isoforms (Barber et al., 2005).

### 1.3.1.5.2 Allosteric control

The activity of ACC1 is acutely controlled by allosteric modulators. Exposure of cells to insulin *in vivo* or *in vitro* leads to rapid activation of ACC1 (Stansbie et al., 1976); conversely catecholamines or glucagons rapidly inhibit the enzyme (Brownsey et al., 1979; Hardie, 1992). The allosteric effectors of ACC1; which include citrate, other carboxylic acids and glutamate, activate the enzyme by promoting polymerization of ACC, resulting in the formation of high order oligomers (Lane et al., 1974; Boone et al., 2000; Thampy and Wakil, 1985). On the other hand, long chain fatty acids such as malonyl-CoA, free-CoA and fatty acyl-CoA esters inhibit the enzyme and promote disassociation of these oligomers (Ogiwara et al., 1978; Moule et al., 1992). However, while allosteric ligands clearly influence ACC1 activity, the effects of insulin and catecholamines are still apparent

following enzyme isolation, indicating the presence of additional control mechanisms (Brownsey et al., 2006).

ACC2 is also activated by citrate and glutamate but does not polymerize as readily as ACC1. In spite of this it has been suggested that allosteric control of ACC2 by citrate is crucial in skeletal muscle (Saha et al., 1999).

### 1.3.1.5.3 Multisite phosphorylation

Experiments involving metabolic labeling with [<sup>32</sup>P]-phosphate demonstrate ACC1 is phosphorylated on at least four hormone-responsive sites in fat and liver cells (Brownsey et al., 2006). Exposure of these cells to adrenaline or glucagon leads to rapid phosphorylation and inactivation of ACC1 (Brownsey et al., 2006). The effects of the inhibitory hormones are mediated by cAMP, whereas the phosphorylation of ACC1 at Ser-79, Ser-1200 and Ser-1215 is carried out largely by AMPK (Hardie, 1992). Purified ACC2, like ACC1, has been demonstrated as a substrate for AMPK in heart and skeletal muscle (Kudo et al., 1995; Winder et al., 1997). The regulation of ACC by AMPK is summarised in Figure 1.5.

In skeletal muscle AMPK is activated by an increase in energy expenditure (exercise) and by fuel deprivation and hypoxia (Winder and Hardie, 1999; Ruderman et al., 1999). Activated AMPK phosphorylates ACC (to its inactive form) and phosphorylates and activates malonyl-CoA decarboxylase, resulting in a decrease in malonyl-CoA levels thus relieving the tonic inhibition of CPT1 and thereby increasing mitochondrial import and oxidation of long chain fatty acids (Park et al., 2002). In fat-fed rats a single injection of AICAR (Iglesias et al., 2002) or prior exercise (Oakes et al., 1997), both of which increase AMPK activity, has been demonstrated to cause sustained decreases in malonyl-CoA, and furthermore increase insulin stimulated glucose uptake by the muscle of control rats (Iglesias et al., 2002; Fisher et al., 2002).

A number of reports has demonstrated that a malonyl-CoA fuel sensing mechanism, similar to that in muscle, is in operation in the endothelial cell (Dagher et al., 1999; Dagher et al., 2001). In HUVEC deprived of glucose for several hours (i.e. no ATP derived from glycolysis), a 3 fold increase in AMPK activity and fatty acid oxidation occurred, and the concentration of malonyl-CoA was diminished by 80 % (Dagher et al., 2001). Notably ATP levels were not reduced in these cells indicating fatty acids can be utilised as their sole fuel. This is contradictory to the previously accepted belief that fatty acid oxidation

was of limited importance as a fuel source in the endothelial cell (Krutzfeldt et al., 1990). Indeed it has been reported that HUVEC oxidise fatty acids from both extracellular and intracellular sources, and when considered together can account for a substantial percentage of calculated ATP production in these cells (Dagher et al., 2001).

It has also been demonstrated that HUVEC incubated in hyperglycemic conditions (30mM glucose) exhibit an increase in apoptosis (72h) an effect preceded at 24h by an elevated concentration of malonyl-CoA, inhibition of fatty acid oxidation, increased diacylglycerol synthesis, an increase in caspase 3 activity and an impaired ability of insulin to activate PKB (Ido et al., 2002). Addition of the AMPK activator AICAR both increased AMPK activity several fold and completely prevented all the observed abnormalities at 24h (Ido et al., 2002). In addition, HUVEC incubated with excess fatty acid (0.2-0.4 mM palmitate) and normal glucose resulted in greater amount of apoptosis, again an effect prevented by AICAR treatment (Ido et al., 2002). The endotheliopathy associated with insulin resistance syndrome appears to largely result from excessive free fatty acid exposure (McCarty, 2005). Therefore, one could argue, pharmacological activation of endothelial AMPK has the potential to decrease the free fatty acid content of endothelial cells thus improving vascular health.

#### 1.3.1.6 eNOS

In endothelial cells, NO is synthesized from the amino acid L-arginine, through a five electron oxidation step involving the formation of the intermediate N<sup>G</sup>-hydroxy-L-arginine, by the constitutively expressed eNOS (Palmer et al., 1988; Zembowicz et al., 1991). The substrates for eNOS-mediated NO production are the amino acid arginine, molecular oxygen, NADPH, while the cofactors BH<sub>4</sub>, FAD and FMN are also required for NO production (Govers and Rabelink, 2001).

Structurally, eNOS consists of oxygenase and reductase domains with an interposed Ca<sup>2+</sup>/calmodulin binding domain (Figure 1.6). While Ca<sup>2+</sup>/calmodulin binding is required for eNOS activity eNOS regulation also involves multiple levels of post-translational regulation including numerous protein–protein interactions (Kone et al., 2003; Fulton et al., 2001) and tightly regulated multi-site phosphorylation involving numerous kinases and phosphatases (Figure 1.7), feedback inhibition from NO, the bioavailability of BH<sub>4</sub> and by association with caveolins (Andrew and Mayer, 1999; Stuehr, 1999).



As the main producer of endothelial NO, eNOS is of major importance to the normal function of the cardiovascular system. eNOS is activated by hormonal factors, including insulin (Sobrevia et al., 1996; Zeng and Quon, 1996), catecholamines and vasopressin, as well as by mechanical forces such as shear stress generated by blood flow through the blood vessels and increases in intracellular  $\text{Ca}^{2+}$  (Andrew and Mayer, 1999).

### 1.3.1.7 Post translational modification of eNOS

Reversible phosphorylation of eNOS, which occurs on Ser-114, -615, -633, and -1177 and Thr-495 (human amino acid sequence) in response to a range of stimuli, is important in regulating enzyme activity, which varies depending on the protein kinase and residue involved.

eNOS activity is increased by phosphorylation at both Ser-1177 and Ser-633 (Chen et al., 1999; Fulton et al., 1999; Bauer et al., 2003; Michell et al., 2002). In contrast, phosphorylation of Thr-495 in the  $\text{Ca}^{2+}$ /calmodulin binding domain is inhibitory (Chen et al., 1999; Matsubara et al., 2003). The effect of eNOS Ser-114 and Ser-615 phosphorylation remain controversial (Bauer et al., 2003; Michell et al., 2002).

The phosphorylation of Ser-1177 has been demonstrated as critical for eNOS activation responding to several stimuli, including shear stress, bradykinin, insulin, adiponectin, and statins (Chen et al., 2003; Harris et al., 2004; Montagnani et al., 2001; Boo et al., 2002b). Phosphorylation of eNOS at Ser-1177 increases eNOS activation mediated by  $\text{Ca}^{2+}$ /calmodulin binding (Chen et al., 1999; Bauer et al., 2003), and can also lead to activation of eNOS at resting levels of intracellular  $\text{Ca}^{2+}$  (Bauer et al., 2003; Montagnani et al., 2001).

As discussed in section 1.3.1 (and highlighted in figures 1.3 and 1.4) Ser-1177 of eNOS is a known target for AMPK (Chen et al., 1999). Based on the consensus sequence for PKB sites (RXRXXS/T) Ser-1177 is also a putative phosphorylation site. Indeed a study conducted by (Dimmeler et al., 1999) has confirmed that Ser-1177 is a key phosphorylation site of eNOS in intact cells that is subject to PKB-mediated phosphorylation with a distinct consequence for eNOS activity. As highlighted in figure 1.7 a number of other protein kinases, namely PKA (Michell et al., 2002; Boo et al., 2002a), CaMKII (Fleming et al., 2001a), and PKG (Butt et al., 2000), have also been implicated as potential mediators of eNOS Ser-1177 phosphorylation (Mount et al., 2007).

In addition to its effect on Ser-1177 PKB also phosphorylates eNOS on Ser-615 (Figure 1.7) (Michell et al., 2002; Harris et al., 2004). PKC mediates phosphorylation of Thr-495 resulting in a decrease in eNOS activity (Matsubara et al., 2003; Fleming et al., 2001a; Michell et al., 2001), an effect prevented by PKC inhibitors. Evidence suggests that PKA is the kinase responsible for phosphorylation of eNOS at Ser-633 (Figure 1.7) (Michell et al., 2002; Harris et al., 2004; Boo et al., 2002a).

The eNOS monomer is heavily post-translationally modified by myristoylation, palmitoylation, farnesylation, acetylation and, following activation, phosphorylation reviewed in (Stuehr, 1999). These modifications serve partly to target eNOS to various subcellular locations.

Acetylation, for example, is responsible for targeting eNOS to the plasma membrane (Shaul et al., 1996), where it is anchored to caveolae via the scaffolding proteins caveolin-1 (Garcia-Cardena et al., 1996) and -3 (Garcia-Cardena et al., 1997). The localisation of eNOS within caveolae renders the enzyme inactive with eNOS activity inhibited by caveolin-1 (Ju et al., 1997). The interaction between eNOS and the caveolin-1 scaffolding domain (amino acid residues 82-101) strongly reduces eNOS activity because caveolin-1 interferes with the binding of calmodulin to eNOS when cytosolic  $Ca^{2+}$  levels are low (Michel et al., 1997). In the presence of shear stress (Rizzo et al., 1998), or  $Ca^{2+}$  mobilising agents such as bradykinin (Prabhakar et al., 1998), acetylcholine or  $Ca^{2+}$  ionosphere (Feron et al., 1998) and estradiol (Kim et al., 1999),  $Ca^{2+}$  bound calmodulin associates with eNOS, whereas caveolin-1 is displaced. Another protein that has been demonstrated to interact and enhance the activation of eNOS is the 90 kDa heat shock protein (HSP-90) (Garcia-Cardena et al., 1998) which is rapidly recruited to the eNOS complex by agonists that stimulate production of NO such as VEGF, histamine and fluid shear stress. Not only does fluid shear stress activate eNOS, it causes a rise in the levels of eNOS transcripts, which is also achieved by exercise and hypoxia (Nathan and Xie, 1994). In addition, eNOS is regulated in endothelial cells by reversible and inhibitory interactions with G-protein coupled receptors and these interactions can be modulated by receptor phosphorylation (Marrero et al., 1999).

### 1.3.1.8 NO function

NO is a free radical (Mayer and Hemmens, 1997) with a half-life of only a few seconds *in vivo* (Moncada and Higgs, 1991; Griffith et al., 1984). However, since it is soluble in both aqueous and lipid media, it readily diffuses through the cytoplasm and plasma membranes.

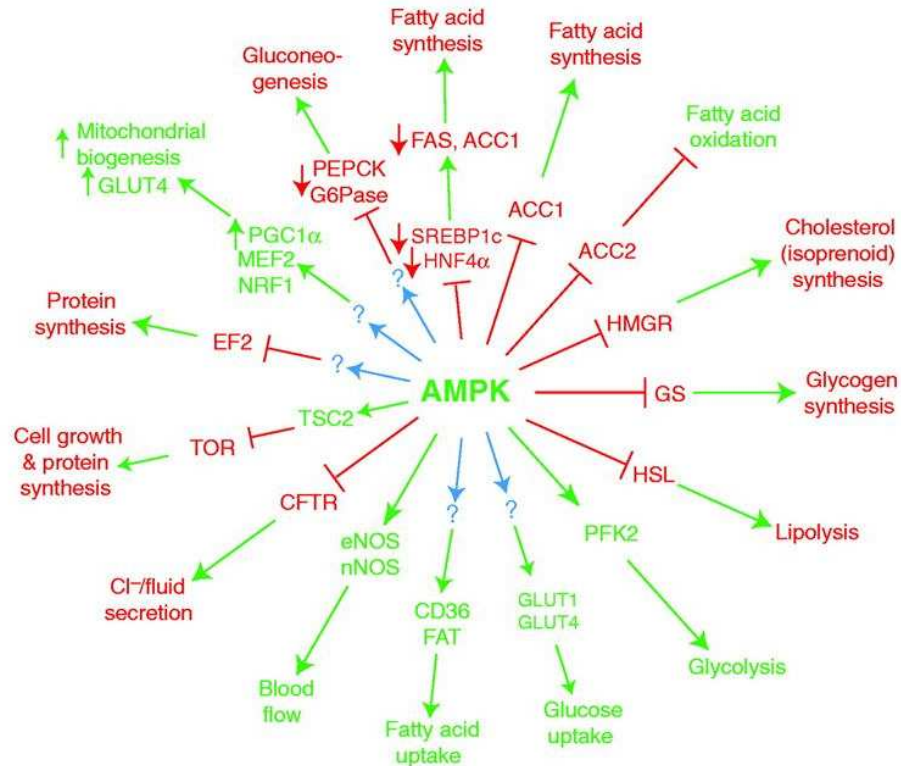
Once produced, NO has several targets, including the enzyme soluble guanylate cyclase (sGC), which catalyses the formation of cyclic guanosine monophosphate (cGMP), a secondary messenger that relays information to cGMP-responsive molecules. In the case of NO-mediated vasodilation, the cGMP-targeted molecules include two cGMP-dependent protein kinases, PKG I and PKG II (also known as cGK-I and -II) (Gewaltig and Kojda, 2002). PKG I mediates vasodilation by phosphorylating various molecules involved in smooth muscle relaxation, including inositol 1,4,5-trisphosphate receptor-associated G kinase (IRAG), myosin light chain (MLC) phosphatase, and phospholamban (Gewaltig and Kojda, 2002). In all these cases, phosphorylation ultimately results in decreased intracellular  $\text{Ca}^{2+}$  levels (Ammendola et al., 2001; Surks et al., 1999; Rivero-Vilches et al., 2003).

A further NO-mediated vasodilatory mechanism is the activation of  $\text{Ca}^{2+}$ -activated  $\text{K}^+$  ( $\text{BK}_{\text{Ca}}$ ) channels by NO through the cGMP-PKG I pathway. Activation of the  $\text{BK}_{\text{Ca}}$  channels can occur either through direct activation of the  $\text{BK}_{\text{Ca}}$  channels by NO (Bolotina et al., 1994) or by PKG I-dependent phosphorylation of the channel protein (Alioua et al., 1998) or of a protein phosphatase (Hall and Armstrong, 2000; Sausbier et al., 2000).  $\text{BK}_{\text{Ca}}$  channel activation increases the  $\text{K}^+$  efflux, thus causing membrane hyperpolarisation and vasodilation (Gewaltig and Kojda, 2002).

eNOS-derived NO is vitally important as a vasodilator: Disruption of the eNOS gene in mice results in mild hypertension (Gewaltig and Kojda, 2002; Huang et al., 1995); and increased blood pressure is also seen in humans when NO synthesis is inhibited pharmacologically (Stamler et al., 1994).

In addition to its vasodilatory effects, NO released from endothelial cells mediates numerous additional functions in the endothelium; inhibiting platelet aggregation, leukocyte adherence and vascular smooth muscle proliferation (Kubes et al., 1991; Gewaltig and Kojda, 2002). Platelet aggregation is prevented by lowering their intracellular  $\text{Ca}^{2+}$  concentration through activation of phospholamban and SERCA via the cGMP-PKG I pathway (Gewaltig and Kojda, 2002). Monocyte adhesion is dependent on the expression of attractant and attachment molecules, including VCAM-1, MCP-1 and cytokines, on the surface of vascular endothelial cells. As leukocyte adherence is an early event in the development of atherosclerosis, NO may protect against the onset of atherogenesis (Forstermann and Munzel, 2006).

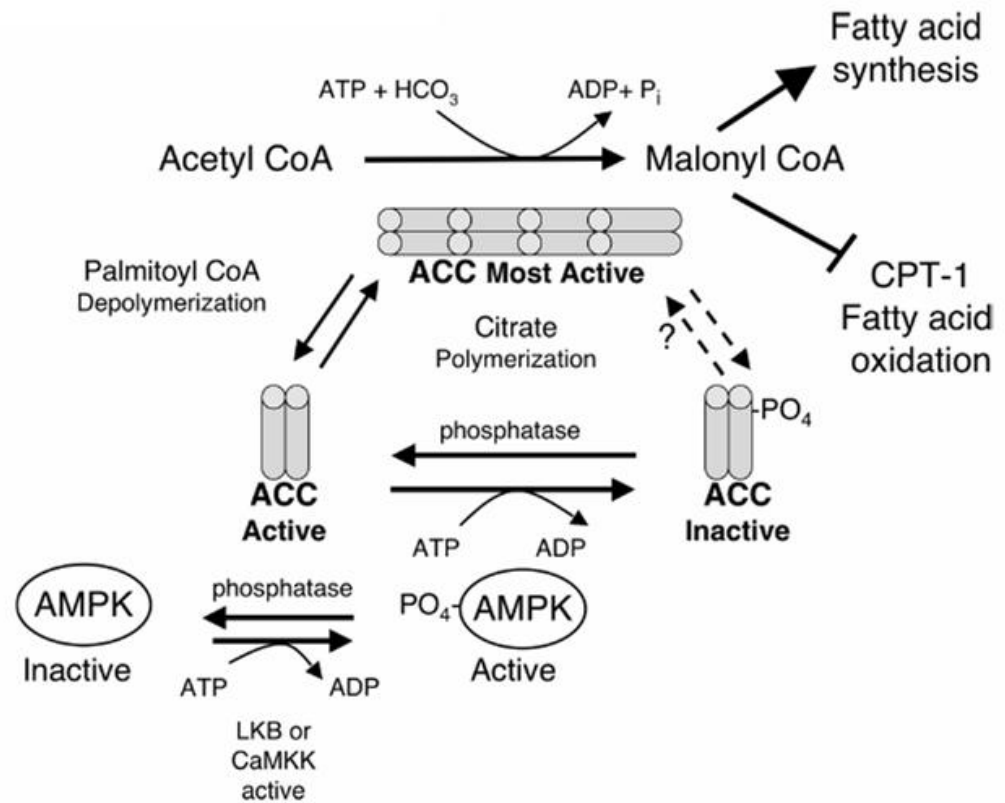
Smooth muscle cell proliferation is inhibited *in vitro* by NO (Garg and Hassid, 1989) through interference with O<sub>2</sub><sup>-</sup>-producing enzymes (e.g. NADPH oxidase) (Clancy et al., 1992). Proliferation may also be blocked by cGMP-dependent activation of PKA and cGMP-inhibition of cAMP phosphodiesterase (PDE III). Activated PKA is thought to inhibit the mitogenic molecule Raf-1, thus preventing smooth muscle cell proliferation (Gewaltig and Kojda, 2002). Unregulated vascular smooth muscle cell proliferation is proatherogenic in that it can lead to artery thickening, thus increasing the risks for hypertension, stroke and infarction (Newby et al., 1995; Newby and George, 1996).



**Figure 1-3 Targets for AMPK**

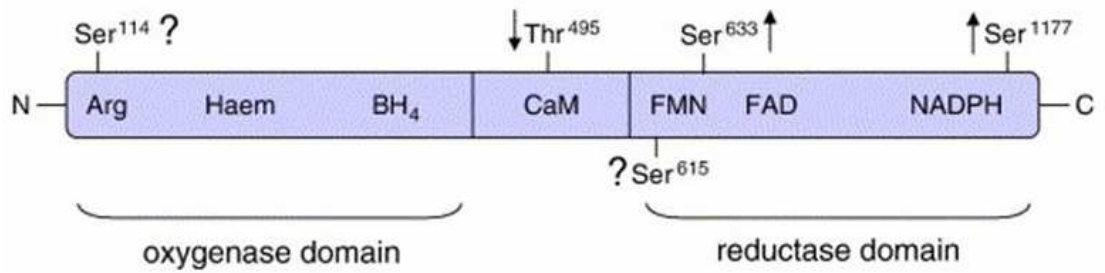
Target proteins and processes activated by AMPK activation are shown in green, and those inhibited by AMPK activation are shown in red. Where the effect is caused by a change in gene expression, an upward-pointing green arrow next to the protein indicates an increase, whereas a downward-pointing red arrow indicates a decrease in expression. Abbreviations: CD36/FAT, CD36/fatty acid translocase; CFTR, cystic fibrosis transmembrane regulator; nNOS, neuronal isoforms of nitric oxide synthase; G6Pase, glucose-6-phosphatase; GLUT1/4, glucose transporters; GS, glycogen synthase; HSL, hormone-sensitive lipase; MEF2, myocyte-specific enhancer factor-2; NRF1, nuclear respiratory factor-1; PEPCK, phosphoenolpyruvate carboxykinase; TOR, mammalian target of rapamycin. (Figure adapted from (Hardie, 2004)).





**Figure 1-5 ACC regulation by AMPK**

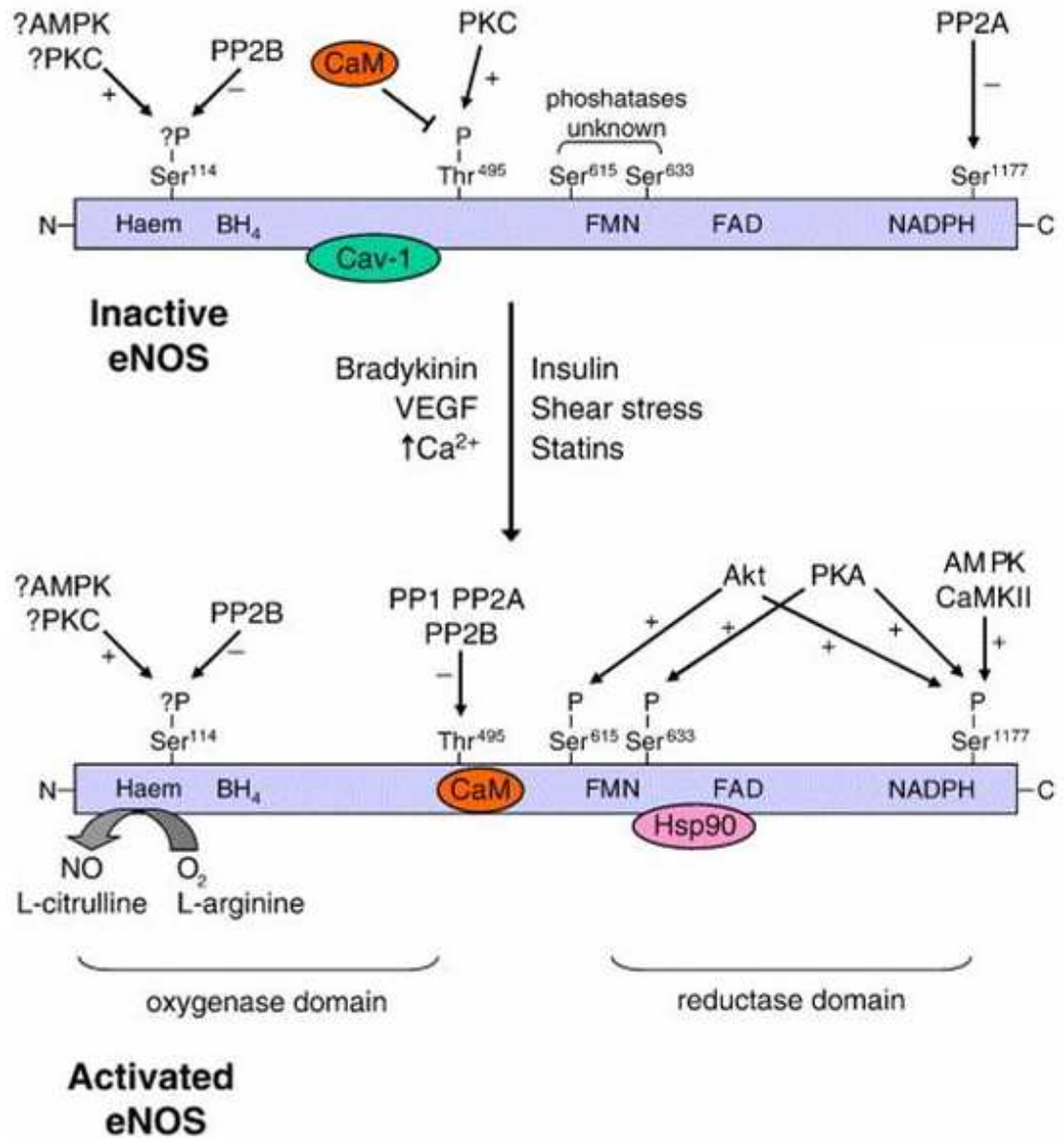
ACC activity is regulated both allosterically (via citrate-induced polymerisation and fatty acid-induced depolymerisation) and covalently (via AMPK and PKA-mediated phosphorylation). ACC activation results in malonyl CoA synthesis, the precursor for fatty acid synthesis in liver and fat and allosteric inhibitor of CPT1, the transferase regulating the transfer of fatty acid into the mitochondria for  $\beta$ -oxidation in skeletal and heart muscle. Not shown here is the regulation of ACC phosphorylation by PKA, which is essentially similar but independent of that of AMPK. (Figure adapted from (Steinberg et al., 2006)).



**Figure 1-6 eNOS phosphorylation sites**

Phosphorylation sites of endothelial nitric oxide synthase. Sites are numbered according to the human eNOS sequence. The arrows indicate the effect of phosphorylation on eNOS activity. Phosphorylation of Ser-633 and Ser-1177 increases eNOS activity, whereas phosphorylation of Thr-495 is inhibitory. The effects of phosphorylation of Ser114 and Ser615 remain controversial. Figure adapted from (Mount et al., 2007)





**Figure 1-7 The role of kinases and phosphatases on eNOS activation**

Activation of NO synthesis by eNOS involves multiple and coordinated phosphorylation events and protein-protein interactions. Important activators of eNOS include bradykinin, VEGF, shear stress and pharmacological stimuli such as statins. Numerous kinases and phosphatases contribute to the regulation of eNOS phosphorylation, although the specific kinases and phosphatases involved can vary depending on either the activating stimulus or the tissue bed. + indicates kinase-mediated phosphorylation. - indicates phosphatase-mediated dephosphorylation. PKB is referred to as Akt in this Figure. This Figure is adapted from (Mount et al., 2007)

## 1.4 Vascular effects of AMPK

AMPK activation is a possible target for the prevention and treatment of disorders associated with insulin resistance, including atherosclerotic vascular disease (Tomas et al., 2002). AMPK is important in the normal development of heart as mutations in AMPK lead to severe heart defects. Greater interest in AMPK as a potential cardiovascular target has arisen with recent evidence that AMPK has a cardioprotective role against myocardial injury and apoptosis in the ischemic heart (Russell, III et al., 2004). Furthermore, there is mounting evidence that AMPK may exert anti-atherosclerotic effects in the vasculature.

A number of reports indicate AMPK activation may benefit the cardiovascular system through various mechanisms. For example, vascular smooth muscle proliferation is a critical event in the development and progression of vascular diseases, including atherosclerosis. It has been reported that AMPK suppresses vascular smooth muscle cell proliferation via cell cycle regulation by p53 upregulation (Igata et al., 2005). It has also been shown that  $\alpha$  lipoic acid improves vascular dysfunction by normalizing lipid metabolism and activating AMPK in endothelial cells (Lee et al., 2005). Furthermore, it has also been demonstrated that activation of AMPK contributes to protection of the heart against hypoxic injury through attenuation of endoplasmic reticulum stress, and the attenuation of protein synthesis via eukaryotic elongation factor 2 inactivation may be the mechanism of cardioprotection by AMPK (Terai et al., 2005).

However, perhaps the importance of AMPK in cardiovascular functions is best demonstrated by recent studies demonstrating that widely used drugs, including statins, metformin and rosiglitazone, which activate AMPK, execute protective effects in the vasculature. The endothelium, the dysfunction of which is associated with the aetiology of disorders including type 2 diabetes, obesity, atherosclerosis and cardiovascular disease (Bonora, 2006), is a key tissue in which AMPK activation may promote improved cardiovascular health.

### 1.4.1 The function of the vascular endothelium

The endothelium, also known as the tunica intima, is located at the interface between the blood and vessel wall, anchored to the underlying basal lamina (Hurairah and Ferro, 2004). The endothelium is composed of flattened cells anchored together by adhesion junctions, including prominent tight junctions that prevent diffusion between cells. The endothelial

layer forms a barrier between the elements of the blood and the tissues by virtue of its direct contact with the circulating blood (Wheatcroft et al., 2003).

In the past the endothelium was seen as a semi-permeable monolayer of inert cells passively allowing passage of water and other small molecules across the vessel wall. However, it is now evident that this dynamic tissue performs many active functions, such as the secretion and modification of vasoactive substances and the contraction and relaxation of vascular smooth muscle (Hurairah and Ferro, 2004; Davies and Hagen, 1993). The intact structure and function of the endothelium is prerequisite for the maintenance of normal vascular function (Davies and Hagen, 1993).

Vascular tone is regulated by a number of endothelium-derived substances with vasodilator or vasoconstrictor function, acting on vascular smooth muscle cells (Ganz and Vita, 2003). The vasodilators include prostacyclin, endothelium derived hyperpolarising factor and NO, whilst the vasoconstrictors include endothelin-1 and thromboxane A<sub>2</sub> (Hurairah and Ferro, 2004; Michiels, 2003). NO released from endothelial cells is an important regulator of vascular homeostasis promoting vasodilatation and inhibiting platelet aggregation, leukocyte adherence and vascular smooth muscle proliferation (Garg and Hassid, 1989; Nabah et al., 2005; de Graaf et al., 1992). Reduced NO production or bioavailability is a characteristic of many cardiovascular disorders (Davignon and Ganz, 2004; Verma et al., 2003). Adrenergic vasoconstrictor tone is unopposed leading to increased vascular tone whilst reduced NO can also lead to increased platelet adhesion and aggregation, and therefore enhanced thrombogenesis. Altered NO bioavailability is a key feature of endothelial dysfunction (Forstermann and Munzel, 2006).

## **1.4.2 Endothelial dysfunction**

Endothelial dysfunction is characterised by an imbalance of the mediators that regulate cardiovascular homeostasis, evidenced by reduced vasodilator/increased vasoconstrictor secretion, increased superoxide production, decreased NO bioavailability and impaired endothelium-dependent vasodilation (Steinberg et al., 2000; Artwohl et al., 2004). Chronic dysfunction of the endothelium is implicated in the pathophysiology of several cardiovascular disorders, including atherosclerosis, hypertension, diabetic vasculopathy and heart failure (Harrison et al., 2003). Several factors including hyperglycemia and free fatty acids (which are induced by insulin resistance syndrome and diabetes) contribute to endothelial damage and dysfunction. Hyperglycemia and free fatty acids can result in a

number of pathophysiological changes at the molecular level, including inflammation (Libby, 2001a), endothelial cell apoptosis (Artwohl et al., 2004; Ido et al., 2002), increases in cell  $\text{Ca}^{2+}$ , ROS (Inoguchi et al., 2000) and NF- $\kappa$ B activation, (Pieper and Riaz, 1997) and decreases in eNOS activity and NO bioavailability, which contribute to or accelerate endothelial damage and dysfunction (Steinberg et al., 2000).

#### 1.4.2.1 AMPK as a therapeutic target in the dysfunctional endothelium

A number of reports have provided evidence that AMPK activation may be anti-atherogenic and function as a protector of endothelial function by alleviating many of these adverse effects associated with endothelial dysfunction and damage.

(i) Induction of the eNOS/NO pathway resulting in increased NO bioavailability;

Ser-1177 on eNOS is a substrate for AMPK (Figure 1.4) (Chen et al., 1999) shown to be phosphorylated in endothelial cells in response to a range of agonists including hypoxia, metformin, adiponectin and shear stress (Chen et al., 2003; Nagata et al., 2003; Zou et al., 2004; Nagata et al., 2003). Furthermore, it has been demonstrated that AICAR causes a time- and dose-dependant stimulation of AMPK activity, with concomitant increase in eNOS Ser-1177 phosphorylation and NO production in human aortic endothelial cells (Morrow et al., 2003). Disruption of the eNOS gene in mice results in mild hypertension (Huang et al., 1995); and increased blood pressure is also seen in humans when NO synthesis is inhibited pharmacologically (Stamler et al., 1994). In addition, infusion of the AMPK activator AICAR into rats has been shown to significantly reduce mean arterial pressure (Foley et al., 1989) and has also been reported to lower blood pressure in hypertensive, obese Zucker rats (Buhl et al., 2002). AMPK therefore provides an additional mechanism whereby local vascular tone may be controlled. Impairment of endothelium-dependent relaxation represents reduced eNOS-derived NO bioavailability and is present in atherosclerotic vessels in advance of any changes in vascular structure (Kawashima and Yokoyama, 2004).

(ii) Suppression of endothelial ROS production induced by deleterious stimuli, such as hyperglycemia or high free fatty acids;

ROS have been shown to cause insulin resistance in adipose and other tissues (Tirosh et al., 2001). Incubation of endothelial cells with excess glucose has been shown to increase  $\text{Ca}^{2+}$  concentration and ROS (Nishikawa et al., 2000; Inoguchi et al., 2000; Pieper and

Dondlinger, 1997), and NF- $\kappa$ B as assessed by increases in its translocation to the nucleus (Pieper and Dondlinger, 1997; Nishikawa et al., 2000). Fatty acids have been shown to have a similar effect on ROS production. Evidence has been provided suggesting AMPK activation may prevent these adverse effects. Hyperglycemic medium and fatty acid (0.4 mM palmitate) increased ROS, an effect inhibited by the AMPK activator AICAR (Ido et al., 2002).

(iii) Improving endothelial free fatty acid oxidation, leading to suppression of lipid accumulation

In cultured endothelial cells, AMPK activation using AICAR or glucose deprivation, stimulates ACC phosphorylation and fatty acid oxidation (Dagher et al., 2001). HUVEC incubated with excess fatty acid (0.2-0.4 mM palmitate) exhibit an increase in apoptosis, an effect prevented by AICAR treatment (Ido et al., 2002).

(iv) Anti-apoptotic and anti-inflammatory effects

Inflammation has been implicated in the early pathogenesis of endothelial cell damage (Ross, 1999; Libby, 2001b) while activation of the transcription factor NF- $\kappa$ B and an increase in circulating vascular adhesion molecules are thought to be early events in the development of atherosclerosis (Razani et al., 2008; Libby, 2001b). Both are prevalent in patients at risk from cardiovascular disease due to diabetes, obesity and metabolic syndrome, all of which have associated increased fatty acid concentrations (Grundy et al., 2004). Incubation of cultured endothelial cells with polyunsaturated fatty acids, such as linoleate, increases the activation of the inflammatory mediators NF- $\kappa$ B and AP1, as well as the expression of VCAM-1 (Hennig et al., 1996). It has been reported that AMPK activation inhibits fatty acid induced increases in NF- $\kappa$ B transactivation (Cacicedo et al., 2004) and cytokine-induced NF- $\kappa$ B activation in vascular endothelial cells (Hattori et al., 2008). In addition, in vascular endothelial cells AMPK activation inhibits TNF $\alpha$ -stimulated leukocyte adhesion by a rapid NO-dependent mechanism associated with reduced MCP-1 secretion (Ewart et al., 2008). As mentioned previously leukocyte adherence is an early event in the development of atherosclerosis. Furthermore, AMPK mediates the inhibitory effect of the high molecular weight form of adiponectin on endothelial cell apoptosis (Kobayashi et al., 2004).

## 1.5 VEGF

VEGF is a major regulator of endothelial cell function that stimulates differentiation, survival, migration, proliferation, vascular permeability and NO production in these cells (Cross et al., 2003). Manipulation of VEGF signalling is seen as a promising therapeutic target for a number of disorders yet its role in the endothelium is incompletely understood.

### 1.5.1 Identification of VEGF

Angiogenesis, a process involving the growth of new blood vessels from pre-existing vessels, and vasculogenesis, the spontaneous development of blood vessels, are processes predominantly regulated by a group of growth factors which act via their associated receptor tyrosine kinases (Ferrara, 2005a). The most prominent of these is the VEGF/VEGF receptor (VEGF-R) family. More than a century ago it was observed that tumour growth can be accompanied by increased vascularity (Ferrara, 2002a), and in 1939 the existence of a tumour-derived blood vessel growth-stimulating factor was postulated on the basis of the strong neovascular response induced by tumours transplanted in transparent chambers (Ide et al., 1939). Compelling evidence for the presence of diffusible angiogenic factors was reported in 1968 when it was demonstrated that transplanted melanoma or choriocarcinoma cells promoted blood vessel proliferation even when a Millipore filter was interposed between the tumour and the host (Greenblatt and Shubi, 1968; Ehrmann and Knoth, 1968). In 1983, a factor was purified from the conditioned medium of a guinea-pig tumour cell line that was shown to increase dye extravasation in the skin (Senger et al., 1983). The authors proposed that this factor, termed “tumour vascular permeability factor (VPF), could be a mediator of the high permeability of tumour blood vessels. In 1989 an endothelial cell-specific mitogen was isolated from medium conditioned by bovine pituitary follicular cells, which was termed “vascular endothelial cell growth factor” (Ferrara and Henzel, 1989). As other endothelial cell mitogens, such as FGF, do not increase vascular permeability it was of great surprise when subsequent work identified that VPF and VEGF were in fact the same molecule (Leung et al., 1989; Keck et al., 1989). The characterisation of VEGF as a potent, diffusible and endothelial cell-specific factor led to the hypothesis that this molecule might play a role in the regulation of physiological and pathological growth of blood vessels (Ferrara and Henzel, 1989; Leung et al., 1989; Ferrara et al., 1991).

## 1.5.2 Activities of VEGF

### 1.5.2.1 Mitogenic effects

Angiogenesis and vasculogenesis are largely regulated by growth factors and their associated receptor tyrosine kinases. Foremost among these is the VEGF family and their associated VEGF receptors. The loss of a single VEGF allele is lethal in the mouse embryo between days 11 and 12 (Carmeliet et al., 1996; Ferrara et al., 1996), with VEGF<sup>+/-</sup> embryos exhibiting significant defects in the vasculature of several organs. Homozygous loss of the VEGF-R1 or VEGF-R2 gene results in embryonic lethality between days 8.5 and 9.5 (Fong et al., 1995; Shalaby et al., 1995). In VEGF-R1 deficient mice this is due to the obstruction of vessels by an overgrowth of endothelial cells (Fong et al., 1995), while loss of the VEGF-R2 gene results of an early defect in the development of haematopoietic and endothelial cells (Shalaby et al., 1995).

In adult endothelial cells, derived from arteries, veins and lymphatics, VEGF has been demonstrated as a potent mitogen both *in vitro*, (reviewed in (Ferrara and Davis-Smyth, 1997), and in a number of *in vivo* models including the chick chorioallantoic membrane (Leung et al., 1989; Plouet et al., 1989), the rabbit cornea (Phillips et al., 1994) and the Matrigel plug in mice (Mesri et al., 1995). VEGF delivery has also been demonstrated to induce lymphangiogenesis in mice (Nagy et al., 2002).

### 1.5.2.2 Pathophysiological angiogenesis

VEGF is expressed in most tumours and is closely linked with their progression (Ferrara, 2005b). Tumour-associated stroma, in addition to tumour cells, is an important source of VEGF (Fukumura et al., 1998). VEGF mRNA is abundantly expressed in hypoxic tumour cells adjacent to necrotic areas (Ferrara and Davis-Smyth, 1997), facilitating the growth of new blood vessels into tumours which would otherwise become necrotic and apoptotic in the absence of an adequate vasculature (Holmgren et al., 1995). It appears that VEGF-R1 signalling plays a more prominent angiogenic role under pathological, rather than physiological, conditions (Hiratsuka et al., 2001; Carmeliet et al., 2001). Indeed VEGF-R1 is involved in tumour metastasis, being linked to the induction of MMP-9 in lung endothelial cells and to the facilitation of lung-specific metastasis (Hiratsuka et al., 2002). VEGF has also been demonstrated to impair the endothelial barrier, which facilitates tumour cell extravasation and metastasis (Weis et al., 2004a); and furthermore induces the

disruption of hepatocellular tight junctions, which may promote tumour invasion (Schmitt et al., 2004). Inhibition of VEGF-R2 has been shown to stabilize the endothelial barrier function and suppresses tumour cell extravasation *in vivo* (Weis et al., 2004a). Capturing of VEGF or blocking of its signalling receptor VEGF-R2 by a VEGF-R tyrosine kinase inhibitor, antisense oligonucleotides, vaccination or neutralizing antibodies has been demonstrated to reduce tumour angiogenesis and growth in preclinical studies (Ferrara et al., 2004). The addition of a humanized anti-VEGF monoclonal antibody (bevacizumab) to fluorouracil-based combination chemotherapy results in a significant improvement in survival among patients with metastatic colorectal cancer (Hurwitz et al., 2004).

### 1.5.2.3 Vascular permeability

VEGF was originally known as VPF based on its ability to induce increased vascular permeability of microvessels to circulating macromolecules (Senger et al., 1983; Dvorak et al., 1995; Bates et al., 2002b). Increased vascular permeability is often observed in areas of pathological angiogenesis in solid tumours, wounds and chronic inflammation (Gratton et al., 2003; Potgens et al., 1995). VEGF has also been shown to induce an increase in hydraulic conductivity of isolated microvessels; an effect mediated by increased  $\text{Ca}^{2+}$  influx (Bates and Curry, 1997). Consistent with this role, VEGF induces endothelial fenestrations in some vascular beds (Roberts and Palade, 1995) and cultured adrenal endothelial cells (Esser et al., 1998), and furthermore promotes the extravasation of ferritin by way of the vesiculo-vacuolar organelle (Bates and Harper, 2002) and disorganization of endothelial junctional proteins such as VE-cadherin and occludin (Kevil et al., 1998).

### 1.5.2.4 Survival

VEGF is a survival factor for endothelial cells *in vitro* and *in vivo* (Alon et al., 1995; Gerber et al., 1998b; Benjamin et al., 1999; Gerber et al., 1998a; Yuan et al., 1996) and has been demonstrated to prevent against serum-starvation induced apoptosis of endothelial cells by a PI3K/PKB-dependent mechanism (Gerber et al., 1998b; Fujio and Walsh, 1999). In addition, VEGF induces the expression of the anti-apoptotic proteins Bcl-2 and A1 (Gerber et al., 1998a). Inhibition of VEGF has been demonstrated to increase apoptosis of endothelial cells isolated from liver in neonatal but not adult mice (Gerber et al., 1999), and VEGF dependence of endothelial cells has been demonstrated in newly formed but not of established vessels within tumours (Benjamin et al., 1999; Yuan et al., 1996). It is believed that coverage by pericytes is an important factor in the loss of VEGF dependence with development (Benjamin et al., 1999).



### 1.5.2.5 Haematopoiesis and blood cell function

VEGF has also been observed to have effects on bone marrow-derived cells. VEGF promotes monocyte chemotaxis (Clauss et al., 1990), and has been demonstrated to have haematopoietic effects inducing colony formation by mature subsets of granulocyte-macrophage progenitor cells (Broxmeyer et al., 1995). Delivery of VEGF to adult mice inhibits dendritic cell development (Gabrilovich et al., 1996), increases production of B cells and the generation of immature myeloid cells (Hattori et al., 2001). VEGF has been reported to regulate haematopoietic stem cell survival during haematopoietic repopulation in lethally irradiated hosts (Gerber et al., 2002). Interestingly in *Drosophila*, which are devoid of a vascular system, a VEGF-dependent pathway is important in the regulation of blood cell migration (Cho et al., 2002) and proliferation (Munier et al., 2002) indicating an ancestral conserved role for VEGF-regulated blood cell function (Evans et al., 2003).

### 1.5.2.6 Vasodilatation

VEGF induces vasodilatation *in vitro* in a dose-dependent fashion (Ku et al., 1993; Yang et al., 1996), and produces transient tachycardia, hypotension and a decrease in cardiac output when injected intravenously in rats (Yang et al., 1996). These effects appear to result from reduction in venous return mediated primarily by endothelial-derived NO (Yang et al., 1996). Indeed, hypotension was a dose-limiting side effect in human trials where VEGF was systemically administered (Henry et al., 2003). Conversely, administration of anti-VEGF monoclonal antibodies to cancer patients has been shown to cause elevated blood pressure (Yang et al., 2003).

### 1.5.2.7 NO

It should be noted that in addition to vasodilation NO underlies a number of VEGF-mediated effects. For example it is clear that NO plays a critical role in VEGF-induced vascular permeability and angiogenesis (Parenti et al., 1998; Ziche et al., 1997; Morbidelli et al., 1996). Angiogenesis, vessel diameter, blood flow rate and vascular permeability are proportional to NO levels and are impaired in eNOS<sup>-/-</sup> mice (Fukumura et al., 2001).

## 1.5.3 VEGF Isoforms

The VEGF gene family consists of VEGF-A (referred to as VEGF throughout this study), PlGF, VEGF-B, VEGF-C and VEGF-D. A number of VEGF-related proteins have also been discovered that are encoded by viruses (VEGF-E) or in the venom of some snakes

(svVEGFs) (Takahashi and Shibuya, 2005). Each VEGF isoforms exhibits variable selectivity for three related receptor tyrosine kinases (VEGF-R1, VEGF-R2 and VEGF-R3) (summarised in Figure 1.8).

### 1.5.3.1 VEGF

VEGF has significant homology to PDGF, and all the eight cysteines found in the A and B chains of PDGF are conserved in VEGF (Leung et al., 1989; Keck et al., 1989). The human VEGF gene is organized in eight exons, separated by seven introns (Houck et al., 1991; Tischer et al., 1991) and is localized in chromosome 6p21.3 (Vincenti et al., 1996). Alternative exon splicing results in the generation of four different isoforms, having 121, 165, 189, and 206 amino acids, respectively, after signal sequence cleavage (VEGF<sub>121</sub>, VEGF<sub>165</sub>, VEGF<sub>189</sub>, VEGF<sub>206</sub>) (Houck et al., 1991; Tischer et al., 1991). VEGF<sub>165</sub>, the predominant isoform, lacks the residues encoded by exon 6, whereas VEGF<sub>121</sub> lacks the residues encoded by exons 6 and 7. Less frequent splice variants have been also reported, including VEGF<sub>145</sub> (Poltorak et al., 1997), VEGF<sub>183</sub> (Jingjing et al., 1999), VEGF<sub>162</sub> (Lange et al., 2003), and VEGF<sub>165b</sub>, a variant reported to have paradoxically an inhibitory effect on VEGF-induced mitogenesis (Bates et al., 2002a). VEGF<sub>165b</sub> has a 3'-splicing structure that predicts a novel COOH-terminal peptide sequence. Usually, the COOH terminus of VEGF consists of six amino acids encoded by the first 18 nucleotides of exon 8. In VEGF<sub>165b</sub>, that is replaced by six amino acids coded for by an 18-nucleotide open-reading frame formed from a more distal splice site selection in exon 8. Native VEGF is a heparin binding homodimeric glycoprotein of 45 kDa (Ferrara and Henzel, 1989). Such properties closely correspond to those of VEGF<sub>165</sub>, the predominant VEGF isoform which potently stimulates angiogenesis (Houck et al., 1992) (and is the VEGF subtype used in the present study unless stated otherwise).

The amino acids encoded by exons 1-5 are conserved in all isoforms but alternative splicing can occur in exons 6-7 which encodes 2 heparin binding domains that influence receptor binding and solubility. The isoforms that encode exon 6 are highly basic and bind to heparin with high affinity and thus are tightly bound to the cell surface (VEGF<sub>145</sub>, VEGF<sub>189</sub>, and VEGF<sub>206</sub>). VEGF<sub>165</sub>, which lacks exon 6 has intermediate properties, in that it is secreted yet a significant fraction remains bound to the cell surface and extracellular matrix (Park et al., 1993). VEGF<sub>121</sub>, which lacks both exon 6 and 7, is an acidic polypeptide that fails to bind heparin and is freely released from the cell (Houck et al., 1992). The ECM-bound isoforms may be released into a diffusible form by heparin or heparinase which displaces them from binding to heparin like moieties or by plasmin

cleavage at the COOH terminus which generates a bioactive fragment 110 amino acids long (Houck et al., 1992). This proteolytic mechanism is important in regulating the local activity and bioavailability of VEGF. The loss of the heparin-binding domain results in the mitogenic activity of VEGF.

It seems the intermediate properties of VEGF<sub>165</sub> enable optimal bioavailability and biological potency. An important feature of VEGF is its increased expression during conditions of hypoxia which is regulated by a hypoxia-responsive element in its promoter (discussed in detail in section 1.3.4.1). Thus, VEGF is crucial for vascular development as well as physiological and pathophysiological angiogenesis. VEGF is particularly important because loss of even a single VEGF allele results in embryonic lethality at days 11 to 12 (Ferrara et al., 1996; Carmeliet et al., 1996).

### 1.5.3.2 VEGF-B

VEGF-B is a highly basic heparin binding growth factor which is structurally similar to VEGF. Although widely expressed VEGF-B is particularly abundant in tissues such as heart, skeletal muscle and pancreas and may act in paracrine fashion to regulate endothelial cell function (Olofsson et al., 1996). Human VEGF-B has two isoforms generated by alternative splicing; VEGF-B<sub>167</sub> and VEGF-B<sub>186</sub>. The VEGF-B isoforms bind and activate VEGF-R1 and can also bind to NRP-1 (discussed in detail in section 1.3.4.5) (Olofsson et al., 1999). Whilst VEGF-B<sup>-/-</sup> mice are viable and fertile one study has reported that these animals have smaller hearts, dysfunctional coronary arteries and an impaired recovery from experimentally induced myocardial ischemia (Bellomo et al., 2000). However, another group has demonstrated that these mice only display a subtle cardiac phenotype, and have suggested that VEGF-B is not required for proper development of the cardiovascular system either during development or angiogenesis in adults (Aase et al., 2001). Further work using VEGF-B<sup>-/-</sup> mice has demonstrated a role for VEGF-B in pathological vascular remodeling in inflammatory arthritis (Mould et al., 2003) and protection of the brain from ischemic injury (Sun et al., 2004).

### 1.5.3.3 VEGF-C and VEGF-D

VEGF-C is closely related to VEGF-D by virtue of the presence of N- and C-terminal extensions that are not found in other VEGF family members (Lohela et al., 2003). Both VEGF-C and VEGF-D bind and activate VEGF-R3 (a member of the VEGF-R family that does not bind VEGF), as well as VEGF-R2, and are mitogenic for cultured endothelial

cells. VEGF-C also binds to NRP-2 (Lohela et al., 2003). Proteolytic cleavage, which is at least partly regulated by the serine protease plasmin (McColl et al., 2003), is required to produce the fully processed form of VEGF-C and VEGF-D, which are initially produced as preproteins (Lohela et al., 2003). It has been demonstrated that VEGF-C (VEGF-C<sup>-/-</sup>) is required for the initial steps in lymphatic development and both VEGF-C alleles are required for normal lymphatic development (Karkkainen et al., 2004). Overexpression of VEGF-C in the epidermis of transgenic mice results in the development of a hyperplastic lymphatic vessel network (Jeltsch et al., 1997). VEGF-D has been shown to induce the formation of lymphatics within tumours and promote the metastasis of tumour cells (Stacker et al., 2001).

#### 1.5.3.4 PIGF

Although initially characterized in the placenta (Maglione et al., 1991), where it is highly expressed at all stages of gestation, PIGF, a VEGF-R1 specific ligand (Sawano et al., 1996; Park et al., 1994), is expressed in a wide variety of cells, tissues, and organs including heart, lung, thyroid gland and skeletal muscle (Persico et al., 1999). The crystal structure of human PIGF has shown that this protein is structurally similar to VEGF (Iyer et al., 2001). However, unlike VEGF, N-glycosylation in PIGF plays an important role in VEGF-R1 binding (Errico et al., 2004). PIGF deficiency (PIGF<sup>-/-</sup>) does not affect embryonic angiogenesis in mice, however, loss of PIGF impairs angiogenesis, plasma extravasation and collateral growth during ischemia, inflammation, wound healing and cancer (Carmeliet et al., 2001).

#### 1.5.3.5 VEGF-E

VEGF-E, a viral VEGF homologue encoded by an Orf virus, which affects sheep, goats and occasionally humans, is structurally similar to VEGF and binds only to VEGF-R2 (Lyttle et al., 1994). The lesions induced in sheep and humans after infection with the virus show extensive dermal vascular endothelial proliferation and dilatation, and it is likely that this is a direct effect of the expression of the VEGF-like gene. VEGF-E seems to be as potent as VEGF at stimulating endothelial cell proliferation despite lacking a heparin-binding basic domain (Takahashi and Shibuya, 2005).

#### 1.5.3.6 svVEGFs

A group of proteins structurally related to VEGF have been identified in svVEGFs, including svVEGF from *Bothrops insularis* (Junqueira, I et al., 2001) and TfsvVEGF

(*Trimeresurus flavoviridis* svVEGF) (Takahashi et al., 2004) from pit vipers, in addition to hypotensive factor (Komori et al., 1999), increasing capillary permeability protein (Gasmi et al., 2002) and vavmin (Yamazaki et al., 2003) from vipers. Vavmin binds VEGF-R2 with high affinity (Yamazaki et al., 2003), whereas TfsvVEGF binds VEGF-R1 with high affinity resulting in increased vascular permeability but a weak stimulation of endothelial cell proliferation (Takahashi et al., 2004). Both vavmin and TfsvVEGF are unable to bind VEGF-R3 or NRP-1.

## 1.5.4 Regulation of VEGF production

### 1.5.4.1 Oxygen tension

Hypoxia is a major stimulator of VEGF expression (Shweiki et al., 1992). Hypoxia-induced transcription of VEGF mRNA is largely mediated by the binding of HIF-1 to a HIF-1 binding site located in the VEGF promoter (Levy et al., 1995; Liu et al., 1995). In addition to the induction of transcription, hypoxia promotes the stabilization of VEGF mRNA by proteins that bind to sequences located in the 3' untranslated region (Stein et al., 1995; Stein et al., 1995; Damert et al., 1997; Claffey et al., 1998; Levy et al., 1998) and the 5' untranslated region of VEGF mRNA (Stein et al., 1998; Akiri et al., 1998). Furthermore, VEGF expression can be regulated at the translational level. The 5' untranslated region of VEGF mRNA contains two functional internal ribosome entry sites that maintain efficient cap-independent translation and ensure efficient production of VEGF, even under unfavourable stress conditions such as hypoxia (Xie et al., 2004). Under normal oxygenation conditions, HIF-1 is scarcely detectable because it is targeted for rapid destruction by an E3 ubiquitin ligase containing vHL. However, under hypoxic conditions HIF-1 protein accumulates thus increasing the transcription of VEGF (Gerald et al., 2004). Interestingly the production of other VEGF family members such as VEGF-B, VEGF-C, and PlGF does not seem to be potentiated by hypoxia even though some of these factors such as VEGF-C are strong angiogenic factors (Jeltsch et al., 1997; Enholm et al., 1997).

### 1.5.4.2 Tumour suppressors

Inactivation of tumour suppressors, such as vHL and p53, is an additional mechanism that leads to overexpression of VEGF in tumour cells. Wild-type vHL inhibits the production of several hypoxia-regulated proteins including VEGF via transcriptional and post transcriptional mechanisms (Mukhopadhyay et al., 1997; Iliopoulos et al., 1996). At the

post transcriptional level, vHL inhibits the activity of PKC  $\zeta$  and  $\delta$  (Pal et al., 1997). Mutation of the vHL gene in tumour cells may be a mechanism for increased angiogenesis in these cells (Maher and Kaelin, Jr., 1997; Siemeister et al., 1996; Stratmann et al., 1997). The loss of the wild-type p53 is associated with increased angiogenesis in developing tumours (Van Meir et al., 1994). Wild-type p53 has been reported as an inhibitor of VEGF production (Mukhopadhyay et al., 1995), and mutated p53 has been demonstrated to enhance VEGF expression (Kieser et al., 1994). However, Agani and coworkers have reported findings which indicate wild-type p53 may not function as an inhibitor of VEGF expression (Kieser et al., 1994).

#### **1.5.4.3 Growth factors, cytokines, oncogenes and extracellular molecules**

Cytokines, growth factors, and gonadotropins that do not stimulate angiogenesis directly can modulate angiogenesis (positively or negatively) via the modulation of VEGF expression. Factors that can potentiate VEGF production include FGF-4 (Deroanne et al., 1997), PDGF (Finkenzeller et al., 1997), TNF $\alpha$  (Ryuto et al., 1996), TGF- $\beta$  (Pertovaara et al., 1994), KGF (Frank et al., 1995), IGF-1 (Goad et al., 1996), IL-1 $\beta$  (Li et al., 1995), and IL-6 (Cohen et al., 1996). Cytokines such as IL-10 and IL-13 can inhibit the release of VEGF (Matsumoto et al., 1997). The observation that inflammatory cytokines such as IL-1 $\alpha$  and IL-6 induce expression of VEGF in several cell types is in agreement with the hypothesis that VEGF may be a mediator of angiogenesis and permeability in inflammatory disorders (Neufeld et al., 1999). Another small molecule that up-regulates VEGF expression is NO. The production of NO is in turn up-regulated by VEGF, indicating that a positive feedback loop exists between these two factors (Hood et al., 1998; Dembinska-Kiec et al., 1997). Specific transforming events also result in induction of VEGF gene expression. Oncogenic mutations or amplification of Ras lead to VEGF up-regulation (Grugel et al., 1995; Okada et al., 1998). These studies indicate that mutant Ras-dependent VEGF expression is necessary, albeit insufficient, for progressive tumour growth *in vivo*.

#### **1.5.5 The VEGF receptors**

Initially, VEGF binding sites were identified on the cell surface of vascular endothelial cells *in vitro* and *in vivo*. Subsequently, it became apparent that receptors for VEGF also occur on bone marrow-derived cells (Ferrara and Davis-Smyth, 1997). VEGFs bind with different affinities to three related receptor tyrosine kinases, VEGF-R1 (also known as fms-like tyrosine kinase), VEGF-R2 (also known as fetal liver kinase 1-murine

homologue/kinase inset domain containing receptor-human homologue) and VEGF-R3 (also known as flt-4). Whereas VEGF-R1 and VEGF-R2 are primarily restricted to vascular endothelial cells VEGF-R3 is predominantly found in lymphatic endothelium. Each of these VEGF receptors have seven immunoglobulin like domains in the extracellular domain, a single transmembrane region and a consensus tyrosine kinase sequence interrupted by a kinase-insert domain (Shibuya et al., 1990; Terman et al., 1991). In addition to these RTKs, VEGF interacts with a family of co-receptors, the neuropilins. Binding of VEGF to receptor tyrosine kinases leads to receptor dimerisation, activation and subsequent autophosphorylation on certain tyrosine residues, which in turn triggers intracellular signalling cascades mediated by several effectors.

The importance of the VEGF-R family is highlighted by a number of genetic studies. VEGF-R2<sup>-/-</sup> mice die at embryonic days 8.5 to 9.5 due to a defect in the development of haematopoietic and endothelial cells resulting in impaired vasculogenesis (Shalaby et al., 1995). VEGF-R1<sup>-/-</sup> mice die due to an overgrowth of endothelial cells and disorganization of blood vessels. However the observation that there is normal vascular development in mice lacking the tyrosine kinase domain of VEGF-R1 (Hiratsuka et al., 1998) indicates VEGF-R2 is the major positive signal transducer, whereas VEGF-R1 has a negative regulatory role in angiogenesis early in embryogenesis. NRP-1 is important not only for neuronal development, but also for vascular formation (Kawasaki et al., 1999). NRP-1<sup>-/-</sup> mice suffer from severe defects in the cardiovascular system in addition to a disorganized neural development, resulting in the death of homozygous embryos by embryonic day 14 (Kawasaki et al., 1999). Studies conducted using NRP-2<sup>-/-</sup> mice indicate NRP-2 is selectively required for the formation of small lymphatic vessels and capillaries during development whereas arteries, veins and larger collecting lymphatic vessels develop normally (Yuan et al., 2002).

#### 1.5.5.1 VEGF-R1 signalling

VEGF-R1, originally cloned from a placental cDNA library (Shibuya et al., 1990), is a 180 kDa transmembrane protein that binds VEGF, PlGF and VEGF-B (Park et al., 1994). In addition to endothelial cells VEGF-R1 is also expressed on monocytes, osteoblasts, macrophages, pericytes, haematopoietic stem cells, vascular smooth muscle cells and colorectal tumour cells (Barleon et al., 1996; Zachary and Glicki, 2001; Ishida et al., 2001; Fan et al., 2005). The second Ig domain of VEGF-R1 is the major binding site for VEGF and PlGF (Ferrara and Davis-Smyth, 1997; Wiesmann et al., 1997; Christinger et al.,

2004). VEGF-R1 binds VEGF with at least 10-fold higher affinity than VEGF-R2 ( $K_d=10\text{-}30$  pM) (Ferrara and Davis-Smyth, 1997). However, the effects of VEGF-R2 activation on endothelial cells, such as those on cell survival and proliferation, can be induced only weakly or slightly by treatment with VEGF-R1-specific ligands. VEGF-R1 is a negative regulator of angiogenesis during early development, but seems to play a more prominent regulatory role in adult tissues. Under some circumstances, VEGF-R1 may transmit a pro-survival signal in endothelial cells, possibly mediated by induction of the anti-apoptotic gene survivin (Adini et al., 2002). It has also been demonstrated that vascular permeability, which is largely regulated by VEGF-R2 (discussed in detail in section 1.3.5.2.4), is enhanced by activation of VEGF-R1 (Takahashi et al., 2004). VEGF-R1-blocking antibodies have been demonstrated to prevent the migration, but not proliferation, of HUVEC in response to VEGF (Kanno et al., 2000). In addition, VEGF-R1-mediated signalling appears to preferentially modulate the reorganization of actin via p38 MAPK, whereas VEGF-R2 contributes to the re-organization of the cytoskeleton by phosphorylating FAK and paxillin, suggesting a different contribution of the two receptors to the chemotactic response. Monocyte migration (Barleon et al., 1996), recruitment of endothelial cell progenitors (Lyden et al., 2001), haematopoietic stem cell survival (Gerber et al., 2002), and release of growth factors from liver endothelial cells (LeCouter et al., 2003) have also been shown to be mediated by VEGF-R1.

VEGF-R1 signalling is of particular interest in relation to a number of pathological disorders (Hiratsuka et al., 2001; Carmeliet et al., 2001). VEGF-R1-dependent induction of MMP-9 expression in premetastatic lung endothelial cells and macrophages has been reported to promote lung metastasis (Hiratsuka et al., 2002). Moreover, it has been shown that VEGF-R1 activates ERK1/2 and stress-activated protein kinase/c-Jun NH<sub>2</sub>-terminal kinase (Fan et al., 2005) and Src family kinases (Lesslie et al., 2006) to mediate growth and migration of human colorectal carcinoma cells. Furthermore, a recent study has shown that activation of VEGF-R1 in breast cancer cells supports their growth and survival (Wu et al., 2006). An alternatively spliced form of VEGF-R1 that encodes a soluble truncated form of the receptor (sVEGF-R1), containing only the first six Ig domains, has been cloned from a HUVEC cDNA library (Ferrara and Davis-Smyth, 1997). sVEGF-R1 sequesters VEGF from signalling receptors and forms non-signalling heterodimers with VEGF-R2 thus inhibiting VEGF activity (Kendall et al., 1996). Elevated plasma levels of sVEGF-R1 have been reported in individuals with cancer, ischemia and pre-eclampsia (Toi et al., 2002; Scheufler et al., 2003; Levine et al., 2004). Increased circulating levels of sVEGF-



R1 in patients with pre-eclampsia are associated with decreased circulating levels of free VEGF and PlGF, resulting in general endothelial dysfunction (Maynard et al., 2003).

### 1.5.5.2 VEGF-R2 signalling

VEGF-R2 is a 230 kDa glycoprotein that binds VEGF (with high affinity  $K_d = 75-125$  pM) (Terman et al., 1992), VEGF-C, VEGF-D and VEGF-E (Takahashi and Shibuya, 2005). The binding site for VEGF has been mapped to the second and third Ig domains (Fuh et al., 1998). It is generally accepted that VEGF-R2 is the primary mediator of VEGF signalling (Gille et al., 2001). In addition to endothelial cells, haematopoietic stem cells, megakaryocytes, retinal progenitor cells, and vascular smooth muscle cells express VEGF-R2. VEGF-R2, along with VEGF-R1, has also been identified in tumour cell lines, non-small cell lung carcinomas, breast, neuroblastoma and gastric cancer cells (Neufeld et al., 1999; Ishida et al., 2001; Price et al., 2001; Meister et al., 1999; Tian et al., 2001). The key role of VEGF-R2 in developmental angiogenesis and haematopoiesis is evidenced by a lack of vasculogenesis and failure to develop blood islands and organized blood vessels in VEGF-R2-null mice, resulting in death in utero between days 8.5 and 9.5 (Shalaby et al., 1995). VEGF-R2 is accepted as the major mediator of the mitogenic, angiogenic and permeability-enhancing effects of VEGF. Tyrosine autophosphorylation sites for human VEGF-R2 bound to VEGF are Y951 and Y996 in the kinase insert domain (Matsumoto et al., 2005), Y1054 and Y1059 in the kinase domain (Kendall et al., 1999; Dougher and Terman, 1999), and Y1175 and Y1214 in the C-terminal tail of VEGF-R2 (Takahashi et al., 2001). In addition Y1305, Y1309 and Y1319 in the C-terminal tail are thought to be minor phosphorylation sites (Matsumoto et al., 2005).

#### 1.5.5.2.1 Cell proliferation

Phosphorylation of tyrosine Y1175 leads to activation of PLC- $\gamma$ 1 which indirectly mediates activation of the PKC/Raf/MEK signalling pathway. This signalling pathway (summarised in Figure 1.9) represents a major component of VEGF-stimulated cell proliferation. A knock in mutation of Y1173 in mice (corresponding to Y1175 in human VEGF-R2) to phenylalanine in the VEGF-R2 gene is embryonically lethal due to severe defects in endothelial and haematopoietic stem cells similar to those observed in VEGF-R2-null mice (Sakurai et al., 2005). VEGF mutants that bind selectively to VEGF-R2 are fully active endothelial cell mitogens, whereas mutants specific for VEGF-R1 are devoid of such activity (Takahashi et al., 1999). The activation of the PI3K/p70 S6 kinase pathway by VEGF-R2 is also involved in VEGF-induced endothelial cell proliferation (Vinals et al.,

1999). Puromycin-intensive leucyl-specific aminopeptidase plays a crucial role in the activation of this pathway via the binding and modification of PDK1 (Yamazaki et al., 2004). Various other downstream mediators of VEGF-induced angiogenic signalling include DAG kinase  $\alpha$  and serum response factor (Baldanzi et al., 2004), SREBP (Zhou et al., 2004) and IQGAP1 (Yamaoka-Tojo et al., 2004). There are a number of possible endogenous feedback inhibitors for VEGF-induced angiogenesis. Vasohibin and DSCR1 are significantly induced by VEGF in endothelial cells (Watanabe et al., 2004; Hesser et al., 2004). Up-regulation of DSCR1 in endothelial cells inhibits the nuclear localization of NFAT, proliferation and tube formation (Minami et al., 2004). A naturally occurring soluble truncated form of VEGF-R2 has been detected in mouse and human plasma (Ebos et al., 2004). Similar to sVEGF-R1, sVEGF-R2 (soluble VEGF-R2) may have regulatory consequences with respect to VEGF-mediated angiogenesis.

#### **1.5.5.2.2 Cell migration**

Phosphorylated Y1175 creates a binding site for Shb (Holmqvist et al., 2004) which mediates the activation of PI3K and the assembly of focal adhesions, thus regulating VEGF-stimulated migration (Holmqvist et al., 2004). Y1214, is involved in activation of Cdc42 and MAPK (Lamalice et al., 2004), a pathway which also contributes to the regulation of cell migration. Mice with a mutation of Y1212 (corresponding to Y1214 in human VEGF-R2) to phenylalanine are viable and fertile indicating Y1214 signalling is not essential for vascular development in mouse embryos (Sakurai et al., 2005). Another important tyrosine autophosphorylation site Y951 binds and mediates tyrosine phosphorylation of the T-cell-specific adaptor protein (TSA<sub>d</sub>), which is expressed in endothelial cells. Y951-mediated coupling of VEGF-R2 and TSA<sub>d</sub>, which forms a complex with Src when activated (Matsumoto et al., 2005), is critical for VEGF-induced cell migration and actin reorganization, but not for VEGF-induced cell proliferation of endothelial cells (Matsumoto et al., 2005). VEGF-R2 intracellular signalling for vascular endothelial cell migration is summarised in Figure 1.10.

#### **1.5.5.2.3 Cell survival**

VEGF-R2 also activates PI3K which results in increased levels PIP<sub>3</sub> and the activation of several important intracellular molecules, including PKB and the small GTP-binding protein Rac. The PKB pathway regulates cell survival by inhibiting involvement of proapoptotic pathways, such as BAD and caspase 9 (Gerber et al., 1998b). However, it seems likely that another pathway may be involved, since the signal to activate PI3K by VEGF-

R2 is usually relatively weak. Indeed it has been reported that the activation of VEGF-R2 by VEGF results in the PI3K/PKB-dependent activation of several integrins, leading to enhanced cell adhesion and migration (Byzova et al., 2000). This synergic interaction with integrins is required for productive signalling from VEGF-R2. VEGF-R2 intracellular signalling for vascular endothelial cell survival is summarised in Figure 1.11.

#### 1.5.5.2.4 Vascular permeability

VEGF mutants that bind selectively to VEGF-R2, unlike those for VEGF-R1, are fully active permeability-enhancing agents (Takahashi et al., 1999). A number of signalling mechanisms have been reported to underlie VEGF-stimulated vascular permeability. In mesenteric microvessels VEGF increases vascular permeability by activation of VEGF-R2 on endothelial cells with subsequent activation of PLC. This causes increased production of DAG that results in influx of  $\text{Ca}^{2+}$  (Bates and Harper, 2002). In mice deficient in Src family kinases ( $\text{Src}^{-/-}$  or  $\text{Yes}^{-/-}$ ) VEGF-dependent vascular permeability is ablated (Eliceiri et al., 1999). Moreover, it seems that the activity of specific Src family kinases is essential for the VEGF-induced enhancement of vascular permeability through the disruption of the VEGF-R2/cadherin/catenin complex (Weis et al., 2004b). The PKB pathway, which activates eNOS (Fulton et al., 1999; Dimmeler et al., 1999), thus generating NO is recognised as another important mediator of VEGF-induced vascular permeability. Furthermore, it has been reported that inhibition of MAPK activity abrogates VEGF-induced vascular permeability *in vivo* and *in vitro*, thus suggesting a role for this kinase in the regulation of vascular permeability (Issbrucker et al., 2003).

There is some debate as to whether there is a link between vascular permeability and angiogenesis. Whilst the extravasation of proteins such as fibrin may act as a scaffold for endothelial cell proliferation and migration (Dvorak et al., 1987), growth factors such as bFGF, which are potent inducers of angiogenesis, have no effect on vascular permeability. Indeed vascular leakage is not necessarily accompanied by angiogenesis (Ferrara, 1995; Murata et al., 1992). It has also been reported that vascular permeability and angiogenesis are differentially regulated by members of the Src family, and furthermore it has been demonstrated that VEGF-vascular permeability signalling may be interrupted without affecting VEGF-stimulated angiogenesis (Eliceiri et al., 1999).

#### 1.5.5.2.5 Lymphangiogenesis

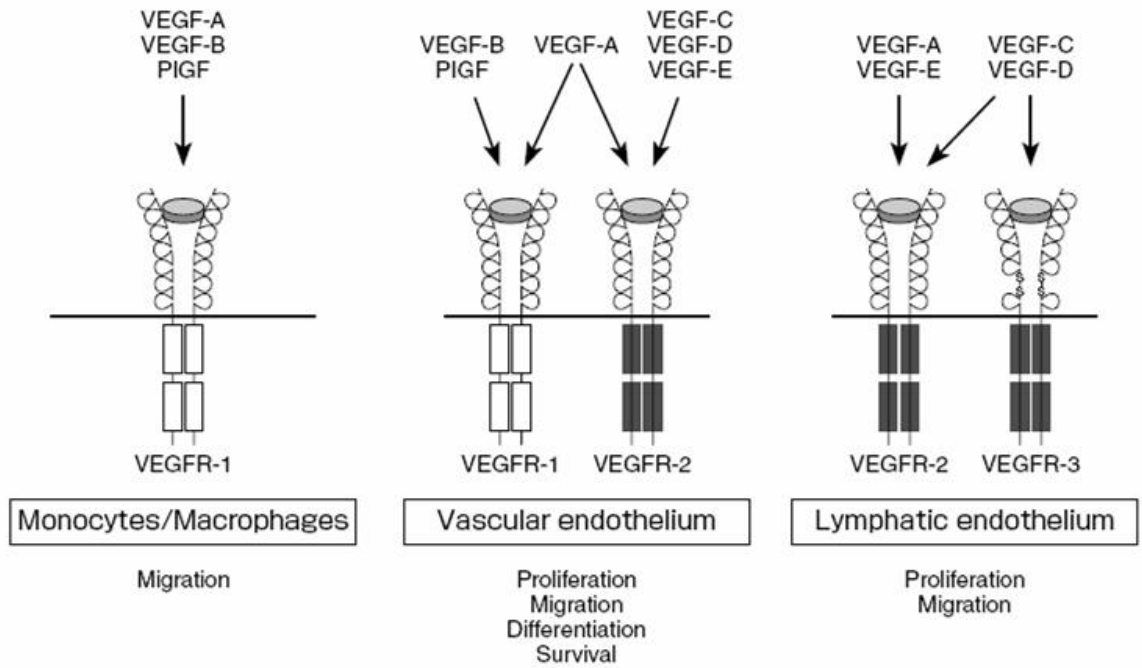
A number of reports have further indicated that the activation of VEGF-R2 also promotes lymphangiogenesis (Nagy et al., 2002; Hong et al., 2004).

### **1.5.5.3 VEGF-R3 signalling**

VEGF-R3, a 170 kDa glycosylated protein was first cloned from human erythroleukemia cells and placental cDNA libraries (Galland et al., 1993). VEGF-R3, which binds VEGF-C and VEGF-D, is expressed on embryonic endothelial cells, though its expression levels decrease with development and thus is restricted to lymphatic endothelium in adult tissues (Kaipainen et al., 1995). Several tyrosine residues have been predicted to become autophosphorylated on activation and dimerisation of VEGF-R3 (Olsson et al., 2006). However, a limited number of signalling effectors have been shown to act downstream of this receptor. Y1337 is required for Grb2 and SHC-mediated transforming capacity of VEGF-R3 (Fournier et al., 1995). Moreover, VEGF-R3 mediates anti-apoptotic effects as well as proliferation and migration of lymphatic endothelial cells through PKB and the PKC-MAPK signalling cascade (Makinen et al., 2001).

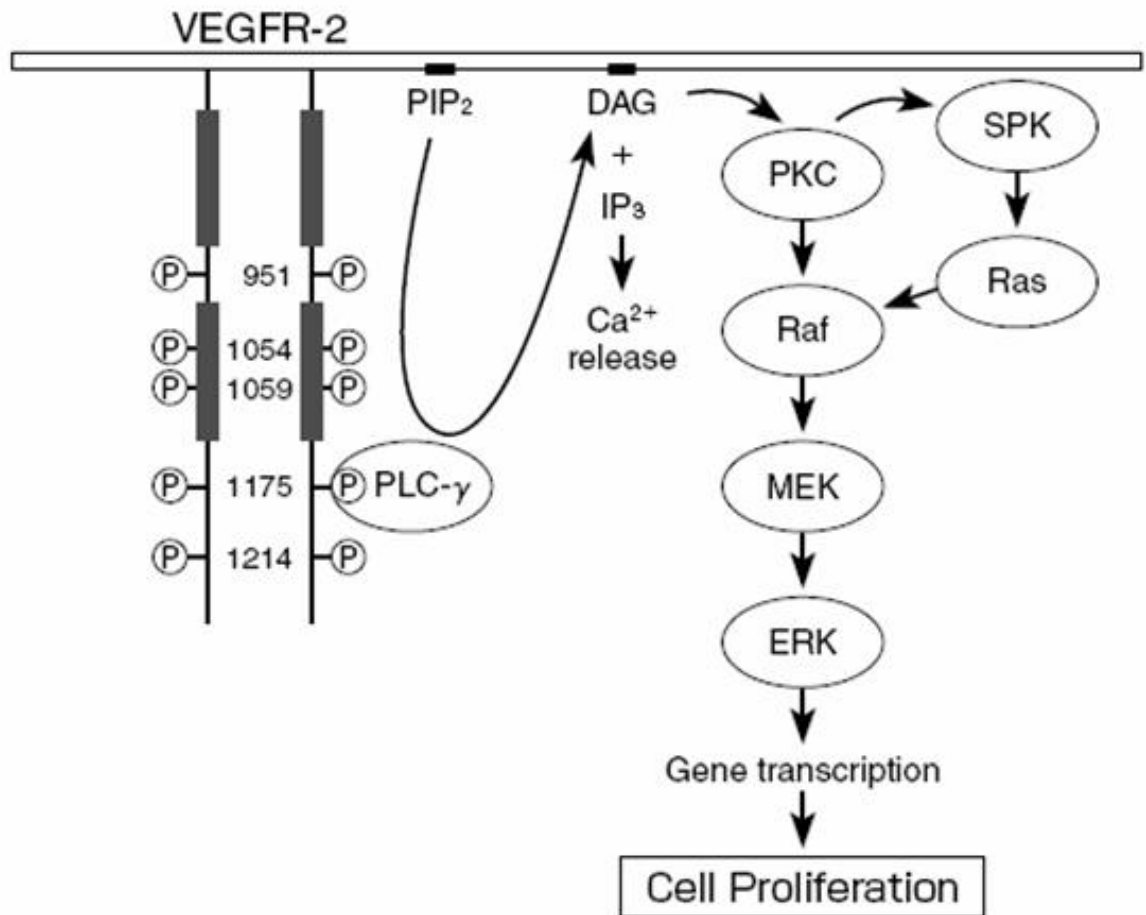
### **1.5.5.4 The neuropilins**

NRP-1, a cell-surface glycoprotein first identified as a semaphorin receptor involved in neuronal guidance (Neufeld et al., 2002), has also been identified as an isoform-specific receptor for VEGF (Soker et al., 1998). NRP-1 is able to bind VEGF, VEGF-B, PlGF-2 and some VEGF-E variants. NRP-2 which was identified based on sequence homology with NRP-1 (Neufeld et al., 2002) can bind VEGF, VEGF<sub>145</sub>, PlGF-2 and VEGF-C. The intracellular domains of NRPs are not sufficient for the independent transduction of biological signals subsequent to semaphorin or VEGF binding. However, VEGF-induced proliferation and migration of cells expressing VEGF-R2 is enhanced in the presence of NRP-1. Recent studies have demonstrated that this effect is the result of the formation of a complex between VEGF-R2 and NRP-1 (Whitaker et al., 2001; Soker et al., 2002).



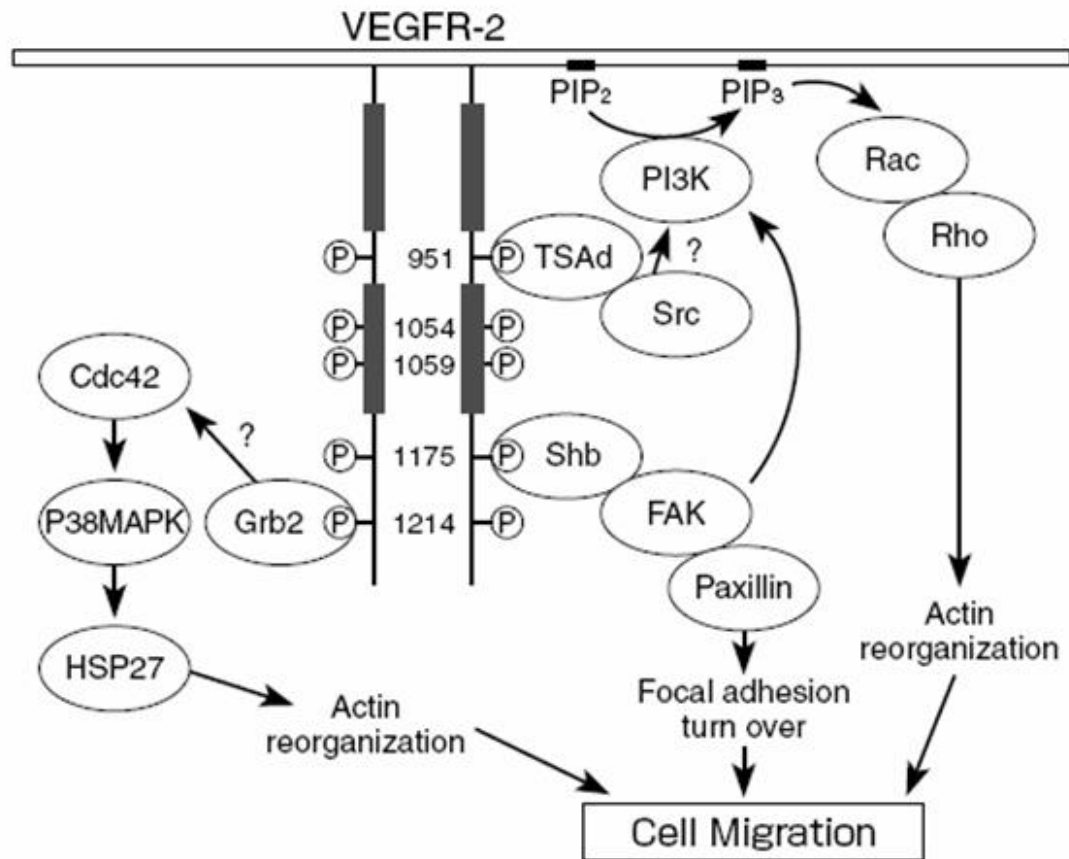
**Figure 1-8 Interactions of VEGF family members with their receptors**

The VEGF ligand family includes VEGF-A, -B, -C, -D, -E and PlGF, all of which bind in a specific manner to three different receptor tyrosine kinases, VEGFR-1, -2 and -3. Both PlGF and VEGF-B are selective ligands for VEGFR-1, whereas VEGF-A binds to both VEGFR-1 and VEGFR-2. VEGF-E is a selective ligand for VEGFR-2. VEGF-C and -D interact with both VEGFR-2 and VEGFR-3. Although both VEGFR-1 and VEGFR-2 are expressed in the vascular endothelium, their angiogenic activities are mainly transduced through VEGFR-2. VEGFR-1 is also expressed on monocytes and macrophages, and its activation leads to cell migration. VEGFR-3 is expressed mainly in lymphatic endothelium and is involved in lymphangiogenesis. (Adapted from (Matsumoto and Mugishima, 2006).



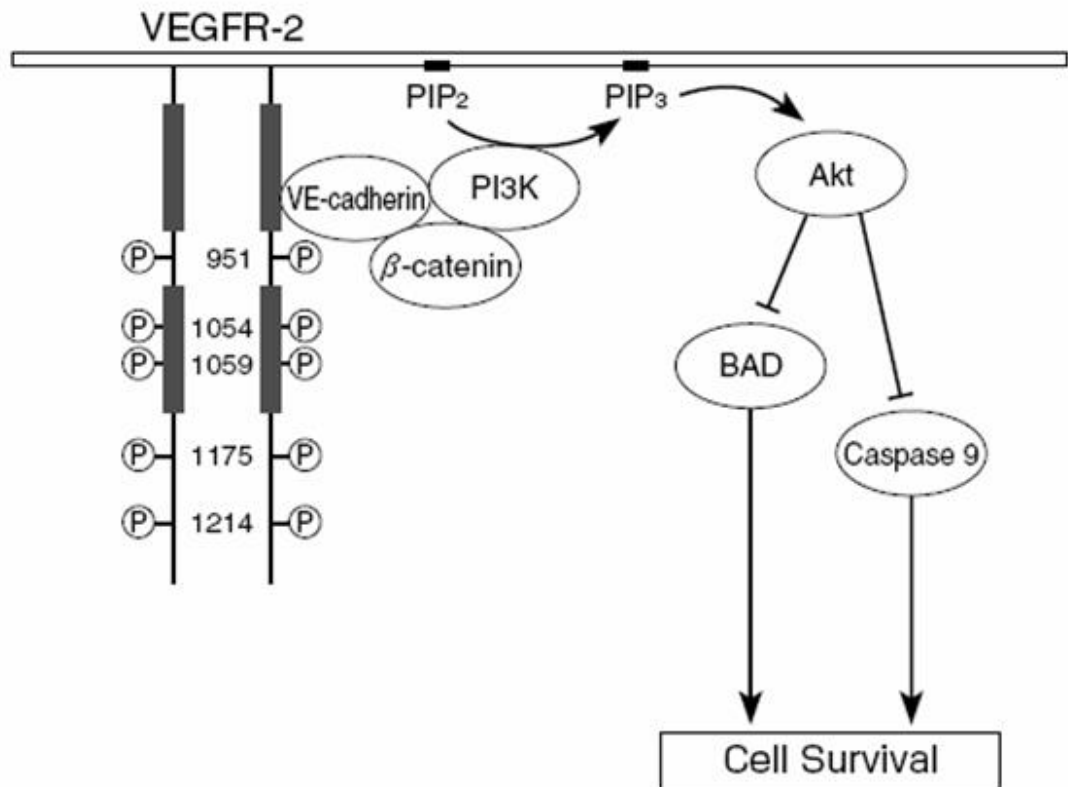
**Figure 1-9 VEGF-R2 signalling and vascular endothelial cell proliferation**

Ligand binding to the extracellular domain induced the dimerisation and autophosphorylation of specific intracellular tyrosine residues (Y951, Y1054, Y1059, Y1175 and Y1214). Phosphorylation of Y1175 creates a binding site for PLC-γ1 and leads to activation of the protein. PLC-γ1 activation results in the hydrolysis of PIP<sub>2</sub> creating IP<sub>3</sub> and DAG which stimulate the release of Ca<sup>2+</sup> from internal stores and activate PKC respectively. Activation of PKC plays a crucial role in VEGF mitogenic signalling via the raf-MEK-ERK pathway. Ras-dependent and independent pathways have been reported in PKC-induced Raf activation. (Figure adapted from (Matsumoto and Mugishima, 2006))



**Figure 1-10 VEGF-R2 signalling and vascular endothelial cell migration**

Activated VEGF-R2 is associated with adaptor proteins TSAAd, Shb and Grb2 via Y951, Y1175 and Y1214, respectively. Phosphorylated TSAAd binds to Src and regulates VEGF-induced actin reorganisation and cell migration. The PI3K/Rac/Rho pathway is thought to be downstream of TSAAd. Other components implicated in VEGF-R2-dependent cytoskeletal regulation and cell migration include FAK and p38 MAPK. Activated Shb mediates VEGF-induced FAK phosphorylation followed by the recruitment of actin-anchoring proteins to focal adhesion plaque. VEGF-induced activation of p38 MAPK is required for Y1214 phosphorylation. Thus, multiple pathways appear to regulate endothelial cell migration. (Figure adapted from (Matsumoto and Mugishima, 2006).



**Figure 1-11 VEGF-R2 signalling and vascular endothelial cell survival**

Activated PI3K induces PKB phosphorylation. The PKB pathway regulates cellular survival by inhibiting pro-apoptotic pathways, such as those involving BAD and caspase 9. Complex formation of VE-cadherin, VEGF-R2, β-catenin and PI3K in response to VEGF is required for PKD-dependent cell survival signalling. (Figure adapted from (Matsumoto and Mugishima, 2006).



## 1.6 Project aim

The overall objective of this project is to characterise the role of AMPK in human vascular endothelial cell function in response to VEGF exposure. Both VEGF and AMPK are important signalling molecules in the endothelium, and a number of reports demonstrate that activation of either has the potential to promote NO production.

VEGF stimulates NO production, proposed to be a result of phosphorylation and activation of eNOS at Ser-1177, a site also phosphorylated after activation of AMPK in cultured endothelial cells. Therefore, whether AMPK, at least in part, underlies VEGF-stimulated NO production formed the initial basis of this study.

As reduced NO bioavailability characterises the dysfunctional endothelium, a disorder closely linked with the aetiology of type 2 diabetes, obesity and cardiovascular disease, the signal transduction pathways which regulate NO bioavailability, which are incompletely understood, are of particular clinical interest.

## **2 MATERIALS AND METHODS**

## 2.1 Materials

### 2.1.1 General reagents

#### **Acros Organics (Loughborough, Leicestershire, UK)**

Glacial acetic acid (nitrogen-flushed), sodium nitrite (NaNO<sub>2</sub>), tetrasodium pyrophosphate (NaPPi)

#### **Alexis Biochemicals**

RHC-80267 (DAG lipase inhibitor)

#### **Beckman Coulter (High Wycombe, UK)**

Ultra-Clear™ ultracentrifuge tubes

#### **BOC gases, Manchester, UK**

N<sub>2</sub>

#### **Calbiochem (Nottingham, UK)**

A23187 (from *Streptomyces chartreusensis*), L-NAME, BAPTA-AM, compound C, DAG kinase inhibitor II (R-59949), OAG, SU1498, U73122

#### **Fisher Scientific UK Ltd (Loughborough, UK)**

Acetone, dimethylsulfoxide (DMSO), ethanol, D-glucose, glycine, NaOH, Tris (hydroxymethyl)-aminoethane (Tris base)

#### **Fisons Scientific Equipment, Loughborough, Leicestershire, UK**

1,1,2-trichlorotrifluoroethane (arclone P)

#### **Formedium, Hunstanton, UK**

Bacto-agar, tryptone, yeast extract

#### **GE Healthcare UK Ltd (Little Chalfont, Buckinghamshire, UK)**

ECL HRP-linked secondary antibodies, prepacked glutathione sepharose 4B, Protein G Sepharose 4 Fast Flow

#### **Hopkin & Williams (Chadwell Heath, UK)**

Sodium azide ( $\text{NaN}_3$ )

**Inverclyde Biologicals (Bellshill, Lanarkshire, UK)**

Nitrocellulose membrane (0.45  $\mu\text{m}$  pore size)

**Invitrogen Ltd (Paisley, UK)**

AlexaFluor488- and alexaFluor 568-linked secondary antibodies, MLV-RT, One Shot TOP10 Chemically Competent Cells, S.O.C medium

**Kodak Industrie (Chalon-sur-Saône, France)**

Kodak MXB film

**Melford Laboratories Ltd (Ipswich, UK)**

DTT, IPTG

**Millipore (formerly Upstate Biotech) (Livingston, UK)**

Streptavidin immobilised beads

**National Diagnostics (Hessle-Hull, UK)**

Scintillation fluid

**Neuro Probe, Inc., Gaithersburgh, MD, USA; via Receptor Technologies Ltd., Adderbury, UK**

Polyvinylpyrrolidone (PVP)-free polycarbonate track-etch (PCTE) membranes for migration assays (8  $\mu\text{m}$  pore size)

**New England Biolabs (Hitchin, UK)**

Prestained protein marker

**Pepceutical Ltd (Nottingham, UK)**

SAMS peptide (HMRSAMSGHLVKRR)

**Premier International Foods Ltd (Spalding, UK)**

Dried skimmed milk

**Promega (Southampton, UK)**

Taq DNA polymerase, molecular grade  $\text{MgCl}_2$ , dATP, dCTP, dGTP, dTTP

**Qiagen (Crawley, West Sussex, UK)**

DNase I

**RELIAtect GmbH (Braunschweig, Germany)**

VEGF-E

**Sartorius Biotech GmbH (Göttingen, Germany)**

Sterile syringe filters (0.2 µm)

**Schleicher & Schuell**

Protran nitrocellulose membrane, Whatman P81 chromatography paper

**Severn Biotech Ltd (Kidderminster, Worcester, UK)**

Acrylamide:bisacrylamide (37.5:1; 30 % (w/v) acrylamide)

**Sigma-Aldrich Ltd (Poole, Dorset, UK, Steinheim, Germany; Seelze, Germany; St Louis, MO, USA), including all Riedel-de-Haën chemicals**

AMP, ampicillin, 2-APB, APS, ATP, bromophenol blue, BSA, Comassie brilliant blue, fatty acid free BSA, CsCl, benzamidine, L-carnitine, coumaric acid, Dowex 1X8-200, D-mannitol, DMSO, EDTA, EGTA, gadolinium, glycerol, H<sub>3</sub>PO<sub>4</sub>, hexanucleotide primers, isopropanol, L-carnitine, L-NAME, luminol, lysozyme, methanol, NaF, NaHCO<sub>3</sub>, Na<sub>2</sub>HPO<sub>4</sub>, NaH<sub>2</sub>PO<sub>4</sub>, NaI (nitrogen-flushed), Na<sub>4</sub>VO<sub>3</sub>, protein G peroxidase, RT-PCR primers, SBTI, SDS, TCA, Triton X-100, trypan blue, tween-20, Type IV collagen, U73122, VEGF, VEGF-B

**Tocris (Avonmouth,UK)**

STO-609 acetate

**Toronto Research Chemicals (Toronto, ON, Canada)**

AICAR

**VWR International (Lutterworth, Leicestershire, UK), including all BDH chemicals**

CaCl<sub>2</sub> solution, 30% (v/v) H<sub>2</sub>O<sub>2</sub>, HEPES, KCl, KH<sub>2</sub>PO<sub>4</sub>, MgCl<sub>2</sub>, NaCl, Na<sub>2</sub>HPO<sub>4</sub>, NaH<sub>2</sub>PO<sub>4</sub>, Nickel

### **2.1.2 Kits**

#### **Cell Biolabs (San Diego, USA)**

QuickTiter™ Adenovirus Titer Immunoassay Kit

#### **Promega (Southampton, UK)**

CellTiter 96® AQueous One Solution Cell Proliferation Assay Kit

#### **Qiagen (Crawley, West Sussex, UK)**

Qiafilter™ Plasmid Maxi Kit, RNeasy mini kit

### **2.1.3 Specialist equipment and suppliers**

#### **Analytix Ltd (Durham, UK)**

Sievers® Nitric Oxide Analyzer 280, exmire microsyringe and needles for NO analysis

#### **Beckman Coulter™ (High Wycombe, UK)**

Optima™ XL-80K ultracentrifuge, SW40TI rotor

#### **Bio-Rad Laboratories (Hemel Hempstead, UK)**

Agarose gel (Mini-Sub/Wide Mini-Sub Cell GT), protein gel casting and Western blotting equipment (Mini Protean III)

#### **Herolab (Wiesloch, Germany)**

UVT-28 MP UV transilluminator

#### **Optika Microscopes (Ponteranica, Italy)**

XDS-1B light microscope

#### **Helena Biosciences (Sunderland, UK)**

Proteus thermal cycler

#### **Shimadzu Europa GmbH (Duisburg, Germany)**

UV-1201 spectrophotometer

#### **WPA (Cambridge, UK)**

S2000 spectrophotometer

## 2.1.4 Radiochemicals

### **GE Healthcare Life Sciences (Little Chalfont, Buckinghamshire)**

$\gamma$ -[<sup>32</sup>P]-ATP, [9,10(n)-<sup>3</sup>H]-palmitic acid

### **Perkin Elmer Life and Analytical Sciences (Beaconsfield, Buckinghamshire, UK)**

$\gamma$ -[<sup>32</sup>P]-ATP

## 2.1.5 Tissue culture plasticware and reagents

### **Becton Dickinson Biosciences (Oxford, UK)**

Corning cell culture flasks, 10 cm-diameter cell culture dishes and multiwell plates

### **Corning, NY, USA**

CoStar 25 ml, 10 ml, 5 ml and 1 ml sterile pipettes, 75 cm<sup>2</sup> tissue culture flasks, 150 cm<sup>2</sup> tissue culture flasks

### **Invitrogen Ltd (Paisley, UK)**

FCS (EU origin), L-glutamine, Lipofectamine<sup>TM</sup>2000, penicillin and streptomycin, trypsin (0.05 % (v/v) in 0.53 mM EDTA•4Na), zeocin, blastacidin

### **Sigma-Aldrich Ltd (Poole, Dorset, UK)**

Tetracycline

### **VWR International (Lutterworth, Leicestershire, UK)**

Falcon<sup>TM</sup> 10 cm-diameter cell culture dishes and multi-well plates

### 2.1.6 Cells and media

Cell type & supplier	Basal media & supplier	Additions for complete media formulation
HEK293	DMEM Invitrogen Catalogue # 41965	5 % (v/v) FCS 2 mM glutamine (final concentration) 100 µg/ml streptomycin 100 U/ml penicillin
HeLa	DMEM	10 % (v/v) FCS 2 mM glutamine (final concentration) 100 µg/ml streptomycin 100 U/ml penicillin
HeLa <sub>LKB1-KD/WT</sub> (A generous gift provided by Prof. D. Alessi, University of Dundee)	EMEM Lonza (Walkersville, MD, USA) Catalogue # BE12-611F	10 % (v/v) FCS 2 mM glutamine (final concentration) 100 µg/ml streptomycin 100 U/ml penicillin 2 mM non-essential amino acids 5 µg/ml blasticidin 100 µg/ml zeocin
HAEC Lonza, PromoCell (Heidelberg, Germany), TCS CellWorks (Botolph Claydon, UK)	Large vessel endothelial cell basal medium TCS cellworks Catalogue # ZHM-2951 Endothelial cell basal medium (EBM®-2) Lonza Catalogue # CC-3162	Large vessel endothelial cell growth supplement (ZHS-8945) and antibiotic supplement (ZHR-9939) were added to ZHM-2951 Bullet kit (growth factors) CC-3156 & CC-4176 were added to CC-3162

**Table 2-1 Cells and media**

Routinely used cell types and their associated basal and complete culture media formulations.



## 2.1.7 Antisera

### 2.1.7.1 Primary antibodies for Western blotting

Epitope	Host species	Dilution	Source
AMPK $\alpha$ 1/ AMPK $\alpha$ 2	Sheep (P)	1:1000 in 1 % (w/v) MP	A generous gift from Prof. D.G. Hardie, University of Dundee (Woods et al., 1996)
AMPK Thr-172	Rabbit (P)	1:1000 in 5 % (w/v) BSA	Cell Signalling Technology (# 2531)
ACC Ser-80	Rabbit (P)	1:1000 in 5 % (w/v) BSA	Cell Signalling Technology (# 3661)
ACC1/ACC2	Sheep (P)	1:1000 in 5 % (w/v) MP	These were a kind gift from Prof. D.G. Hardie, University of Dundee
CaMKK $\alpha$	Mouse (M)	1:1000 in 5 % (w/v) MP	Santa Cruz Biotechnology (# F-2 sc-17827)
CaMKK $\beta$	Goat (P)	1:500 in 5 % (w/v) MP	Santa Cruz Biotechnology ( # L:19: sc-9629)
eNOS	Rabbit (P)	1:5000 in 1 % (w/v) BSA	Sigma (# N-2643)
eNOS Ser-1177	Rabbit (P)	1:1000 in 5 % (w/v) BSA	Cell Signalling Technology (# 9571)
FLAG	Mouse (M)	1:1000 in 1 % (w/v) MP	Sigma (# F-3165)
GAPDH (clone 6C5)	Mouse (M)	1:1500 in 5 % (w/v) MP	Ambion (# 4300)

PKB	Rabbit (P)	1:1000 in 5 % (w/v) MP	Cell Signalling Technology (# 9272)
PKB Ser-473	Rabbit (M)	1:1000 in 5 % (w/v) BSA	Cell Signalling Technology (# 4058)
VEGF-R1	Mouse (M)	1:200 in 5 % (w/v) MP	Abcam (# ab11934)
VEGF-R2	Rabbit (M)	1:1000 in 5 % (w/v) BSA	Cell Signalling Technology (# 2479)

**Table 2-2 Primary antibodies for Western blotting and their conditions of use**

Monoclonal (M) or polyclonal (P) antibodies are denoted in brackets. All BSA or MP solutions were made in TBST (made as described in section 2.2.1).

**2.1.7.2 Secondary detection agents for Western blotting**

<b>Linked molecule</b>	<b>Epitope</b>	<b>Host species</b>	<b>Dilution</b>	<b>Source</b>
HRP	Mouse IgG	Sheep	1:1000-1:2000 in 1 % (w/v) MP	GE Healthcare (# NA931)
HRP	Rabbit IgG	Donkey	1:1000-1:2000 in 1 % in (w/v) MP	GE Healthcare (# NA934)
HRP	Streptococcus sp. Protein G	n/a	1:1000-1:2000 in 1 % (w/v) MP	Sigma (# P8170)

**Table 2-3 Secondary detection agents for Western blotting**

Secondary detection agents for Western blotting and their conditions of use.

## 2.2 Buffers

### 2.2.1 General buffers and solutions

Unless stated all buffers and reagents were made up with distilled water

#### **Bradford's reagent**

35 mg/L Coomassie brilliant blue, 5.0 % (v/v) ethanol, 5.1 % (v/v) H<sub>3</sub>PO<sub>4</sub>

Bradford's reagent was filtered and stored in the dark

#### **Earles-HEPES**

20 mM HEPES (pH 7.4), 5.5 mM glucose, 1.8 mM CaCl<sub>2</sub>, 0.8 mM MgSO<sub>4</sub>, 5.3 mM KCl, 116 mM NaCl, 1 mM NaH<sub>2</sub>PO<sub>4</sub>

#### **Enhanced chemiluminescence (ECL) detection reagents**

Solution 1: 0.1 mM Tris-HCl (pH 8.5), 450 mg/L luminol in 2 % (v/v) DMSO, 130 mg/L coumaric acid in 1 % (v/v) DMSO

Solution 2: 0.1 mM Tris-HCl (pH 8.5), 0.06 % (v/v) H<sub>2</sub>O<sub>2</sub>

#### **HEPES-Brij buffer**

50 mM HEPES-NaOH (pH 7.4 at 4°C), 1 mM DTT, 0.02 % (v/v) Brij-35

#### **HEPES-Brij buffer (high salt)**

50 mM HEPES-NaOH (pH 7.4 at 4°C), 1 mM DTT, 0.02 % (v/v) Brij-35, 850 mM NaCl

#### **Immunoprecipitation buffer**

50 mM Tris-HCl pH 7.4 (at 4°C), 150 mM NaCl, 50 mM NaF, 5 mM NaPPi, 1 mM EDTA, 1 mM EGTA, 1 % (v/v) Triton X-100, 1 % glycerol, 1 mM DTT, 1 mM Na<sub>3</sub>VO<sub>4</sub>, 0.1 mM benzamidine, 0.1 mM PMSF, 5 µg/ml SBTI

#### **Krebs-Ringer-HEPES (KRH) buffer**

20 mM HEPES-NaOH (pH 7.4), 119 mM NaCl, 5 mM NaHCO<sub>3</sub>, 5 mM glucose, 4.8 mM KCl, 2.5 mM CaCl<sub>2</sub>, 1.2 mM MgSO<sub>4</sub>, 1.2 mM NaH<sub>2</sub>PO<sub>4</sub>, 0.1 mM L-arginine

#### **KRH buffer (Ca<sup>2+</sup>-free)**

20 mM HEPES-NaOH (pH 7.4), 119 mM NaCl, 5 mM NaHCO<sub>3</sub>, 5 mM glucose, 4.8 mM KCl, 1.2 mM MgSO<sub>4</sub>, 1.2 mM NaH<sub>2</sub>PO<sub>4</sub>, 0.1 mM L-arginine, 1 mM EGTA

**KRH buffer (High K<sup>+</sup>)**

20 mM HEPES-NaOH (pH 7.4), 59.5 mM NaCl, 5 mM NaHCO<sub>3</sub>, 5 mM glucose, 64.3 mM KCl, 2.5 mM CaCl<sub>2</sub>, 1.2 mM MgSO<sub>4</sub>, 1.2 mM NaH<sub>2</sub>PO<sub>4</sub>, 0.1 mM L-arginine

**Lysis buffer**

50 mM Tris-HCl (pH 7.4 at 4°C), 50 mM NaF, 1 mM NaPPi, 1 mM EDTA, 1 mM EGTA, 1 % (v/v) Triton X-100, 250 mM mannitol, 1 mM DTT, 1 mM Na<sub>3</sub>VO<sub>4</sub>, 0.1 mM benzamidine, 0.1 mM PMSF, 5 µg/ml SBTI

**Phosphate-buffered saline (PBS) (pH 7.2)**

137 mM NaCl, 2.7 mM KCl, 10 mM Na<sub>2</sub>HPO<sub>4</sub>, 1.8 mM KH<sub>2</sub>PO<sub>4</sub>

**Ponceau S stain**

0.2 % (w/v) Ponceau S, 1 % (v/v) acetic acid

**SDS-PAGE running buffer**

190 mM Glycine, 62 mM Tris, 0.1 % (w/v) SDS

**SDS sample buffer**

200 mM Tris-HCl pH 6.8, 40 % (v/v) glycerol, 8 % (w/v) SDS, 0.4 % (w/v) bromophenol blue

The above recipe for 4X SDS sample buffer was used neat, or diluted with distilled water to 2X or 1X working concentration as required. DTT was added to a final concentration of 200 mM before use (i.e. 20 % (v/v) from 1 M stock).

**Tris-buffered saline + Tween-20 (TBST)**

20 mM Tris-HCl (pH 7.4), 137 mM NaCl, 0.1 % (v/v) Tween-20

**Transfer buffer**

25 mM Tris base, 192 mM glycine, 20 % (v/v) ethanol

**2.2.2 Molecular biology solutions****2YT media**

1.6 % (w/v) tryptone, 1 % (w/v) yeast extract, 0.5 % (w/v) NaCl

2YT media was autoclaved before use

**LB broth**

1 % (w/v) tryptone, 0.5 % (w/v) yeast extract, 1 % (w/v) NaCl

LB media was autoclaved before use

**6X DNA loading buffer**

0.5 % (w/v) bromophenol blue, 15 % (w/v) ficoll

**TAE buffer (50x)**

40 mM Tris, 1 mM EDTA, 5.71 % (v/v) glacial acetic acid

## 2.3 Molecular biology protocols

### 2.3.1 Preparation of agar plates

2 % (w/v) bacto-agar was added to 2YT media (as described in section 2.2.2), autoclaved and allowed to cool to 50°C, prior to the addition of 50 µg/ml ampicillin (YT-amp). 2 % (w/v) bacto-agar was added to LB media (as described in section 2.2.2) containing 2 % (w/v) glucose and 50 µg/ml ampicillin (LB-amp). In both cases media were mixed to ensure equal distribution of antibiotic and approximately 25 ml poured per 10 cm<sup>2</sup> plate. The plates were left to set at room temperature then stored at 4°C.

### 2.3.2 Transformation of competent bacterial cells

An aliquot of competent bacteria (One Shot TOP10 Competent Cells) was allowed to thaw on ice. 5-10 ng of DNA was then added to “TOP10” cells and incubated on ice for 15 minutes. The cells were subjected to heat shock at 42°C for 45 seconds and returned to ice for 1 minute. 250 µl S.O.C. medium was added to the cells and placed in a shaking incubator at 37°C for 1h. 100 µl of the reaction was spread onto an agar plate (YT-amp) (as described in section 2.3.1) and incubated upside down at 37°C overnight. Plates were removed the following day and stored at 4°C.

### 2.3.3 Preparation of plasmid DNA

The Qiagen Qiafilter kit (as described in section 2.1.2) was used to produce large scale DNA samples. Purification of DNA was carried in accordance with the manufacturer's instructions. A single colony from a freshly streaked plate was used to inoculate 50 ml 2YT media containing 50 µg/ml ampicillin and incubated for 16h. The transformed bacteria were harvested by centrifugation at 1,370 x g for 20 minutes at 4°C. All traces of media were removed and the bacterial pellet re-suspended in 10 ml of chilled buffer P1 (50 mM Tris-HCl pH 8.0, 10 mM EDTA, 100 µg/µl Rnase A). Cell lysis was achieved by the addition of 10 ml buffer P2 (200 mM NaOH, 1 % (w/v) SDS) for 5 minutes at room temperature. 10 ml of buffer P3 (3 M potassium acetate pH 5.5) was added to neutralise the reaction and the solution immediately applied to a QIAfilter cartridge and left for 10 minutes at room temperature to settle. Meanwhile, a Qiagen tip 500 was equilibrated by the addition of buffer QBT (750 mM NaCl, 50 mM MOPS pH 7.0), 15 % (v/v) isopropanol). The DNA was eluted by the addition of buffer QF (1.25 M NaCl, 50 mM Tris-HCl pH 8.5,

15 % (v/v) isopropanol) to the tip. DNA was precipitated by the addition of 10.5 ml of isopropanol and pelleted by centrifugation at 1,370 x g for 1h at 4°C. The DNA pellet was washed with 5 ml of room temperature 70 % (v/v) ethanol and then centrifuged at 1, 370 x g for 15 minutes at 4°C. The supernatant was carefully removed and the pellet allowed to air dry prior to resuspension in 200 µl DEPC treated sterile water.

### **2.3.4 Quantification of DNA**

Quantification of DNA samples was performed by examining the absorbance of a 1:100 dilution of the sample at 260 nm. An  $A_{260}$  value of 1 unit was taken to correspond to 50 µg/ml of double stranded DNA.

### **2.3.5 RT-PCR**

#### **2.3.5.1 RNA extraction**

HAEC were grown to 80-100 % confluency in 10 cm petri dishes. Total RNA was isolated according to the Qiagen RNeasy Mini Kit centrifugation protocol, utilising 15 second centrifugation steps (at 16,352 x g in a bench top centrifuge) for the lysate application and wash steps, discarding the flowthrough between each step. Cells were lysed by addition of 0.6 ml lysis (RLT) buffer and subsequently collected with a cell scraper. Lysates were homogenised by passing at least five times through a sterile 23-gauge needle and syringe. The lysate was mixed gently with an equal volume of 70 % (v/v) ethanol and applied to a RNeasy mini columns in 700 µl aliquots until the entire lysate volume had been passed over the column. Columns were washed with 350 µl of wash buffer 1 (RW1) before treatment with DNase I (Qiagen): 10 µl of DNase I stock premixed with 70 µl dilution buffer (RDD) were pipetted directly onto the silica gel membrane of each column and incubated at room temperature for 15 minutes. Columns were washed again with 350 µl buffer RW1 and were transferred to fresh 2 ml collection tubes prior to addition of 2 x 500 µl wash buffer 2 (RPE). After discarding the flowthrough, columns were centrifuged at 27,635 x g for 2 minutes to dry the RNeasy silica gel membrane and to eliminate any chance of accidental carryover of buffer RPE. Columns were transferred to fresh, 1.5 ml collection tubes and RNA was eluted with 30-50 µl RNase-free water by spinning for 1 minute at 27,635 x g. The RNA concentration was determined at  $A_{260}$  in a UV-1201 spectrophotometer (Shimadzu) prior to storage at -80°C.



### 2.3.5.2 First strand cDNA synthesis

For cDNA synthesis, total RNA extracted from HAEC was reverse transcribed using MLV RT as described by the manufacturer. In each reaction ~1 µg DNase-free RNA was added to 2.5 µl random hexamers (corresponding to 250 ng total), 1 µl 10mM dNTP mix (10mM each dATP, dGTP, dCTP, dTTP) and sterile distilled water added to a final volume of 13 µl. The mixture was heated at 65°C for 5 minutes and after a quick chill on ice the contents were collected by brief centrifugation. 4 µl of 5X first strand buffer and 2 µL 0.1 M DTT was then added, the contents mixed and incubated at 37°C for 2 minutes. 1 µl of M-MLV RT was then added, gently mixed and incubated at 25°C for 10 minutes. A further incubation period of 50 minutes at 37°C was carried out. The reaction was inactivated by heating at 70°C for 5 minutes. Resulting cDNA was stored at -20°C.

### 2.3.5.3 PCR and gel resolution of PCR products

In order to amplify cDNA from HAEC, the relevant human gene sequences were retrieved from the NCBI database ([www.ncbi.nlm.nih.gov](http://www.ncbi.nlm.nih.gov)). PCR primers were designed with “FastPCR” software and are detailed in table 2.4.

For amplification of gene transcripts, equal volumes of cDNA (not exceeding 10 % of the total reaction volume of 50 µl) were mixed with a PCR mastermix (see below), and gene-specific primers to the transcript of interest were added. The PCR mastermix contained equal amounts (0.2 µM each) of the gene-specific forward and reverse primer (Sigma-Proligo), 1 µl of 10 mM dNTPs, 3 µl of 25 mM MgCl<sub>2</sub>, 5 µl of 10X Taq polymerase buffer, 0.4 µl Taq polymerase (Promega), and water to a total volume of 50 µl. PCR reactions were carried out in a gradient thermocycler under the following conditions. Pre-heating (2 minutes, 94°C), thirty five cycles of [denaturation (1 minute, 94°C), primer annealing (1 minute, 58°C), extension (50 sec, 72°C)], and final extension (5 minutes, 72°C). PCR products were stored at -20°C. Analysis of PCR products was performed on 2 % (w/v) agarose gels, resolved for 20-25 minutes at 100 V, using a Mini-Sub or Wide Mini-Sub Cell GT gel system (Bio-Rad). PCR products were mixed 5:1 with 6x DNA loading buffer, and equal volumes (18-20 µl/ lane) of PCR products were applied to the agarose gels.

### 2.3.5.4 Visualisation of DNA bands

DNA gels were visualised on a UVT-28 MP transilluminator (Herolab), photographed and scanned into Adobe Photoshop software using a Mercury 1200c scanner.

Primer name	5'-3' Sequence of primer	Tm (°C)	GC content (%)	Product size (bp)
TRPC3 forward	AAGCCCATCCCTGAGACGCATGAC	57	58	
TRPC3 reverse	GCTCAGAGTGAGACGCTTGCTG	62	59	400
TRPC6 forward	GGATCTGACAACAGACTGGCTCAC	57	54	
TRPC6 reverse	TTGCTGGAGTTCAGACTGGCTA	54	50	435
TRPC7 forward	CCGCCTACATGTTCAACGAGAAGG	61	54	
TRPC7 reverse	GTGCACGATCTCATACTCCTGGCA	60	54	458
18S RNA forward	AAA CGG CTA CCA CAT CCA AG	64.1	50	
18S RNA reverse	CGCTCCCAAGATCCAACACTAC	63.9	55	250

**Table 2-4 Primer sequences for RT-PCR**

nts: nucleotides; Tm: melting temperature. Accession numbers; TRPC3 NM\_003305, TRPC6 NM\_004621, TRPC7 NM\_020389.

## 2.4 Cell culture

### 2.4.1 Cryopreservation of cells

HEK293, HAEC, HeLa or HeLa<sub>LKBI-KD/WT</sub> cells (~80% confluence) cells were cryopreserved after centrifugation at 146 x g for 5 minutes and re-suspended in 90 % (v/v) complete medium and 10 % (v/v) DMSO, by overnight incubation in a polycarbonate freezing container at -80°C. The following day, cells were transferred to liquid nitrogen for storage.

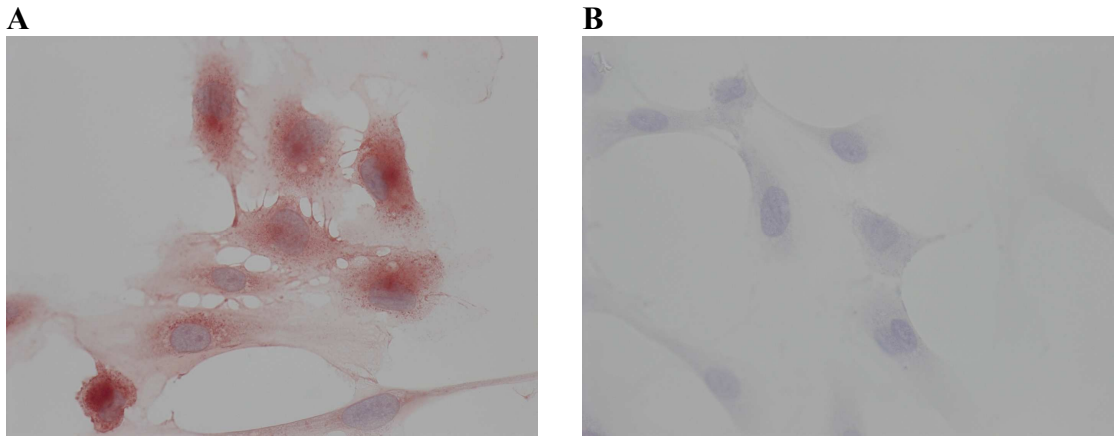
### 2.4.2 Revival and culture of cryopreserved cells

Aliquots of cryopreserved HAEC were rapidly thawed in a 37°C water bath. Cells were then added to complete cell medium pre-equilibrated in a humidified incubator containing 5 % (v/v) CO<sub>2</sub>, 95 % (v/v) air, at 37°C. The cell suspension was divided equally between 6-10 25 cm<sup>2</sup> cell culture flasks and incubated at 37°C in a humidified incubator supplemented with 5 % (v/v) CO<sub>2</sub>, 95 % (v/v) air, at 37°C. Cell medium was replaced after 24h, and every 48h thereafter with fresh complete medium. After thawing HeLa, HeLa<sub>LKBI-KD/WT</sub> and HEK293 cells were divided into 2-3 75 cm<sup>2</sup> flasks, but otherwise treated as described above.

### 2.4.3 Determination of endothelial cell phenotype of cultured HAEC

In order to prevent differentiation from the endothelial phenotype of HAEC, only HAEC in passages 4-6 were used for experiments. To assess the maintenance of the endothelial phenotype, the presence of the endothelial cell marker CD31 in cultured HAEC was ascertained by immunocytochemistry. Dr Ian Montgomery (University of Glasgow) carried out these studies. For immunocytochemistry, HAEC were grown on coverslips and fixed with methanol for 10 minutes. After washing, the coverslips were attached to glass slides using vaseline and were circled using a Dako PAP pen to form a watertight seal. The cells were rinsed in PBS and blocked with 1:30 goat serum in PBS. Anti-CD31 primary antibody (1:40 dilution in PBS + 1 % (w/v) BSA) was incubated in a humidified chamber on an orbital shaker for 1h. After washing in PBS, the coverslips were incubated with biotinylated goat anti-mouse IgG (1:20 dilution in PBS + 1 % (w/v) BSA) for 30 minutes. After thorough washing in PBS, cells were incubated for a further 30 minutes in ExtrAvidin Peroxidase (1:20 dilution in PBS + 1 % (w/v) BSA). Following washing in

PBS, the AEC substrate reagent was prepared and incubated for 5-10 minutes. Coverslips were then rinsed with distilled water, stained with haematoxylin for 1 minute and rinsed gently. The coverslips were carefully removed from the glass slides and mounted on clean slides using Aquamount mounting medium and left to dry overnight. Stained cells were examined using a Zeiss Axiophot microscope and images were captured using a JVC video camera and AverCAP video card in a Viglen computer. The cytoplasm of CD31-positive cells was rose-red to brownish-red and the nucleus was stained pale blue/purple. As a negative control, HAEC were stained in the absence of primary antibody.



**Figure 2-1 HAEC stained with anti-CD31 antibody and haematoxylin**

In order to confirm the endothelial phenotype of HAEC, immunocytochemistry was carried out by Dr Ian Montgomery as described in section 2.4.3. HAEC positive for the endothelial cell marker CD31 show red-stained cytoplasm (panel A), while the cytoplasm of unlabelled control cells does not stain (panel B). Cell nuclei appear blue after staining with haematoxylin (both panels).

#### 2.4.4 Passaging of cells

Cells were routinely subdivided 1/3-1/6 in 75 cm<sup>2</sup> tissue culture flasks. HeLa, HeLa<sub>LKB1-KD/WT</sub> and HEK293 cells were subdivided 1/5-1/10 in 75 cm<sup>2</sup> tissue culture flasks. For passaging, cells (~80% confluence) were washed once in 2-4 ml of basal medium and detached with 1-2 ml trypsin (0.05 % (v/v) in EDTA). Cells were briefly incubated at 37°C until the cells fully detached upon tapping of the cell culture flask. Trypsin was neutralised by addition of 1-3 ml of complete medium. Cells were pooled in a 50 ml centrifuge tube and centrifuged at 146 x g for 5 minutes. The supernatant was aspirated and the cell pellet resuspended in 5 ml complete medium prior to dilution into an appropriate volume of complete medium. The cell suspension was divided between 75 cm<sup>2</sup> tissue culture flasks and 10 cm-diameter cell culture dishes and/or multiwell plates, as required.

#### 2.4.5 LKB1 kinase dead and LKB1 wild type expressing HeLa cell lines

HeLa cells expressing the Tet-ON tetracycline repressor (Sapkota et al., 2002) were kindly provided by Professor Dario Alessi (University of Dundee). Kinase dead (HeLa<sub>LKB1-KD</sub>) or wild type (HeLa<sub>LKB1-WT</sub>) FLAG-epitope tagged LKB1 was induced in these cell lines by the addition of 1 µg/ml tetracycline for 24h prior to lysis.

#### 2.4.6 Transient transfection of HeLa cells

VEGF-R1 and VEGF-R2 cDNA (Cheng et al., 2006; Fragozo et al., 2006) was kindly provided by Professor Segio Dias (Angiogenesis laboratory, CIPM, Lisbon, Portugal) and Professor David Bates (University of Bristol, UK) respectively. VEGF-R1 and VEGF-R2 cDNA was transfected into HeLa cells using Lipofectamine<sup>TM</sup>2000, in accordance with the manufacturer's instructions. Cells were grown to 90-95 % confluency in 6-well plates in complete media containing no antibiotics. For each sample 4 µg of DNA was diluted in 250 µl of Opti-MEM (Gibco # 31985-047) reduced serum medium and gently mixed. Lipofectamine<sup>TM</sup>2000 was mixed before use then 10 µl added to 250 µl of Opti-MEM and incubated for 5 minutes at room temperature. The diluted DNA was combined with the diluted Lipofectamine<sup>TM</sup>2000 and incubated for 20 minutes at room temperature. Complexes were then added to each well containing cells and medium and incubated at 5 % (v/v) CO<sub>2</sub>, 95 % (v/v) air, at 37°C for 24-36h prior to testing for transgene expression. Opti-MEM was exchanged for complete medium 6h after complexes had been added.

### 2.4.7 Lysate preparation

Unless otherwise stated, all treatments were carried out in duplicate for each experiment. Cells, stimulated as indicated in the Figure legends, were serum-starved by aspirating the cell culture medium, washing the cells once with 10 ml of fresh, pre-warmed KRH buffer and replacing it with 5 ml/10 cm dish of fresh KRH buffer. Cells were incubated for 2-3h at 37°C. Thereafter, the buffer was aspirated and dishes were placed on ice. To each dish, 0.4 ml fresh, ice-cold lysis buffer was added and cells were scraped off using a cell lifter. Resulting cell lysates were transferred to pre-cooled 1.5 ml-microcentrifuge tubes, vortexed for 30 seconds and centrifuged at 17,530 x g for 3 minutes at 4°C. Lysate supernatants were transferred to fresh, pre-cooled 1.5 ml-microcentrifuge tubes and stored at -20°C short-term or at -80°C long-term. The protein concentration of each sample was determined by spectrophotometric analysis at 595 nm according to the method of (Bradford, 1976). Duplicates of 2 µg, 4 µg and 6 µg BSA were made up to 100 µl with H<sub>2</sub>O and utilised as reference standards. Duplicates of 5 µl from each sample were added to 95 µl distilled H<sub>2</sub>O. To all samples and reference standards, 1 ml Bradford's reagent (made as described in section 2.2.1) was added and spectrophotometric analysis performed in a WPA 2000 spectrophotometer within 10 minutes of reagent addition. The mean absorbance for each sample duplicate was calculated and the protein concentration determined by comparison to the calculated mean  $A_{595}/\mu\text{g}$  BSA derived from the linear portion of the BSA reference standard curve.

### 2.4.8 Specific inhibitors

A number of specific inhibitors, added to HAEC or HeLa cell lines prior to lysate preparation (as described in the figure legends), were used to determine the contribution of certain molecules in the VEGF-AMPK signalling cascade. Table 2.5 is an overview of these inhibitors.

Compound/formula	Description	References
2-APB (C <sub>6</sub> H <sub>5</sub> )BOCH <sub>2</sub> CH <sub>2</sub> NH <sub>2</sub>	Membrane-permeable modulator of intracellular IP <sub>3</sub> -induced calcium release.	(Maruyama et al., 1997; Ascher-Landsberg et al., 1999)
Compound C C <sub>24</sub> H <sub>25</sub> N <sub>5</sub> O	A cell-permeable compound that acts as a potent, selective, reversible, and ATP-competitive inhibitor of AMPK. Does not affect the activities of ZAPK, Syk, PKC $\theta$ , PKA, or JAK3.	(Zhou et al., 2001)
L-NAME C <sub>7</sub> H <sub>15</sub> N <sub>5</sub> O <sub>4</sub> .HCl	Cell permeable. More soluble analog of arginine and a competitive, slowly reversible inhibitor of eNOS.	(Moore et al., 1990; Moncada et al., 1991)
PP1 C <sub>19</sub> H <sub>19</sub> N <sub>5</sub>	A potent and cell-permeable inhibitor of Src-family tyrosine kinases.	(Bishop and Shokat, 1999; Bishop et al., 2000; Hanke et al., 1996)
R-59949 C <sub>28</sub> H <sub>25</sub> F <sub>2</sub> N <sub>3</sub> OS	Inhibits DAG kinase in isolated platelet membranes and in intact platelets by binding to the catalytic domain.	(de Chaffoy de et al., 1989; Rodriguez-Linares et al., 1991).
RHC-80627 C <sub>20</sub> H <sub>34</sub> N <sub>4</sub> O <sub>4</sub>	Selective inhibitor of diacylglycerol lipase activity.	(Sutherland and Amin, 1982)
STO-609 C <sub>19</sub> H <sub>10</sub> N <sub>2</sub> O <sub>3</sub> .C <sub>2</sub> H <sub>4</sub> O <sub>2</sub>	Selective, cell-permeable inhibitor of CaMKK $\alpha/\beta$ ; competes for the ATP-binding site. Displays > 80-fold selectivity over CaMKI, CaMKII, CaMKIV, MLCK, PKC, PKA and MAPK.	(Tokumitsu et al., 2002)



SU1498	A potent and selective inhibitor of VEGF-R2 that has a weak inhibitory effect on PDGFR, EGFR and HER-2 kinases	(Strawn et al., 1996)
U73122 C <sub>29</sub> H <sub>40</sub> N <sub>2</sub> O <sub>3</sub> .xH <sub>2</sub> O	Acts as an inhibitor of agonist-induced phospholipase C activation in human platelets and neutrophils.	(Thompson et al., 1991; Yule and Williams, 1992; Stam et al., 1998)
Wortmannin C <sub>23</sub> H <sub>24</sub> O <sub>8</sub>	A specific irreversible inhibitor of PI3K catalytic activity.	(Powis et al., 1994)

**Table 2-5 Specific inhibitors**

An overview of the actions of the specific inhibitors utilised in the present study. These agents are discussed in further detail in the subsequent results chapters as appropriate.

## 2.5 Recombinant AMPK adenovirus preparation

Control and recombinant adenovirus expressing dominant negative and constitutively active AMPK, described previously in (Woods et al., 2000), were generous gifts from Dr. F. Foufelle, Centre Biomédical des Cordeliers, Paris.

### 2.5.1 Propagation

Thirty confluent 150 cm<sup>2</sup> flasks of HEK293 cells were infected with crude viral extract retained from previously infected HEK293 cells. Once the cytopathic effects of the virus had caused the cells to detach from the flasks, cells were harvested and pelleted by centrifugation at 146 x g for 5 minutes. The supernatant was removed and the cell pellet re-suspended in 10 ml sterile PBS and frozen at -20°C, ready for viral harvesting and purification.

### 2.5.2 Purification

Cell pellets were defrosted on ice and 10 ml of Arklone P was added and shaken for 10 seconds. The adenovirus was centrifuged at 252 x g for 15 minutes at room temperature. The top layer containing the adenovirus was extracted and purified by CsCl density centrifugation. 2.5 ml of sterile CsCl was dissolved in 5 mM Tris-HCl, 1 mM EGTA pH 7.8 at a density of 1.33 g/ml (0.44 g/ml CsCl) and added to a sterile Beckman Coulter Ultraclear™ centrifuge tube. 1.5 ml of sterile 1.45 g/ml CsCl (made as above by adding 0.6 g/ml CsCl) was slowly added below the less dense CsCl layer. The clarified adenovirus was then carefully added to the top of the CsCl gradient and centrifuged in an Optima XL-80K ultracentrifuge using a Beckman SW40TI rotor at 100,000 x g, 8°C for 90 minutes with the deceleration set to zero resulting in the production of a translucent white band between the two layers of CsCl, representing pure adenovirus. This viral band was extracted into a sterile 2.5 ml syringe using a 21-gauge needle. The viral band was then transferred to a slide-a-lyzer dialysis cassette (Pierce) and dialysed in 500 ml cold 5 mM Tris-HCl, 1 mM EDTA buffer pH 7.8 overnight. The following day the virus was further dialysed for 2-4 hours against fresh 5 mM Tris, 1 mM EDTA buffer containing 10 % (v/v) glycerol, then removed, aliquoted and stored at -80°C.

### 2.5.3 Titration

Purified adenovirus was titered using the Cell Biolabs QuickTiter™ Adenovirus Titer Immunoassay Kit as recommended by the manufacturer. HEK293 cells were harvested and re-suspended in complete medium at  $2.5 \times 10^5$  cells/ml. 1 ml was seeded in each well of a 24 well plate and incubated in 5 % (v/v) CO<sub>2</sub>, 95 % (v/v) air, at 37°C for 1h. Immediately prior to infection a 10-fold serial dilution of viral sample from  $10^{-3}$  to  $10^{-7}$  was set up. 100 µl of the viral sample, and a negative control, was added to duplicate wells. Infected cells were incubated in 5% (v/v) CO<sub>2</sub>, 95% (v/v) air, at 37°C for 2 days. Culture medium was removed from wells, infected cells fixed with 0.5 ml of cold methanol, then incubated at -20°C for 20 minutes. Fixed cells were washed 3 times with PBS then blocked with 1 % (w/v) BSA in PBS for 1h. Cells were then immunostained for 1h with 0.25 ml of α-hexon antibody (supplied). The hexon proteins are the largest and most abundant of the structural proteins of the adenovirus capsid. After 3 PBS washes secondary antibody (HRP-conjugated) was added for a further hour. Binding of the HRP-conjugated antibody was subsequently detected by incubation with a solution of the HRP substrate, diaminobenzidine (DAB: supplied). DAB undergoes oxidative polymerisation in the presence of HRP to produce a dark brown precipitate. Adenovirus-infected cells stained rapidly and were clearly visible under light microscopy as discrete brown patches in the cell monolayer. Positively stained cells were counted in ten fields at a virus dilution that gave 5-50 positive cells/field when viewed using a 10 x objective. The mean result was determined and used to calculate the number of infected cells per ml of the original adenovirus preparation to give a titre value in plaque forming units/ml (pfu/ml).

### 2.5.4 Infection of cells with recombinant adenovirus

HAEC were infected with 25 pfu/cell adenovirus and cultured for 24h prior to experimentation, unless stated otherwise. Within 24h of infection with a GFP-expressing virus, the majority (>95 %) of HAECs expressed GFP.

## 2.6 Biochemical assays

### 2.6.1 SDS-polyacrylamide gel electrophoresis and Western blotting

#### 2.6.1.1 SDS-polyacrylamide gel electrophoresis

Protein samples (as described in section 2.4.7) were resolved by SDS-PAGE. Cell lysate samples were mixed 3:1 with 4X SDS-containing sample buffer and heated to 95°C in a heating block for 4-5 minutes. SDS-gels for cell lysates routinely consisted of 6-10 % (w/v) acrylamide resolving gels overlaid with 5 % (w/v) stacking gels. All gels were cast in Mini-Protean III gel casting equipment (Bio-Rad). Gel lanes were loaded with equal amounts of protein (3-15 µg). Broad range pre-stained molecular weight markers (15 µl) (New England Biolabs) were added to at least one lane. Samples were resolved at 60 V until the samples had reached the resolving gel, then the voltage was increased to 150 V and the samples were resolved until the dye front had migrated the entire length of the gel.

#### 2.6.1.2 Western blotting and immunodetection of proteins

Protein-containing samples were transferred from SDS-gels onto nitrocellulose membranes at 60 V for 2h, or at 40 mA overnight, using Mini-Protean II/III equipment (Bio-Rad). Membranes were briefly stained with Ponceau S to check for equal loading of the gels, and blocked for 1h in TBST containing 5 % (w/v) non-fat dried milk. Following brief washing in TBST, the membranes were incubated overnight with primary antibody, diluted as shown in (2.1.7.1). All washes and incubation steps were carried out under agitation. After primary antibody probing, membranes were washed three times in TBST and incubated for 1h in HRP-linked species-specific secondary antibodies, diluted 1:1000-1:2000 in TBST containing 1 % (w/v) milk (for primary antibodies raised in sheep, a HRP-conjugated protein G secondary detection agent was used (2.1.7.2)). Following three washes with TBST, 2 ml of each of the ECL reagents (as described in section 2.2.1) was added to each nitrocellulose membrane and incubated for 1 minute. Chemiluminescence was detected with a Kodak X-Omat using Kodak MXB blue-sensitive X-Ray film. In order to quantify phosphorylation levels of a protein, the Western blot band intensity of a phosphorylated protein, detected with a site-specific phospho-antibody, was measured and expressed as a ratio to the corresponding total protein within the same sample.

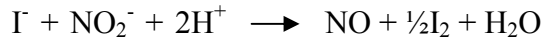
### 2.6.1.3 Densitometric quantification of protein bands

The antibody-detected bands on the developed film were scanned on a Mercury 1200c scanner, using Adobe Photoshop software. The intensity of the immunodetected protein bands on the film was measured using ImageJ software.

## 2.6.2 NO assay

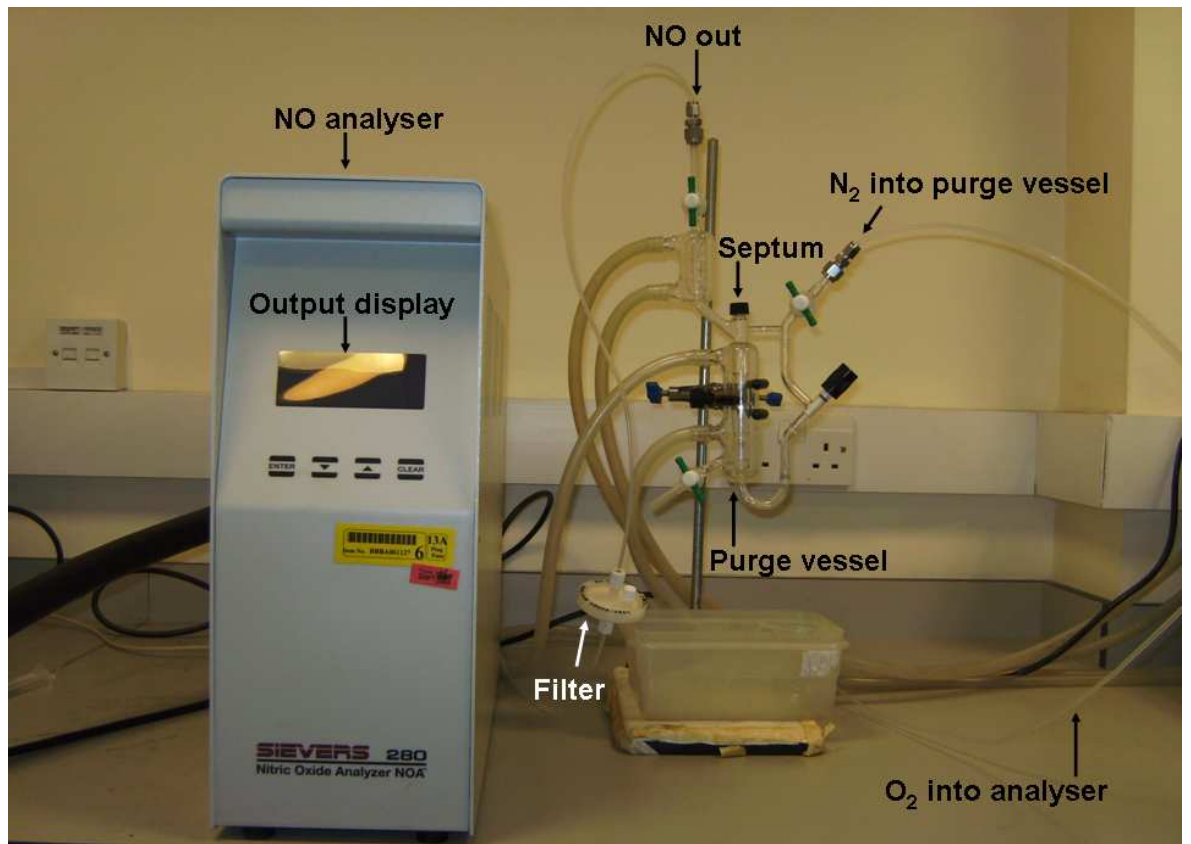
### 2.6.2.1 NO measurement

NO released from cells reacts with dissolved oxygen in the cell culture medium or buffer to form nitrate and, predominantly, nitrite. NO production by HAEC was hence analysed by a nitrite reduction method (described below) using a Sievers Nitric Oxide Analyzer 280 (Figure 2.2). The NO analyser calculates the amount of NO produced by the cells from the amount of nitrite present in the cell culture supernatant sample. To set up the NO analyser for nitrite reduction, a reducing agent (composed of 5 ml nitrogen-flushed glacial acetic acid and 50 mg nitrogen-flushed NaI dissolved in 1.5 ml of de-ionised water) was added to the purge vessel and flushed with N<sub>2</sub> gas to purge any traces of NO<sub>2</sub><sup>-</sup> from the vessel. After 30 minutes of purging, the purge vessel was sealed with a septum and the reducing agent was refluxed under N<sub>2</sub> gas. Prior to each experiment, a nitrite standard curve was prepared: From a standard solution of 100 mM NaNO<sub>2</sub>, serial dilutions of 50 μM, 10 μM, 1 μM and 100 nM were prepared and injected into the purge vessel using an Exmire microsyringe. Under the nitrite-reducing conditions used, nitrite present in the standards was reduced to NO as shown in equation 1. The NO produced was then detected by the NO analyser and reacted with O<sub>2</sub> to produce O<sub>3</sub>, which was detected by chemiluminescence. The chemiluminescence signal was converted to an electrical potential and displayed as mV by the NO analyser. The amount of NO produced by duplicates of each nitrite standard was recorded by the analyser and used to produce a calibration curve. After calculation of the standard curve, cell culture supernatant samples (prepared as described in 2.6.2.2) were injected into the purge vessel using an Exmire microsyringe. Samples were injected at 1 minute intervals to allow the output curve to return to baseline. The output in mV was then related to the amount of nitrite present in the sample using the nitrite standard curve prepared on that day.



**Equation 2-1 Production of nitric oxide from nitrite**

The chemical reaction between sodium iodide and acetic acid results in reducing conditions that cause the reduction of nitrite ( $\text{NO}_2^-$ ) to NO in the liquid sample injected into the Sievers NO meter purge vessel.



**Figure 2-2 The Sievers nitric oxide analyser**

### 2.6.2.2 Preparation of cell culture supernatants for NO analysis

HAEC cultured in 6-well plates were pre-incubated for 2h at 37 °C in 0.5 ml/well KRH buffer at 37°C. The medium was removed and replaced with 0.4 ml fresh KRH buffer in the presence or absence of VEGF (10 ng/ml), wortmannin (100 nM) and/or L-NAME (1 mM) for 15 min. The medium was removed and refluxed in glacial acetic acid containing NaI. Under these conditions, NO<sub>2</sub><sup>-</sup>, a stable breakdown product of NO, is quantitatively reduced to NO. NO-specific chemiluminescence was then analyzed using a Sievers 280A NO meter (Figure 2.2). Values were corrected for NO<sub>2</sub><sup>-</sup> present in media in the absence of cells, and the appropriate control experiments were performed in the presence of the eNOS inhibitor, L-NAME. NO production (adjusted for L-NAME-insensitive production) is expressed per hour per well.

### 2.6.3 AMPK activity assay

Total catalytic AMPK was immunoprecipitated from lysates and AMPK activity assessed by the incorporation of [ $\gamma$ -<sup>32</sup>P]-ATP into a synthetic AMPK substrate, SAMS peptide.

#### 2.6.3.1 Immunoprecipitation of catalytic AMPK

1ml of IP buffer was added to 10  $\mu$ l (per sample) protein G- Sepharose beads and centrifuged at 17,530 x g at 4°C for 30 seconds. The supernatant was removed and the beads washed 3 times with IP buffer. 2  $\mu$ g of AMPK $\alpha$ 1 and  $\alpha$ 2 antibody (per sample) was added to 10  $\mu$ l (per sample) of protein G-Sepharose slurry in IP buffer (50 % v/v) and rotated at 4°C for 2-3h. Immune complexes were then isolated by brief centrifugation at 17,030 x g at 4°C and washed 3 times with 1ml of IP buffer. 10  $\mu$ l of the antibody-protein G-Sepharose mixture (per sample) was added to cell lysates (150-200  $\mu$ g protein), to which an appropriate amount of IP buffer was added leaving each with an equal final volume. Each sample was then rotated for 3h at 4°C to allow antibody-antigen complexes to form. Samples were briefly centrifuged at 17,530 x g at 4°C and pellets washed as follows: twice with 1 ml high salt IP buffer, twice with 1 ml IP buffer and once with 1 ml HBD buffer (buffers were made as described in section 2.2.1). After the final wash the supernatant was completely removed and the pellet stored at -20°C.

#### 2.6.3.2 AMPK activity assay

Immunoprecipitated AMPK pellets (as described in section 2.6.3.1) were re-suspended in 20  $\mu$ l of HBD buffer. 5  $\mu$ l of this immunoprecipitate was added, along with 5  $\mu$ l HBD, 5  $\mu$ l

1mM AMP and 5  $\mu$ l 1mM SAMS peptide to a pre-chilled micro-centrifuge tube. 5  $\mu$ l of 0.2  $\mu$ Ci/ $\mu$ l [ $\gamma$ -<sup>32</sup>P]-ATP made up in HBD, containing 1 mM ATP and 25 mM MgCl<sub>2</sub>, was then added to each sample (giving a final 25  $\mu$ l volume). Samples were assayed in duplicate with an additional sample included where SAMS peptide was replaced with HBD and taken as a blank. All samples were incubated at 30°C for 10 minutes on a vibrating platform. 15  $\mu$ l from each sample was then removed and spotted onto a 1.5 cm x 1.5 cm square of phosphocellulose paper (P81,Whatman) which was dropped into 500 ml (1 % v/v) H<sub>3</sub>PO<sub>4</sub> stopping the reaction. The squares were washed for ~20 minutes as follows: two water washes, one H<sub>3</sub>PO<sub>4</sub> wash and then two final water washes. Squares were then air dried, added to 5 ml scintillation fluid and counted in a Beckman LS6500 scintillation counter. 5  $\mu$ l of the [ $\gamma$ -<sup>32</sup>P]-ATP mix was added to phosphocellulose paper and placed directly into 5 ml of scintillation fluid and measured to assess the total counts.

#### 2.6.4 LKB1 activity assay

5  $\mu$ g of  $\alpha$ -LKB1 antibody (a kind gift from Prof. D.G Hardie, University of Dundee) added to HAEC lysate (100  $\mu$ g protein) was adjusted to a final volume of 300  $\mu$ l with lysis buffer and rotated overnight at 4°C. 5  $\mu$ l of protein G- Sepharose slurry made up in IP buffer (50 % v/v) was then added to each lysates and mixed by rotation at 4°C for 4h. After brief centrifugation 17,530 x g at 4°C the pellet was washed 3 times in ice cold buffer (50 mM HEPES pH 7.4, 1 % Triton X-100). 600 ng of recombinant AMPK $\alpha$ 1 kinase domain (constructed and expressed as described in section 1.6.4.1) was added to the LKB1 immunoprecipitate in a total volume of 25  $\mu$ L assay buffer (50 mM HEPES, 1 mM DTT, 0.1 mM ATP, 5 mM MgCl<sub>2</sub> and shaken at 37°C for 30 minutes. Subsequently, the mixture was centrifuged (17,530 x g at 4°C for 30 seconds) and the supernatant assayed for AMPK activity using the SAMS peptide substrate as described in section 1.6.3.2

##### 2.6.4.1 AMPK $\alpha$ 1 kinase domain

50  $\mu$ l glycerol stock of *E. coli* (BL21) transformed with a plasmid encoding the kinase domain of the  $\alpha$ 1 AMPK subunit (Scott et al., 2002) was plated overnight (LB-amp) (plates described in section 1.3.1). A single colony was placed in 400 ml of LB broth (described in section 1.2.2) containing 2 % (w/v) glucose and ampicillin (50  $\mu$ g/ml) until A<sub>600</sub> reached 0.4. IPTG was added to a final concentration of 1 mM, and the culture was incubated for a further three hours. The cells were harvested by centrifugation and purification was carried out at 4°C. Cell pellets were re-suspended in 8 ml of ice-cold lysis buffer (50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1 mM EDTA, 1 mM DTT, 0.1 mM benzamidine, 0.1 mM PMSF,



5 µg/ml SBTI, 1 mg/ml lysozyme). The suspensions were left on ice for 30 minutes, Triton X-100 added to 2 % (v/v), and the suspension vortex mixed for one minute. The lysate were clarified by centrifugation (45,000 x g for 1h at 4 °C). A pre-packed glutathione-sepharose column (2 ml) was washed with six volumes of lysis buffer and the lysate applied. The column was washed with ten volumes of lysis buffer containing 1 % (v/v) Triton X-100 and 1 M NaCl, followed by six volumes of lysis buffer without Triton X-100. The protein was eluted with 15 ml of elution buffer (50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1 mM EDTA, 1 mM DTT, 0.1 mM benzamidine, 0.1 mM PMSF, 5 µg/ml of SBTI, 20 mM reduced glutathione). The eluate was concentrated by centrifugation in Vivaspin-4 microconcentrators (Vivascience) (4200 x g for 3h at 4 °C). The protein was exchanged into kinase storage buffer (50 mM Na Hepes (pH 7.4), 50 mM NaCl, 1 mM EDTA, 1 mM DTT, 0.1 mM benzamidine, 0.1 mM PMSF, 5 µg/ml of SBTI) and stored at -80 °C.

### 2.6.5 Fatty acid oxidation assay

Palmitate oxidation was measured on the basis of  $^3\text{H}_2\text{O}$  production as described previously (Moon and Rhead, 1987). In order to account for intracellular fatty acid pools, HAEC cultured in 12 well plates until 90-95 % confluent were pre-labelled with 2 µCi/ml [ $^3\text{H}$ ]-palmitic acid 24h prior to the start of the experiment (Dagher et al., 2001). The reaction mixture was prepared by adding 50 µCi of [ $^3\text{H}$ ]-palmitic acid to 300 µl of 2.2 mM unlabelled palmitic acid in absolute ethanol. After complete evaporation of the solvent under  $\text{N}_2$  gas, the fatty acid was re-suspended in 300 µl of Earles-HEPES solution containing 50 µM carnitine (Dagher et al., 2001) and 10 mg/ml fatty acid free BSA. The mixture was incubated at 37°C for 30 minutes in a water bath and for a further 30 minutes with sonication. The reaction mixture was further diluted with 5.7 ml Earles-HEPES (containing 50 µM carnitine) to a final concentration of 110 µM palmitic acid. HAEC were washed twice with warm PBS before being pre-incubated with Earles-HEPES for 30 minutes. Blanks were set by incubating cells with 500 µl methanol for 3-5 minutes before the addition of the reaction mixture. HAEC were incubated with 500 µl of the reaction mixture, to which AICAR or VEGF was added, for 2 hours at 37°C. At the end of the incubation period  $^3\text{H}_2\text{O}$  was separated from the unreacted substrate. 30 µl of 10 % (w/v) fatty acid free BSA was added to the reaction mixture and mixed. 50 µl of 72 % (w/v) TCA was then added to each sample, mixed, and left on ice for 10 minutes before centrifugation at 2000 x g for 10 minutes at 4°C. The supernatant was subsequently removed and the pH adjusted to 7-9 using 1 M NaOH. Each sample then was transferred to a Dowex 1X8-200 ion exchange column constructed set in 5 ml syringes (Becton

Dickinson Plastipak) to separate residual  $^3\text{H}_2\text{O}$ . Samples were eluted with 2 ml of deionised water and collected in scintillation vials to which 10 ml of scintillant was added before counting in a Beckman LS6500 scintillation counter.

### 2.6.6 Proliferation assay

HAEC proliferation was determined using the CellTiter 96® AQueous One Solution Cell Proliferation assay kit. This is a colorimetric method for determining the number of viable cells in proliferation assays. The reagent contains a tetrazolium compound (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulphophenyl)-2H-tetrazolium, inner salt; MTS) and an electron coupling reagent (phenazine ethosulphate; PES). The MTS tetrazolium compound is bio-reduced by cells into a coloured formazan product that is soluble in tissue culture medium. The quantity of the formazan product as measured by the absorbance at 490 nm is directly proportional to the number of living cells in culture.

HAEC were cultured in complete media until 50-60 % confluent (96-well plate) then incubated in serum-free HAEC media for 2h. Media was subsequently removed then VEGF was added for 20 hours in basal endothelial cell media + 0.2 % FCS (no other growth factors were added) in the presence or absence of inhibitors. 20  $\mu\text{l}$  of the AQueous one solution was then added directly to each well for 4 hours and the absorbance measured at 490 nM. Wells which had not been seeded with HAEC were taken as a blank. In experiments where AMPK adenovirus was used HAEC were infected for 24h prior to serum starvation.

### 2.6.7 Migration Assay

Migration assays were performed in a modified Boyden chamber using a 48-well chemotaxis chamber (Neuroprobe). A PCTE membrane was coated with 100  $\mu\text{g/ml}$  type IV collagen in serum free media containing 0.5 % BSA for 1h. The filter was placed over a bottom chamber containing VEGF (10 ng/ml) in serum free media containing 0.5 % BSA. HAEC ( $1 \times 10^6$  cells/ml) were suspended in serum free media containing 0.5 % BSA and 50  $\mu\text{l}$  added to each well in the upper chamber. For experiments using AMPK adenovirus HAEC were infected with 25 pfu/cell adenovirus for 24h before experimentation. The assembled chemotaxis chamber was incubated for 5h at 37°C with 5%  $\text{CO}_2$  to allow cells to migrate through the collagen-coated polycarbonate filter. Non-migrated cells on the upper surface of the filter were removed by scraping, and the filter subsequently stained

with crystal violet (made up to 0.5 % w/v in 20 % methanol). Migrated cells were quantified by counting the average number of cells from 3 different fields at 40X magnification. Each condition was performed in triplicate.

## 2.7 Statistical analysis

The “two-sample Student’s t-test assuming unequal variance” (“Student’s t-test”) was used for the statistical analysis of data as indicated in the results sections. Statistical significance was defined by a two-tailed p-value of less than 0.05.

### **3 AMPK MEDIATES VEGF-STIMULATED NO PRODUCTION**

### 3.1 Introduction

VEGF, a well-known angiogenic factor important for the vascular development and maintenance of all mammalian organs (Breen, 2007), is a key regulator of endothelial cell function, stimulating differentiation, survival, migration, proliferation, and vascular permeability of endothelial cells (Cross et al., 2003; Ferrara et al., 2003). VEGF binds to VEGF receptor tyrosine kinases, which have been demonstrated to stimulate a diverse array of signalling pathways, including PLC- $\gamma$ , PI3K and Src (Cross et al., 2003; Ferrara et al., 2003).

NO, synthesized by eNOS, is an important regulator of cardiovascular homeostasis. Endothelium-derived NO promotes vasodilation and inhibits platelet aggregation, leukocyte adherence, and vascular smooth muscle proliferation (Moncada et al., 1991). These effects exert a profound influence on blood flow, vascular remodeling, and angiogenesis. Upon VEGF stimulation, NO is rapidly synthesized in the endothelium (Gelinas et al., 2002; Papapetropoulos et al., 1997; Michell et al., 1999); a response proposed to be the result of PI3K-mediated activation of PKB, which directly phosphorylates and activates eNOS at Ser-1177 (Michell et al., 1999). However, VEGF-stimulated NO production has been reported as only partially sensitive to wortmannin, suggesting that VEGF-stimulated NO production is mediated by both PI3K/PKB and a PI3K-independent kinase (Papapetropoulos et al., 1997; Gelinas et al., 2002; Thuringer et al., 2002).

In addition to PKB, AMPK has also been shown to phosphorylate and activate eNOS at Ser-1177 in endothelial cells in response to a range of agonists including AICAR, hypoxia, metformin, adiponectin and shear stress (Morrow et al., 2003; Chen et al., 2003; Nagata et al., 2003; Zou et al., 2004; Zhang et al., 2006). Furthermore, commonly used *in vitro* (endothelial cell migration and differentiation assays) and *in vivo* (blood vessel growth in mouse Matrigel plug implantation) angiogenesis models (reviewed in (Auerbach et al., 2003) and discussed further in section 5.1) suggest that AMPK mediates angiogenesis in response to adiponectin (Ouchi et al., 2004; Shibata et al., 2004) and hypoxia (Nagata et al., 2003). Thus, whether the angiogenic factor VEGF stimulates NO production, at least in part, through AMPK was determined in the present study.

These studies were conducted using commercially available HAEC (human aortic endothelial cells) (Section 2.1.6) as a model. These cells were routinely characterised by

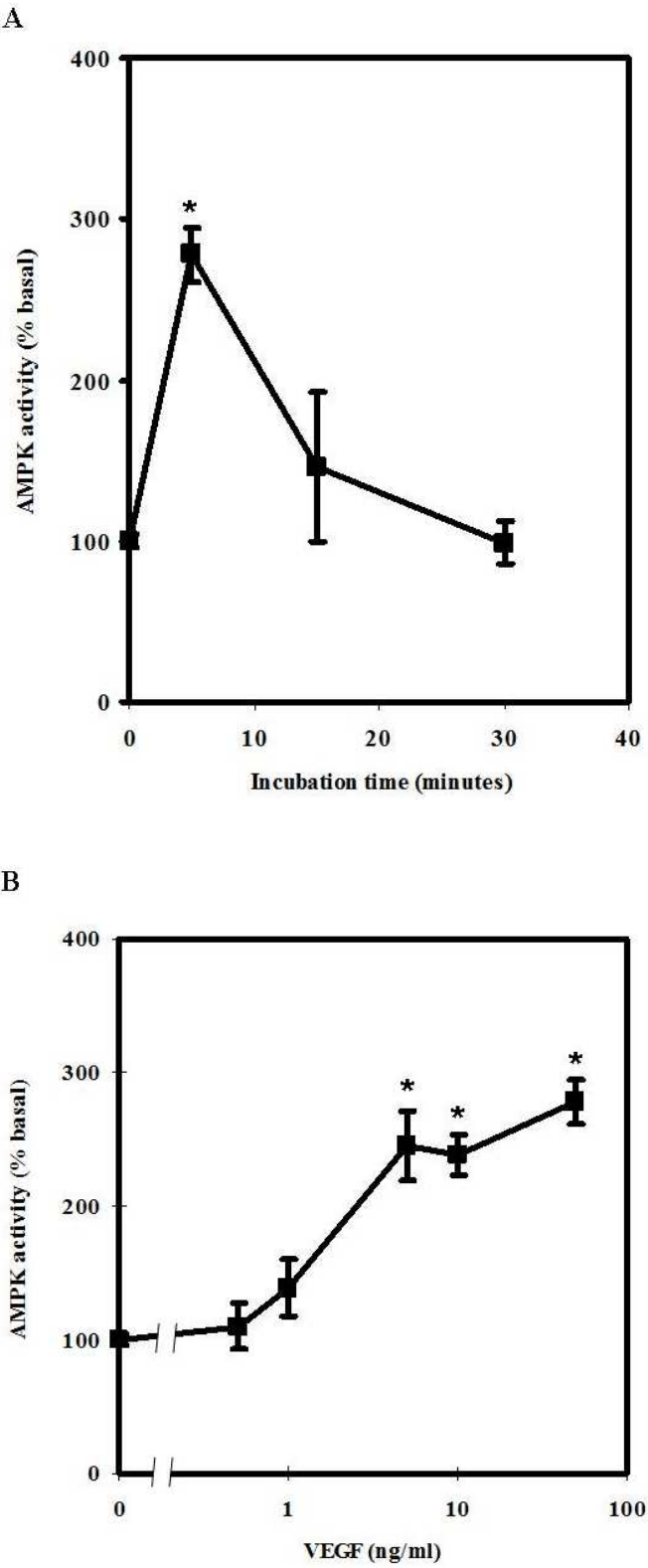
the suppliers using immunofluorescent staining (positive for acetylated low-density lipoprotein and von Willebrand factor, and negative for smooth muscle  $\alpha/\beta$  actin). In addition, expression of the endothelial cell marker CD31 was confirmed in these cells (as described in section 2.4.3) by Dr Ian Montgomery, University of Glasgow. Previous studies conducted in our laboratory have demonstrated that acute incubation of HAEC with AICAR stimulates AMPK activity, eNOS Ser-1177 phosphorylation, and NO production, effects inhibited by the expression of a dominant-negative AMPK mutant (Morrow et al., 2003).

## 3.2 Results

Initially, the effect of VEGF treatment on AMPK activity was assessed in HAEC. Total AMPK activity was assayed in HAEC incubated with 10 ng/ml VEGF for various durations after immunoprecipitation with a mixture of AMPK  $\alpha$ 1 and  $\alpha$ 2 specific antibodies. VEGF elicited a transient activation of AMPK in HAEC reaching maximal  $\sim$ 2.8-fold stimulation after 5 minutes (Figure 3.1A). Thereafter AMPK activity returned to basal levels within 30 minutes (Figure 3.1A). Activation of AMPK by VEGF was dose-dependent with a maximal ( $\sim$ 2.5-fold) stimulation resultant from treatment with 10 ng/ml VEGF (Figure 3.1B), a concentration which was used for all subsequent experiments unless indicated otherwise.

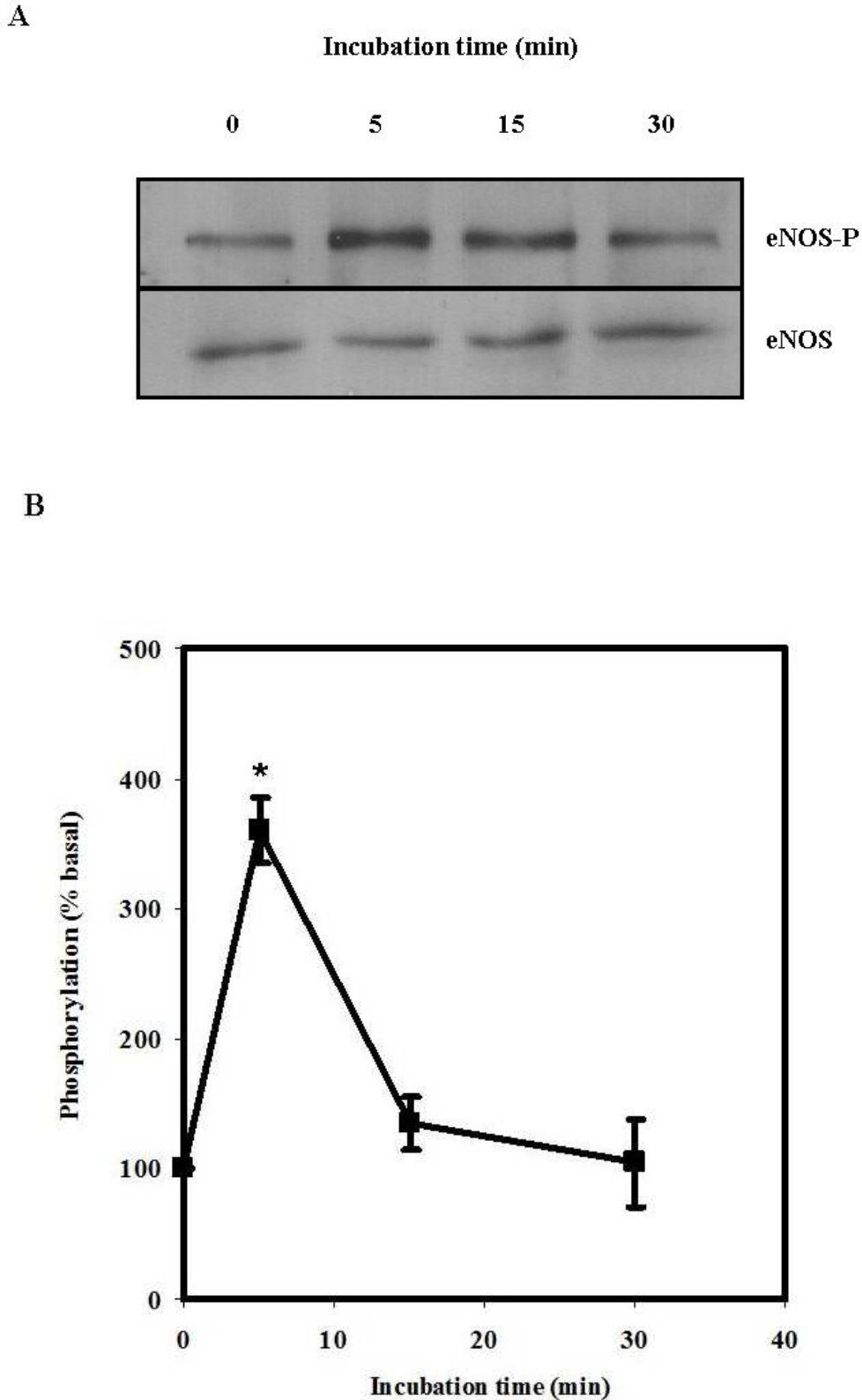
The effect of VEGF stimulation on the AMPK substrate eNOS was subsequently determined. VEGF elicited a transient phosphorylation of eNOS at Ser-1177, which reached maximal  $\sim$ 3.6-fold stimulation after 5 minutes (Figure 3.2). Thereafter, eNOS phosphorylation rapidly returned to basal levels (Figure 3.2). Activation of eNOS at Ser-1177 by VEGF was dose-dependent with maximal stimulation ( $\sim$ 3.6-fold) elicited by 10 ng/ml VEGF (Figure 3.3). VEGF was found to stimulate phosphorylation of eNOS at Ser-1177 in a time- (Figure 3.2) and dose-dependent (Figure 3.3) manner, parallel with VEGF-stimulated AMPK activation (Figure 3.1), suggesting a causal relationship.





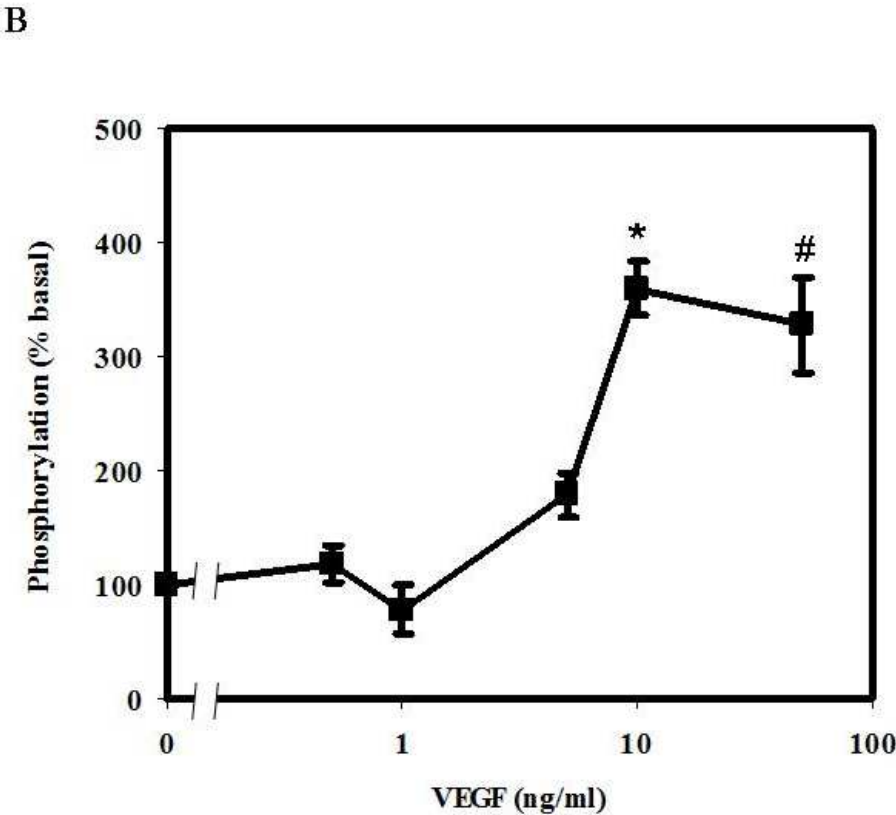
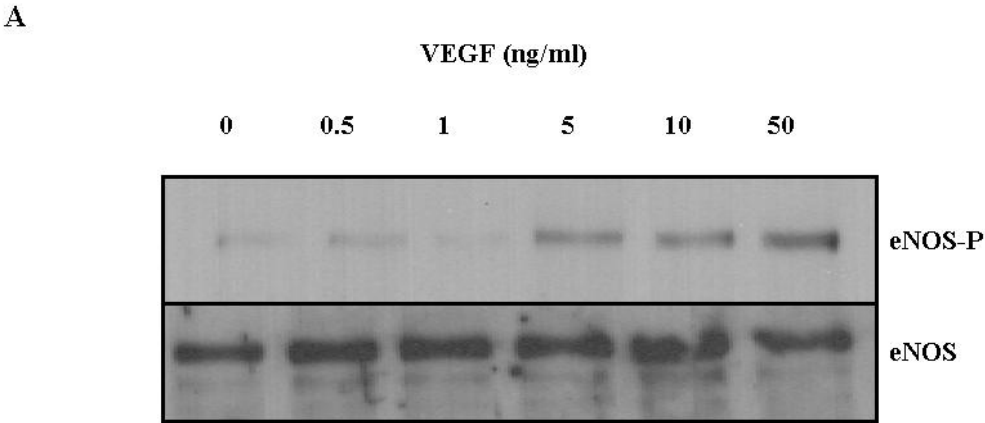
**Figure 3-1 Effect of VEGF treatment on AMPK activity in HAEC**

HAEC were incubated with 10 ng/ml VEGF for various durations (A) or incubated with various concentrations of VEGF for 5 minutes (B) and lysates prepared. Total AMPK was immunoprecipitated from both sets of lysates using AMPK  $\alpha$ 1 and  $\alpha$ 2 specific antibodies then AMPK activity assessed (described in detail in section 2.6.3). The results are expressed as a mean  $\pm$  SD % basal AMPK activity for three independent experiments in each case. \* $p$ <0.01 relative to value in absence of VEGF.



**Figure 3-2 Time course: VEGF-stimulated phosphorylation of eNOS at Ser-1177**

Lysates, prepared from HAEC incubated with 10 ng/ml VEGF for the indicated durations, were subjected to SDS PAGE, transferred to nitrocellulose and probed with the antibodies indicated. Probing of lysates with  $\alpha$ -eNOS and  $\alpha$ -eNOS Ser-1177 resulted in a single band at ~140 kDa in both instances. (A) Representative immunoblots are shown in, repeated with similar results on 3 different samples of lysates. (B) Quantification of eNOS Ser-1177 phosphorylation using NIH image software. The results are expressed as the mean  $\pm$  SD % basal eNOS phosphorylation for three independent experiments. \* $p < 0.01$  relative to value in absence of VEGF.

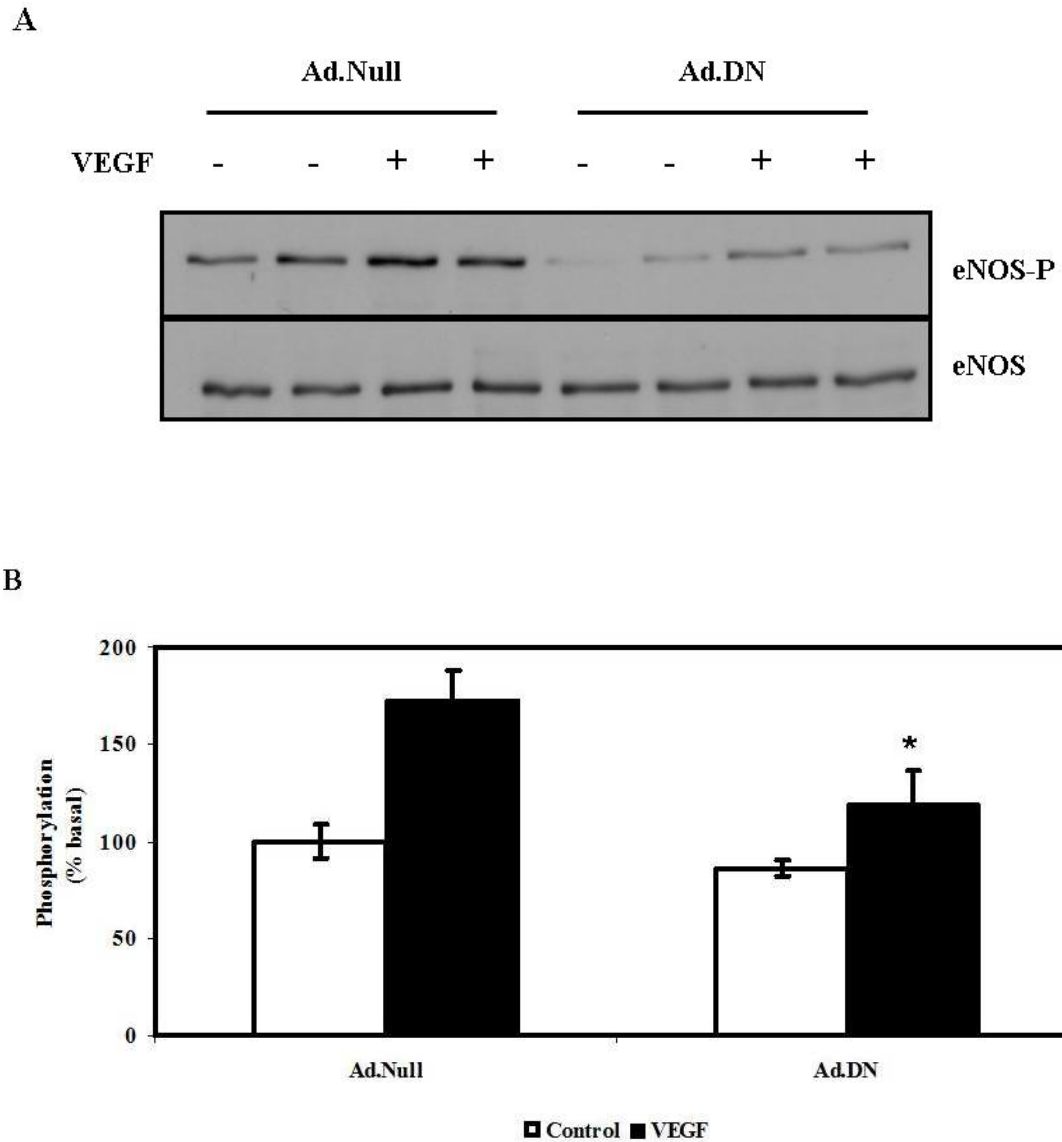


**Figure 3-3 Dose response: VEGF-stimulated phosphorylation of eNOS at Ser-1177**

HAEC were incubated with the indicated concentrations of VEGF for 5 minutes and lysates prepared. Lysates were subjected to SDS PAGE, transferred to nitrocellulose and probed with the antibodies indicated. Probing of lysates with  $\alpha$ -eNOS and  $\alpha$ -eNOS Ser-1177 resulted in a single band at ~140 kDa in both instances. The intensity of the resultant bands was quantified using NIH image software. (A) Representative immunoblots are shown and repeated with similar results on 3 different samples of lysates which are quantified in (B). Quantification of eNOS Ser-1177 phosphorylation was done using NIH image software. The results are expressed as the mean  $\pm$  SD % basal eNOS phosphorylation for three independent experiments. \* $p < 0.01$  relative to value in absence of VEGF. #  $p < 0.05$  relative to value in absence of VEGF.

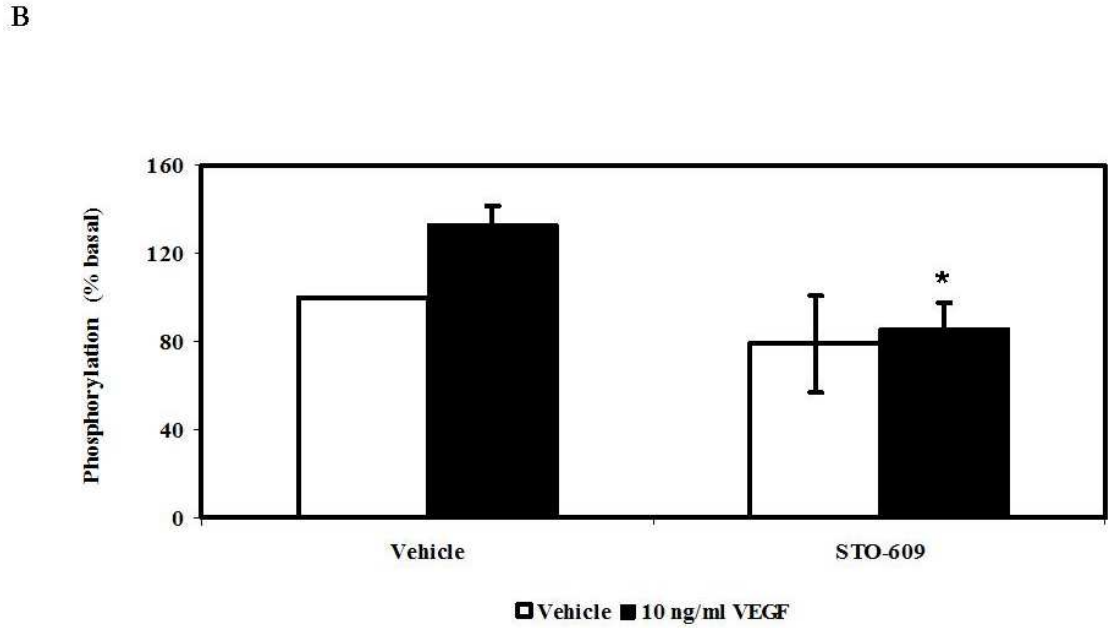
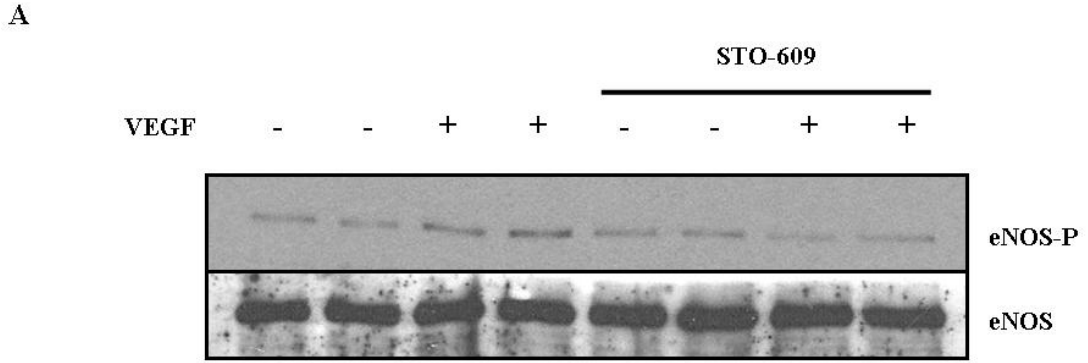
In order to determine the role of AMPK in VEGF-eNOS signalling adenovirus-mediated gene transfer was used to express a mutant form of AMPK (Woods et al., 2000), that acts as a dominant negative inhibitor of endogenous AMPK. Aspartate 157 within the  $\alpha$  subunit, which lies in the conserved DFG motif (subdomain VII in protein kinase catalytic subunits), has been shown as essential for MgATP binding in protein kinases (Johnson et al., 1996). Mutation of this residue to alanine (in either  $\alpha 1$  or  $\alpha 2$ ) yields an inactive kinase, but does not affect the binding of  $\beta\gamma$  subunits within the complex (Stein et al., 2000). This overexpressed mutant form competes with native  $\alpha$  subunits for  $\beta\gamma$  binding thus eliciting its dominant negative effect as the intact heterotrimeric AMPK complex ( $\alpha\beta\gamma$ ) is essential for kinase activity. Infection of HAEC with this mutant (Ad.DN) significantly reduced VEGF-stimulated phosphorylation of eNOS at Ser-1177 (Figure 3.4). Quantification of eNOS phosphorylation status in Ad.DN-infected cells revealed VEGF-stimulated phosphorylation at Ser-1177 was reduced by approximately ~70 % (Figure 3.4B).

Pre-treatment of HAEC with STO-609 (described in table 2.5), a selective inhibitor of the upstream AMPK kinase CaMKK (Tokumitsu et al., 2002), that has been shown to inhibit thrombin-induced AMPK activation in the endothelium (Stahmann et al., 2006), abrogated VEGF-stimulated eNOS phosphorylation at Ser-1177 (Figure 3.5B), supporting a role for CaMKK-AMPK in the regulation of this event in HAEC.



**Figure 3-4 Effect of Ad.DN on VEGF-stimulated phosphorylation of eNOS at Ser-1177**

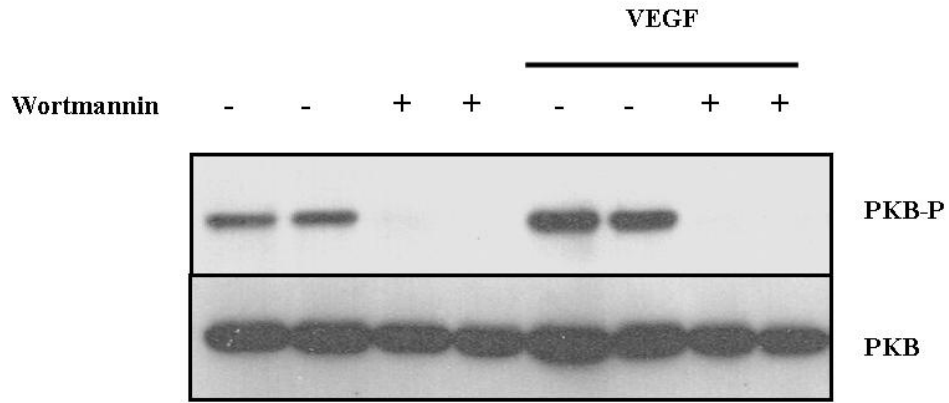
HAEC were infected with Ad.DN or a null AMPK adenovirus (Ad.Null). Subsequently, HAEC lysates were prepared from cells incubated in the presence or absence of 10 ng/ml VEGF. Lysates were resolved by SDS PAGE, transferred to nitrocellulose and probed with the antibodies indicated. Probing of lysates with  $\alpha$ -eNOS and  $\alpha$ -eNOS Ser-1177 resulted in a single band at ~140 kDa in both instances. (A) Representative immunoblots are shown, repeated with similar results on 4 different samples of lysates. (B) Quantification of eNOS Ser-1177 phosphorylation from four independent experiments using NIH image software. \* $p < 0.05$  relative to value in control virus-infected cells. These experiments were performed by Dr I. Salt.



**Figure 3-5 Effect of STO-609 on VEGF-stimulated phosphorylation of eNOS at Ser-1177**

HAEC were treated in the presence or absence of 10 ng/ml VEGF for 5 minutes after pre-incubation with 25  $\mu$ M STO-609 for 15 minutes and lysates prepared. Lysates were resolved by SDS PAGE, transferred to nitrocellulose and probed with the antibodies indicated. Probing of lysates with  $\alpha$ -eNOS and  $\alpha$ -eNOS Ser-1177 resulted in a single band at ~140 kDa in both instances. (A) Representative immunoblots are shown, repeated with similar results on 4 different samples of lysates. (B) Quantification of eNOS Ser-1177 phosphorylation from four independent experiments using NIH image software. \* $p < 0.05$  relative to value in the absence of STO-609.

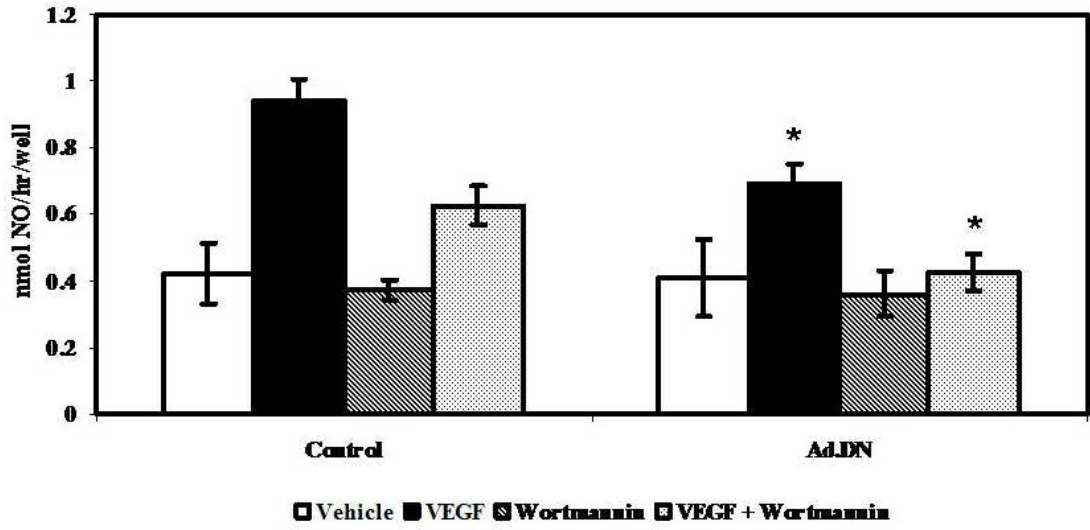
To characterise whether VEGF-stimulated NO production was indeed mediated by AMPK, VEGF-stimulated NO production was assayed in HAEC infected with Ad.DN. VEGF treatment elicited a ~2.2-fold stimulation of NO production (Figure 3.7). In agreement with other studies, pre-incubation of HAEC with the PI3K inhibitor wortmannin incompletely but significantly reduced VEGF stimulated NO production (~65 %) (Figure 3.7), at a concentration that completely inhibited phosphorylation of PKB at Ser-473 (Figure 3.6). These data suggest both PI3K/PKB and a PI3K-independent kinase mediate VEGF-stimulated NO production. Infection of HAEC with Ad.DN caused a ~40 % reduction in VEGF-stimulated NO production (Figure 3.7). Treatment of HAEC with Ad.DN alone was without effect on basal NO production (Figure 3.7). In the presence of wortmannin, infection with Ad.DN significantly reduced VEGF-stimulated NO production to basal levels (Figure 3.7), suggesting that AMPK, as well as PKB, contributes to the regulation of VEGF-stimulated NO production.



**Figure 3-6 Effect of wortmannin on VEGF-stimulated PKB phosphorylation at Ser-473**

Lysates were prepared from HAEC incubated in the presence or absence of 10 ng/ml VEGF for 5 minutes after pre-treatment with 100 nm wortmannin for 40 minutes. Lysates were subjected to SDS-PAGE, transferred to nitrocellulose and probed with the antibodies indicated. Probing of lysates with  $\alpha$ -PKB and  $\alpha$ -PKB Ser-473 resulted in a single band at ~60 kDa in both instances. This experiment was performed by Dr I. Salt.





**Figure 3-7 Effect of Ad.DN on VEGF-stimulated NO production**

HAEC were infected with Ad.Null or Ad.DN adenoviruses. Cells were subsequently incubated in KRH buffer in the presence or absence of 10 ng/ml VEGF and/or 100 nM wortmannin. After 15 minutes, medium was removed and assayed for L-NAME-sensitive  $\text{NO}_2^-$  content. The data shown represent the means  $\pm$  SD NO synthesis from nine independent experiments. \* $p < 0.05$  relative to value in Ad.Null-infected cells. These experiments were performed by Dr I. Salt.

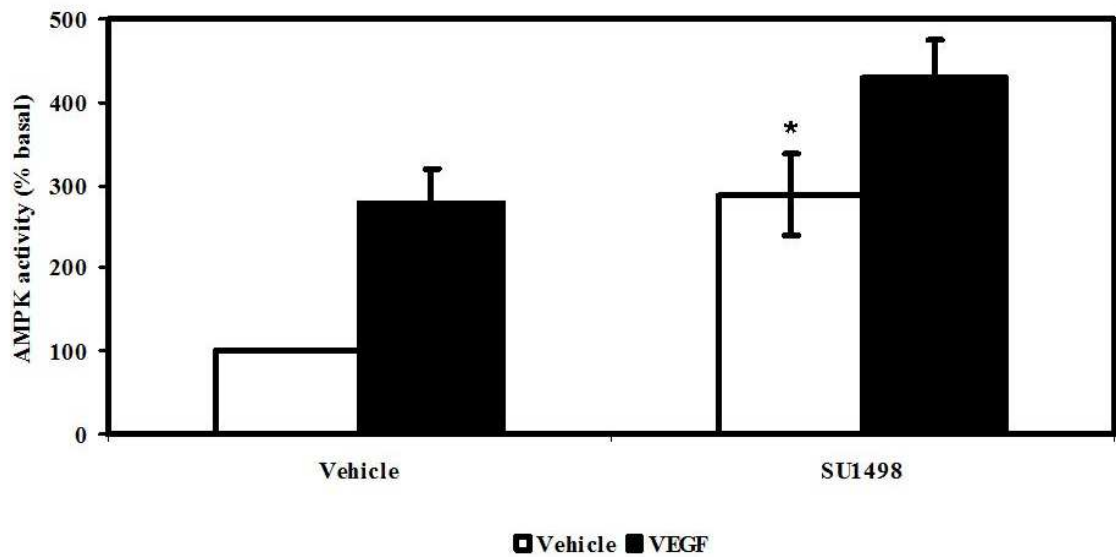
Endothelial cells express VEGF-R1 and VEGF-R2 (Zachary, 2003; Cross et al., 2003). Due to the obstruction of vessels by an overgrowth of endothelial cells VEGF-R1 deficient mice exhibit embryonic lethality (Fong et al., 1995). Interestingly however, deletion of the intracellular domain of VEGF-R1 is compatible with normal vascular development (Hiratsuka et al., 1998; Barleon et al., 1996). As a result of these findings it has previously been postulated that VEGF-R1 acts as a decoy, binding and neutralising VEGF in the absence of catalytic activation. Indeed the majority of known biological responses of VEGF in endothelial cells are reported to be mediated by the activation of VEGF-R2 (Cross et al., 2003; Zachary, 2003).

The VEGF-R2 specific inhibitor SU1498 (described in table 2.5) was used to assess the role of this receptor subtype in VEGF-mediated AMPK activation in HAEC. SU1498 is a small molecule reported to specifically inhibit the enzymatic activity of VEGF-R2 (Strawn et al., 1996). The agent has been shown to act as an angiogenesis inhibitor, as demonstrated by its activity in the chorioallantoic membrane (CAM) assay and in an *in vivo* VEGF-induced permeability assay (Strawn et al., 1996; Jin et al., 2002). Angiogenesis assays are discussed further in section 5.1; for review see (Auerbach et al., 2003)). SU1498 (low  $\mu\text{M}$  range) has been shown to inhibit endothelial VEGF signalling in several studies including (Xu et al., 2006; Feliers et al., 2005; Rajesh et al., 2005). Perhaps surprisingly, pre-treatment of HAEC with 5  $\mu\text{M}$  SU1498 stimulated AMPK activity  $\sim 2.8$ -fold above basal even in the absence of VEGF treatment ( $278 \pm 42 \%$ ,  $n=4$ ,  $p<0.05$ ) (Figure 3.8). Stimulation with VEGF did not significantly increase AMPK activity in the presence of SU1498 (Figure 3.8).

However, as highlighted in section 1.5.5.1, a number of reports suggest VEGF-R1 may play a significant role, particularly in adult endothelia (Ahmed et al., 1997; Kanno et al., 2000; Knight et al., 2000) Intriguingly, one report suggested VEGF-mediated NO synthesis is driven through VEGF-R1 in endothelial cells (Bussolati et al., 2001) Thus, it remained possible that either VEGF-R1 or VEGF-R2 may be responsible for mediating the stimulation of AMPK/eNOS/NO production in response to VEGF treated HAEC. As described in section 1.5.3 and Figure 1.8 VEGF-B is a selective ligand for VEGF-R1, whereas VEGF-E is selective for VEGF-R2. In a preliminary experiment, HAEC treated with either VEGF-B or VEGF-E resulted in an increase in AMPK activity  $174 \pm 16 \%$  and  $193 \pm 9 \%$  respectively compared to vehicle treated controls  $100 \pm 40 \%$  (Figure 3.9), indicating that activation of either receptor alone has the potential to stimulate AMPK.

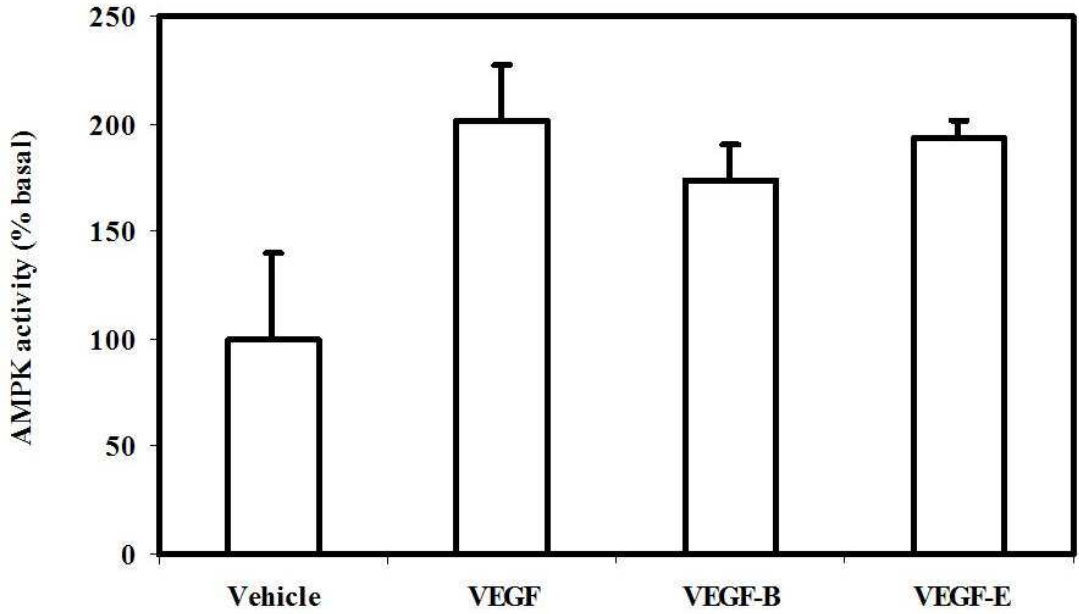
To further assess the role of individual VEGF receptor subtypes, HeLa cells were transfected with either receptor subtype, treated with VEGF and ACC phosphorylation determined as a measure of AMPK activity. As highlighted in section 1.3.1 and Figures 1.3-1.6 ACC is a major substrate for AMPK important in the regulation of fatty acid metabolism.

Neither endogenous VEGF-R1 (data not shown) nor VEGF-R2 (Figure 3.10A) was detected in HeLa lysates when probed with the appropriate receptor specific antibodies. VEGF-R2 was successfully transfected into HeLa cells (Figure 3.10A), whereas probing with a VEGF-R1 specific antibody failed to detect the receptor in HeLa<sub>VEGF-R1</sub> or HAEC lysates (data not shown). Although modest, VEGF treatment was found to increase phosphorylation of ACC at Ser-80 in mock transfected cells (Figure 3.10C), thus suggesting, whilst not detected in the present study, an endogenous expression of VEGF receptor may be present. In the absence of VEGF treatment there appeared to be an increase in phosphorylation of ACC at Ser-80 in HeLa<sub>VEGF-R1</sub> ( $136 \pm 17.9\%$ ,  $n=3$ ,  $p>0.05$ ) and HeLa<sub>VEGF-R2</sub> transfected cells ( $157 \pm 26\%$ ,  $n=3$ ,  $p>0.05$ ) in comparison to mock transfected controls (Figure 3.10C). Similarly, in the presence of VEGF treatment, HeLa<sub>VEGF-R1</sub> ( $167 \pm 28\%$ ,  $n=3$ ) and HeLa<sub>VEGF-R2</sub> ( $174 \pm 26\%$ ,  $n=3$ ) both demonstrated a marked, but non-significant increase in phosphorylation of ACC at Ser-80 in comparison to mock transfected cells ( $111 \pm 2\%$ ,  $n=3$ ) (Figure 3.10C).



**Figure 3-8 Effect of SU1498 on VEGF-stimulated AMPK activity**

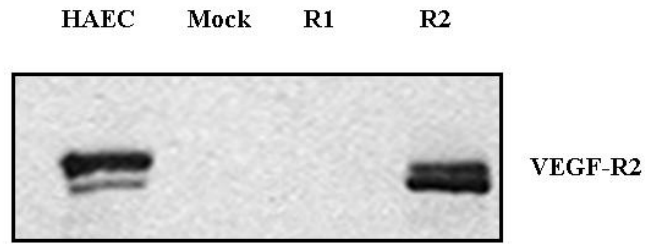
HAEC were incubated in the presence or absence of 10 ng/ml VEGF for 5 minutes after pre-treatment with 5  $\mu$ M SU1498 for 25 minutes and lysates prepared. Total AMPK was immunoprecipitated from HAEC lysates (200  $\mu$ g) and AMPK activity assessed. The results are expressed as a mean  $\pm$  SEM % basal AMPK activity for four independent experiments. \* $p$ <0.05 relative to value in absence of SU1498. These experiments were performed in collaboration with Dr I. Salt.



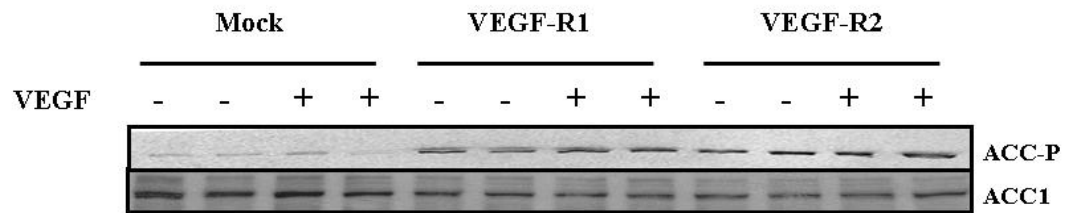
**Figure 3-9 Effect of receptor specific VEGF subtypes on AMPK activity**

HAEC were incubated with 10 ng/ml VEGF, VEGF-B or VEGF-E for 5 minutes and lysates prepared. Total AMPK was immunoprecipitated from HAEC lysates (200µg) and AMPK activity assessed. The results are expressed as a mean ± SEM % basal AMPK activity for one experiment carried out in duplicate.

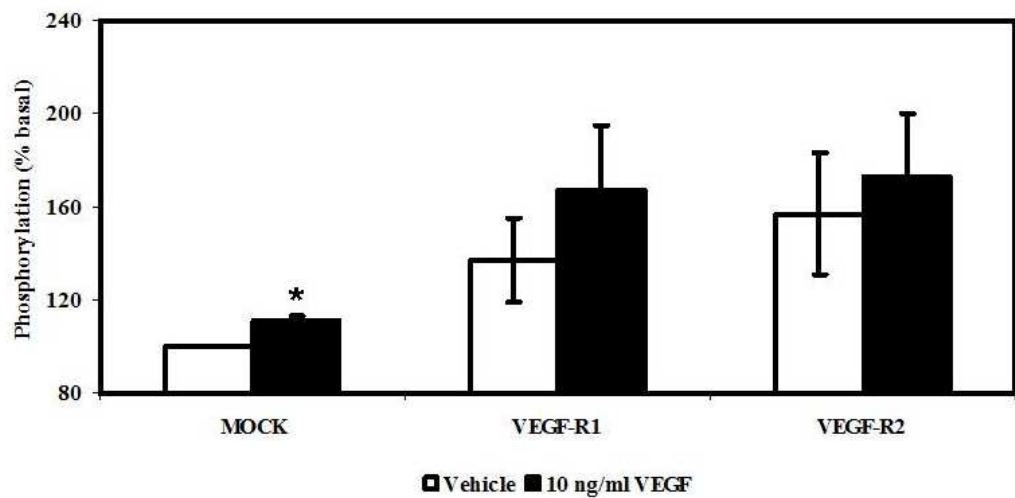
A



B



C



### Figure 3-10 Effect of VEGF receptors in HeLa cells

(A) Lysates were prepared from HeLa cells transfected with mock, VEGF-R1 (R1) or VEGF-R2 (R2) DNA (as described in section 2.4.6). A lysate was also prepared from untransfected HAEC. Lysates were subsequently subjected to SDS PAGE, transferred to nitrocellulose and probed with the antibody indicated. Probing of lysates with  $\alpha$ -VEGF-R2 resulted in two distinct bands at ~210 and 230 kDa. (B) HeLa cells were transfected with mock, R1 or R2 DNA and subsequently treated in the presence and absence of 10 ng/ml VEGF for 5 minutes and lysates prepared. Lysates were then subjected to SDS PAGE, transferred to nitrocellulose and probed with the antibodies indicated. Representative immunoblots are shown, repeated with similar results on 3 different samples of lysates quantified in (C). The results are expressed as the means  $\pm$  SEM % basal eNOS phosphorylation for three independent experiments. \* $p < 0.05$  relative to value in absence of VEGF.

### 3.3 Discussion

A central finding of the present study is the identification of VEGF as a novel activator of AMPK. Physiological concentrations of VEGF stimulate AMPK in a time- (Figure 3.1A) and dose-dependent manner (Figures 3.1B). The magnitude of VEGF-stimulated AMPK activity was found to be similar to that reported in other studies in response to a number of other stimuli (Morrow et al., 2003; Dagher et al., 2001; Zou et al., 2004; Zou et al., 2002). Furthermore, the effect of VEGF was specific to endothelial cells as VEGF had no effect on fibroblasts which do not express VEGF receptors (I.Salt, personal communication).

At the start of this work, one study had examined the effect of VEGF on AMPK phosphorylation in HUVEC (Nagata et al., 2003). Stimulation of HUVEC for 6h had no effect on AMPK phosphorylation (Nagata et al., 2003), in agreement with the present study in which VEGF-stimulated AMPK activity returned to basal levels within 30 minutes. In contrast, it has also been reported by another group that stimulation of BAEC with VEGF for 10 minutes was without effect on AMPK or ACC phosphorylation at Thr-172 or Ser-79 respectively (Zou et al., 2003). In the current study, VEGF significantly stimulated AMPK activity after incubation for 5 minutes, and returned to basal levels within 15 minutes. Thus, it is possible that by the time Zou and colleagues had sampled these cells VEGF-stimulated AMPK activity had returned to basal levels. It is also worth noting that different methods to assess AMPK activity were utilised in the two studies. In the current investigation AMPK activity was assayed using kinase assays whereas AMPK phosphorylation by Western blotting was used by (Zou et al., 2003). Indeed, a more recent study conducted in BAEC has demonstrated that VEGF (10 ng/ml) increased AMPK phosphorylation within 1 minute of agonist addition, reaching a maximum ~2.5-fold increase by 5 minutes, with a gradual return to basal levels within ~30 minutes (Levine et al., 2007).

Previous studies have proposed that VEGF-stimulated eNOS phosphorylation at Ser-1177 is mediated by PI3K/PKB (Gelinias et al., 2002; Fulton et al., 1999; Michell et al., 1999), yet complete inhibition of PKB with the PI3K inhibitor wortmannin only partially inhibited VEGF-stimulated NO production (Papapetropoulos et al., 1997; Thuringer et al., 2002; Gelinias et al., 2002). Consistent with these observations, in the present study wortmannin significantly but incompletely (~65 %) reduced VEGF-stimulated NO production (Figure 3.6), at a concentration that completely inhibited PKB phosphorylation at Ser-473 (Figure 3.6A). These data suggest that both PI3K/PKB and a PI3K-independent mechanism

mediate VEGF-stimulated NO production. Direct activation of AMPK has previously been shown to stimulate eNOS at Ser-1177 in HAEC (Morrow et al., 2003), and adenoviruses expressing dominant negative AMPK or dominant negative PKB have been demonstrated to inhibit VEGF-stimulated migration, eNOS Ser-1177 phosphorylation and tubulogenesis under conditions of hypoxia in endothelial cells (Nagata et al., 2003). Under normoxic conditions however, the dominant negative AMPK adenovirus was without effect (Nagata et al., 2003). In the present study, VEGF was found to stimulate phosphorylation of eNOS at Ser-1177 in a time- (Figure 3.2) and dose- dependent manner (Figure 3.3), concomitant with AMPK activation (Figure 3.1), suggesting that this phosphorylation may be mediated by AMPK in HAEC. Indeed, infection of HAEC with Ad.DN or pre-treatment with the CaMKK inhibitor STO-609 significantly inhibited VEGF-stimulated eNOS phosphorylation (Figures 3.4 and 3.5 respectively), and moreover Ad.DN infection resulted in a significant (~40 %) reduction in NO production (Figure 3.7). In the presence of wortmannin, infection with Ad.DN significantly reduced VEGF-stimulated NO production to basal levels (Figure 3.7). Together these data, unlike those reported by (Nagata et al., 2003), demonstrate both PKB and AMPK, in HAEC at least; mediate VEGF-stimulated phosphorylation of eNOS at Ser-1177 and NO production under normoxic conditions.

A number of recent reports have also provided evidence which supports the finding that AMPK and PKB are both important molecules which regulate VEGF-stimulated eNOS activation. Youn and coworkers have demonstrated that inhibition of AMPK using compound C completely attenuates VEGF-stimulated NO production, while wortmannin elicits a similarly potent effect, suggesting, in BAEC at least, there is some crosstalk between these pathways (Youn et al., 2008). Another study also conducted in BAEC suggests Rac1 may act as a molecular link between AMPK and PKB (Levine et al., 2007). In the present study as incubation of HAEC with wortmannin completely inhibits PKB phosphorylation (Figure 3.6) without altering AMPK activity (Figure 4.1), AMPK activation is not downstream of PI3K in response to VEGF in HAEC. It remains possible that AMPK lies upstream of PKB in VEGF-stimulated eNOS phosphorylation, as is reported in BAEC (Levine et al., 2007), but this seems unlikely as previous work conducted in HAEC reported infection with dominant negative AMPK adenoviruses did not alter PKB phosphorylation (Morrow et al., 2003). Interestingly, a recent study has demonstrated that PKB and AMPK act as two mutually independent kinases which regulate ghrelin-mediated eNOS signalling (Xu et al., 2008). It may be the case that a similar mechanism underlies VEGF-eNOS/NO signalling in HAEC.



SU1498 has been reported as a specific inhibitor of VEGF-R2. Surprisingly, pre-incubation of HAEC with the VEGF-R2 inhibitor SU1498 stimulated AMPK activity even in the absence of VEGF treatment (Figure 3.8). Stimulation with VEGF did not significantly increase AMPK activity in the presence of SU1498 (Figure 3.8). SU1498 has previously been shown to inhibit VEGF-stimulated migration and tubulogenesis of HUVEC (Boguslawski et al., 2004). However it was found that SU1498 also inhibited tubulogenesis of HUVEC in response to sphingosine 1-phosphate, basic fibroblastic growth factor and hepatocyte growth factor (Boguslawski et al., 2004). In addition SU1498 stimulated the accumulation of phosphorylated forms of ERK1/2 but blocked ERK1/2 activity in the presence of VEGF (Boguslawski et al., 2004). These data and the finding that SU1498 increases AMPK activity in the present investigation challenge the specificity of SU1498 as a VEGF-R2 inhibitor.

Further experiments were undertaken to determine the role of VEGF-R1 and VEGF-R2 in VEGF-mediated AMPK activation. Although not statistically significant ( $p > 0.05$ ), even in the absence of VEGF treatment there seemed to be a substantial increase in basal ACC phosphorylation in HeLa cells transfected with both VEGF-R1 and VEGF-R2 (Figures 3.10B and C). It is well documented that tumour cell lines, including HeLa, produce VEGF (Asano et al., 1999; Kondo et al., 1993). Indeed, VEGF was first described as a factor secreted by a hepatocarcinoma cell line that increased dye extravasation into the skin of guinea pigs (Senger et al., 1983). Therefore it is perhaps not surprising that VEGF receptor transfection resulted in increased ACC phosphorylation even in the absence of exogenous VEGF application. VEGF treatment increased phosphorylation of ACC at Ser-80 in mock transfected cells (Figure 3.10C) suggesting an endogenous expression of VEGF receptor(s). It has been reported previously that HeLa cells express cell surface VEGF receptors (Neufeld et al., 1994). The VEGF receptor subtype(s) responsible for this endogenous activation was undetermined in the present study. Neither VEGF-R1 (data not shown) nor VEGF-R2 was detected in HeLa lysates when probed with the appropriate receptor specific antibodies (Figure 3.10A). The VEGF-R2 antibody did detect the receptor in HeLa<sub>VEGF-R2</sub> and in a HAEC lysate. Notably, the VEGF-R1 antibody failed to detect the receptor in HeLa cells, HeLa<sub>VEGF-R2</sub> and in lysates made from various endothelial cell lines (data not shown). It remains possible that VEGF receptors are expressed in HeLa cells below the detection level of the antibodies used. Perhaps more suitable antibodies or an alternative approach such as RT-PCR to confirm VEGF receptor expression may be of value.

A third VEGF receptor subtype, VEGF-R3, which although primarily expressed in the lymphatic endothelium, has been reported in HUVEC, HDMEC and vascular endothelial cells *in vivo* (Makinen et al., 2001). This receptor subtype has also been demonstrated in HeLa cells (Shi et al., 2007). As VEGF activates the VEGF-R1 and VEGF-R2 (Neufeld et al., 1999), but not the VEGF-R3, subtype which is specifically activated by VEGF-C and VEGF-D (Neufeld et al., 1999), it is unlikely that any effects seen in response to VEGF in this study can be attributed to this receptor. It was further observed that in both the presence and absence of VEGF treatment there was an increase in ACC-Ser-80 phosphorylation in HeLa<sub>VEGF-R1</sub> and HeLa<sub>VEGF-R2</sub> (Figure 3.10C), and furthermore, in a preliminary experiment the VEGF-R1 and VEGF-R2 specific ligands, VEGF-B and VEGF-E respectively, had the ability to stimulate AMPK (Figure 3.9). Although the latter data represents a preliminary experiment, together these data may indicate a possible role for both VEGF receptor subtypes in the regulation of VEGF-stimulated AMPK activity, and as such warrant further investigation.

It has previously been shown that in both native and transfected endothelial cells VEGF-R2-mediated autophosphorylation leads to downstream eNOS activation (He et al., 1999; Feng et al., 1999; Wu et al., 1999; Kroll and Waltenberger, 1999; Thuringer et al., 2002). Conversely, another report has documented that VEGF-mediated NO synthesis is driven through VEGF-R1 in HUVEC, whereas specific activation of VEGF-R2 was unable to induce NO release (Bussolati et al., 2001). The addition of a neutralizing anti-VEGF-R1 antibody has been shown to significantly inhibit VEGF-induced NO release in HUVEC (Ahmed et al., 1997), and specific activation of VEGF-R1 using VEGF-B has been demonstrated to increase phosphorylation of eNOS at Ser-1177 and PKB at Ser-473, and furthermore to promote angiogenesis (Silvestre et al., 2003). It therefore remains unclear as to which receptor mediates the effect of VEGF on eNOS/NO production with different studies implicating both endothelial VEGF receptor subtypes. CYP epoxygenases metabolise arachidonic acid to EETs, which activate several signalling pathways to promote endothelial cell proliferation, migration and angiogenesis. A recent report has shown VEGF increases CYP 2C promoter activity and that a dominant negative AMPK mutant prevented the VEGF-induced increase in CYP 2C RNA and protein expression in human endothelial cells (Webler et al., 2008). It was observed that VEGF was able to increase the activity of the CYP 2C9 promoter in cells expressing both VEGF-R1 and VEGF-R2 (Webler et al., 2008). VEGF-R2 siRNA was reported to abrogate AMPK and ACC phosphorylation in response to VEGF in BAEC (Levine et al., 2007). The findings of the present investigation suggest VEGF-R1, in addition to VEGF-R2, may mediate the

effects of VEGF on AMPK activation. Activation of the VEGF-R2 receptor by VEGF in cells devoid of VEGF-R1 results in a mitogenic response, while the activation of VEGF-R1 by VEGF in cells lacking VEGF-R2 fails to induce cell proliferation (Seetharam et al., 1995; Waltenberger et al., 1994). However, activation of VEGF-R1 by VEGF does induce cell migration, a response that is also induced as a result of VEGF-R2 activation by VEGF (Barleon et al., 1996; Yoshida et al., 1996; Soker et al., 1998). These results indicate that the signal transduction cascades induced by VEGF-R1 and VEGF-R2 are somewhat different. It is an intriguing possibility that VEGF acting through different VEGF receptors may confer a specific signal to AMPK which may enable the kinase to direct separate functional effects.

## **4 THE MECHANISM OF VEGF-STIMULATED AMPK ACTIVATION**

## 4.1 Introduction

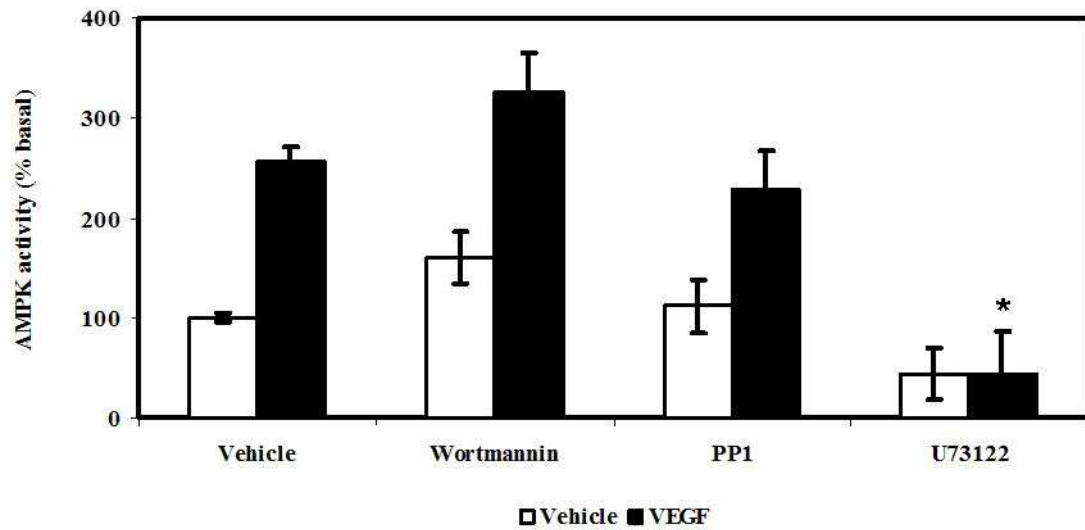
The underlying regulatory mechanisms of VEGF-stimulated AMPK activity remained uncharacterised. The experiments discussed in this chapter were therefore undertaken to elucidate this signalling process.

A number of previous studies provide evidence implicating a range of likely candidates perhaps involved in the regulation of VEGF-stimulated AMPK activity. PI3K, Src and PLC- $\gamma$  are known effectors of VEGF signalling in endothelial cells. It has been suggested that AMPK stimulation is downstream of PI3K and Src activation in peroxynitrite- and metformin-treated BAEC (Zou et al., 2004; Zou et al., 2003). In a recent study also conducted in BAEC an inhibitor of Src diminished VEGF-mediated AMPK and ACC phosphorylation (Levine et al., 2007). In HUVEC, histamine and thrombin have been shown to stimulate AMPK, a process proposed to be mediated by a PLC $\gamma$ -mediated increase in intracellular Ca<sup>2+</sup> (Thors et al., 2004). In this chapter, the role of these signalling effectors in the regulation of VEGF-stimulated AMPK in HAEC was investigated.

LKB1 and CaMKK are AMPKKs, either of which may potentially regulate VEGF-stimulated AMPK activity. LKB1 is responsible for AMPK activation when there is an increase in the cellular AMP:ATP ratio whereas CaMKK stimulates AMPK in an AMP-independent, Ca<sup>2+</sup>-dependent manner. As mentioned above histamine- and thrombin-stimulated AMPK activity may be mediated by a PLC $\gamma$ -dependent increase in intracellular Ca<sup>2+</sup> (Thors et al., 2004). Interestingly, thrombin has recently been demonstrated to activate AMPK in endothelial cells via a pathway involving PLC and CaMKK $\beta$  (Stahmann et al., 2006). It therefore seemed possible that a PLC-Ca<sup>2+</sup>-CaMKK pathway may underlie VEGF-stimulated AMPK activity. However, LKB1 itself may participate in the regulation of this process. There is reduced vascular structure and angiogenesis in LKB1<sup>-/-</sup> mouse embryos and increased concentrations of VEGF, suggesting a possible link between LKB1 and VEGF/angiogenesis (Ylikorkala et al., 2001). In this chapter, the importance of both CaMKK and LKB1 in VEGF-mediated AMPK activity was also determined.

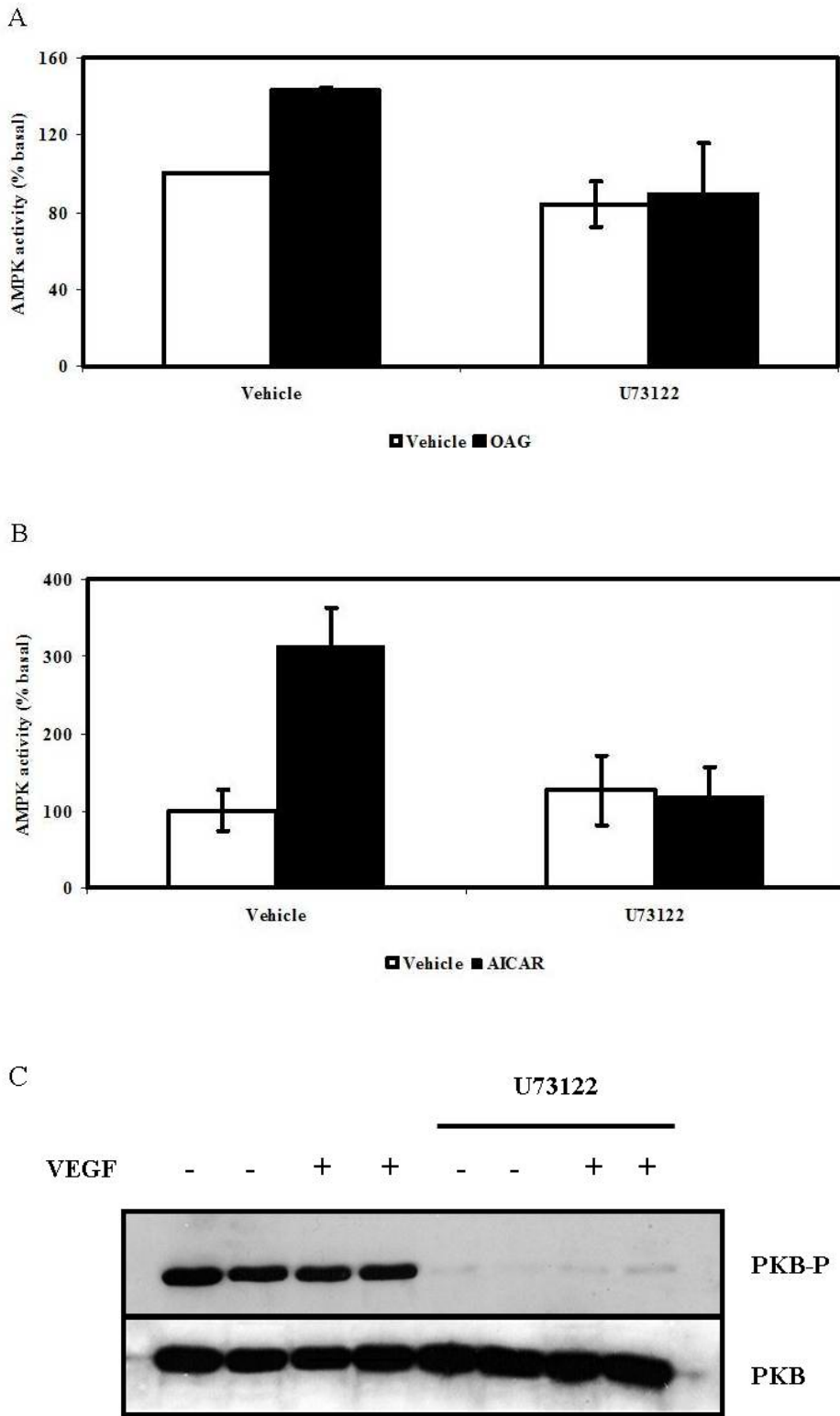
## 4.2 Results

Inhibition of either PI3K or Src, with the inhibitors wortmannin and PP1 (described in table 2.5) respectively, at concentrations previously demonstrated to inhibit PI3K/Src in endothelial cells (Wang et al., 2005; Zeng and Quon, 1996; Klint et al., 1999), had no effect on VEGF-stimulated AMPK activity in HAEC, suggesting that neither of these kinases mediate VEGF-stimulated AMPK activation. Conversely, VEGF-stimulated AMPK activity was reduced to basal levels in HAEC pre-treated with the reported PLC inhibitor U73122 (described in table 2.5) (Figure 4.1). However, further work conducted using U73122 suggests this agent may not be a specific inhibitor of PLC. AMPK activation stimulated by both AICAR and OAG (an agent found to stimulate AMPK activity (Figure 4.12), likely downstream of PLC) were unexpectedly inhibited by U73122 (Figures 4.2B and 4.2A respectively). In addition PKB phosphorylation at Ser-473 was completely abolished by U73122 treatment (Figure 4.2C).



**Figure 4-1 Effect of various inhibitors on VEGF-stimulated AMPK activity**

HAEC were incubated in the presence or absence of VEGF for 5 minutes after pre-incubation with 100 nM wortmannin, 1  $\mu$ M PP1 or 10  $\mu$ M U73122 for 45 minutes and lysates prepared. Total AMPK was immunoprecipitated from HAEC lysates (100  $\mu$ g) and assayed for AMPK activity. The results are expressed as the mean  $\pm$  SD basal AMPK activity for four independent experiments. \* $p < 0.01$  relative to the value in the absence of inhibitor. These experiments were performed by Dr I. Salt.



**Figure 4-2 The action of U73122**

HAEC were incubated in the presence or absence of (A) 100  $\mu$ M OAG for 30 minutes (B) 2 mM AICAR for 30 minutes after pre-incubation with 10  $\mu$ M U73122 for 15 minutes and lysates prepared. Total AMPK was immunoprecipitated from HAEC lysates (100-200  $\mu$ g) and assayed for AMPK activity. The results are expressed as the mean  $\pm$  SEM basal AMPK activity for two independent experiments in each case. (C) HAEC were incubated in the presence or absence of 10 ng/ml VEGF for 5 minutes after pre-treatment with 10  $\mu$ M U73122 for 30 minutes and lysates prepared. Lysates were resolved SDS-PAGE, transferred to nitrocellulose and probed with the antibodies indicated. Probing of lysates with  $\alpha$ -PKB and  $\alpha$ -PKB Ser-473 resulted in a single band at ~60 kDa in both instances.

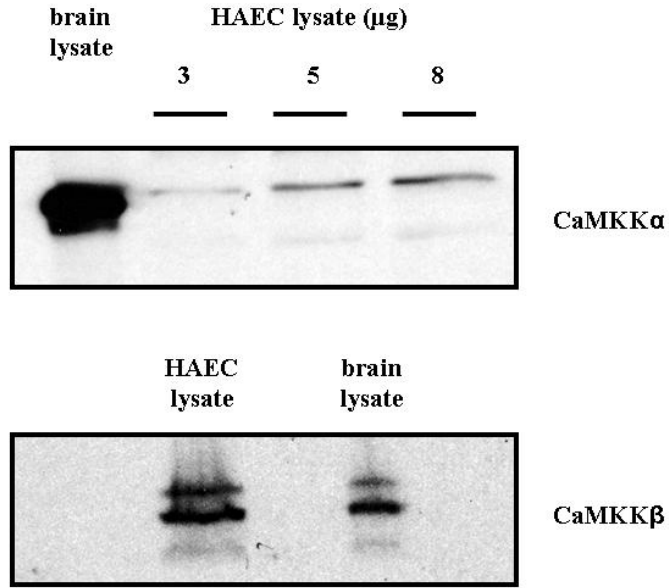


As preliminary work had implicated PLC in VEGF-mediated AMPK activity, and as PLC is known to increase intracellular  $\text{Ca}^{2+}$  levels in response to VEGF treatment (Xia et al., 1996), an intriguing possibility that VEGF stimulated AMPK was regulated by the  $\text{Ca}^{2+}$  dependent upstream kinase CaMKK was highlighted. Using isoform-specific anti-CaMKK antibodies the expression of both isoforms of CaMKK,  $\alpha$  and  $\beta$ , was demonstrated in HAEC (Figure 4.3).

AMPK activity in HAEC was responsive to increased intracellular  $\text{Ca}^{2+}$  levels as treatment with the  $\text{Ca}^{2+}$  ionophore A23187 resulted in a ~3.7-fold increase in AMPK activity (Figure 4.4A). Moreover, VEGF treatment resulted in a ~1.8-fold increase in AMPK activity (Figure 4.4B) in HeLa cells which express CaMKK but are LKB1-deficient (Hurley et al., 2005).

The  $\text{Ca}^{2+}$  chelator BAPTA-AM was employed to further ascertain the role of  $\text{Ca}^{2+}$  in VEGF-stimulated AMPK activation. In HAEC pre-treated with BAPTA-AM AMPK activity was no longer increased in response to VEGF treatment (Figure 4.5). There was however a noticeable, yet non-significant increase, in AMPK activity above basal in BAPTA-AM treated cells even in the absence of VEGF treatment ( $156 \pm 40\%$ ,  $n=5$ ,  $p>0.05$ ) (Figure 4.5). Indeed, VEGF treatment in the presence BAPTA-AM resulted in an AMPK activity not dissimilar to VEGF treatment alone (Figure 4.5).

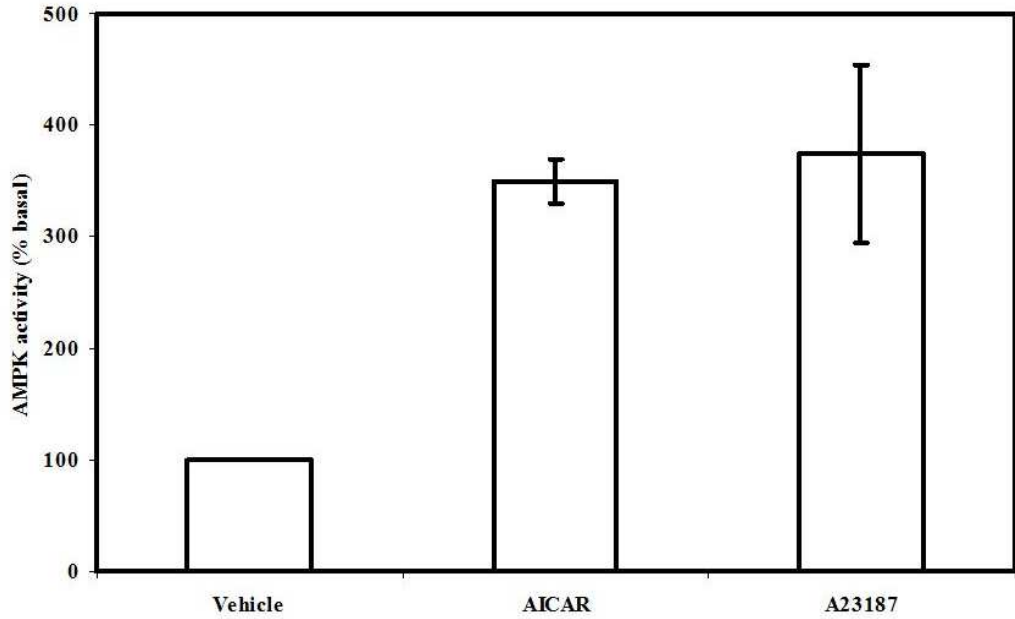
The role of CaMKK in the regulation of VEGF-stimulated AMPK activity was subsequently determined using the inhibitor STO-609. Pre-treatment of HAEC with STO-609 reduced VEGF-stimulated AMPK activity to basal levels, without altering AICAR-stimulated AMPK activity (Figure 4.6). VEGF-stimulated phosphorylation of AMPK at Thr-172 was similarly reduced to basal levels after pre-incubation with STO-609 (Figure 4.7).



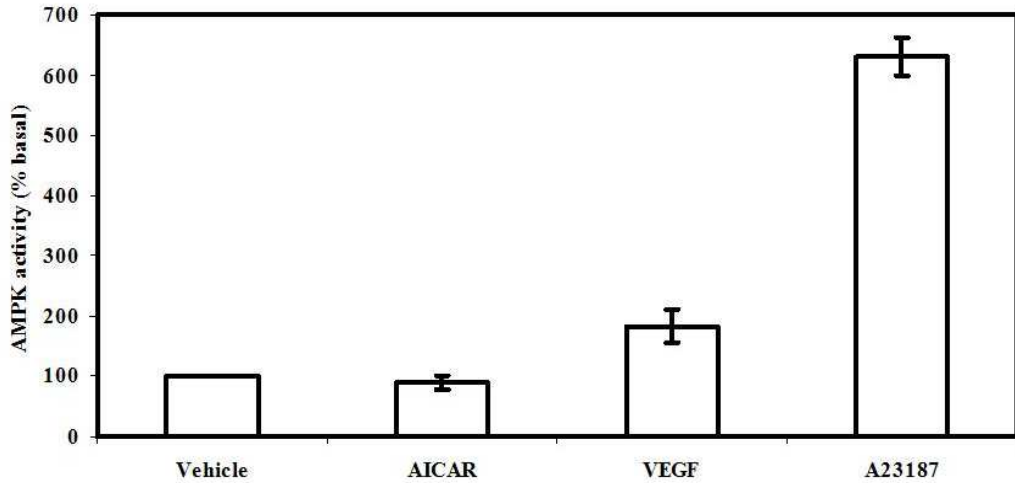
### Figure 4-3 CaMKK isoform expression

HAEC lysates were resolved by SDS PAGE, transferred to nitrocellulose and probed with anti-CaMKK $\alpha$  antibodies. CaMKK $\beta$  was immunoprecipitated from HAEC lysate, subjected to Western blotting and probed with anti-CaMKK $\beta$  antibodies. A lysate prepared from whole rat brain was used as a positive control. Representative immunoblots are shown. Probing of lysates with  $\alpha$ -CaMKK $\alpha$  resulted in a band at ~63 kDa whereas probing with  $\alpha$ -CaMKK $\beta$  resulted in a two distinct bands at ~66 kDa.

A

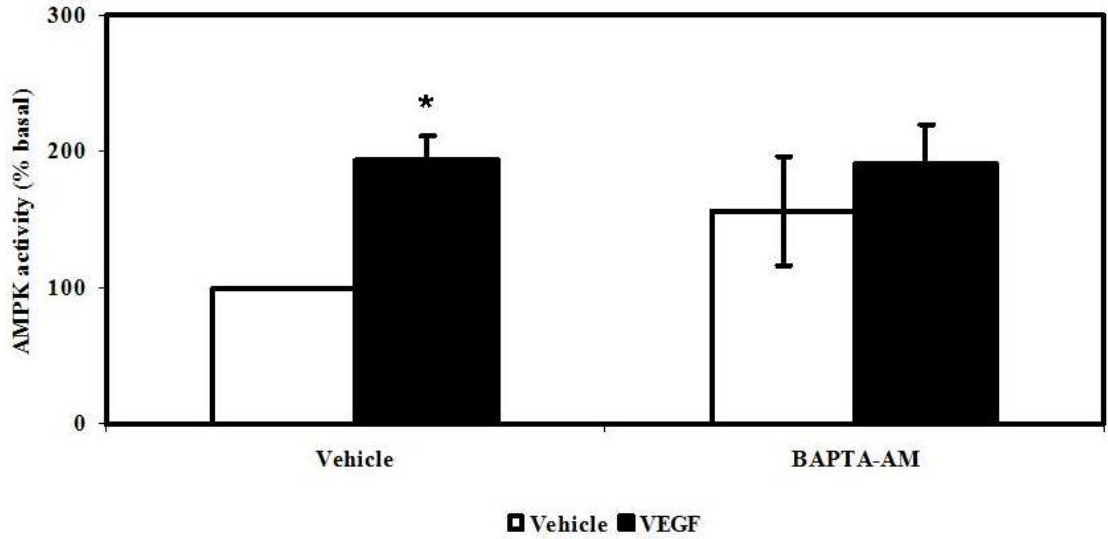


B



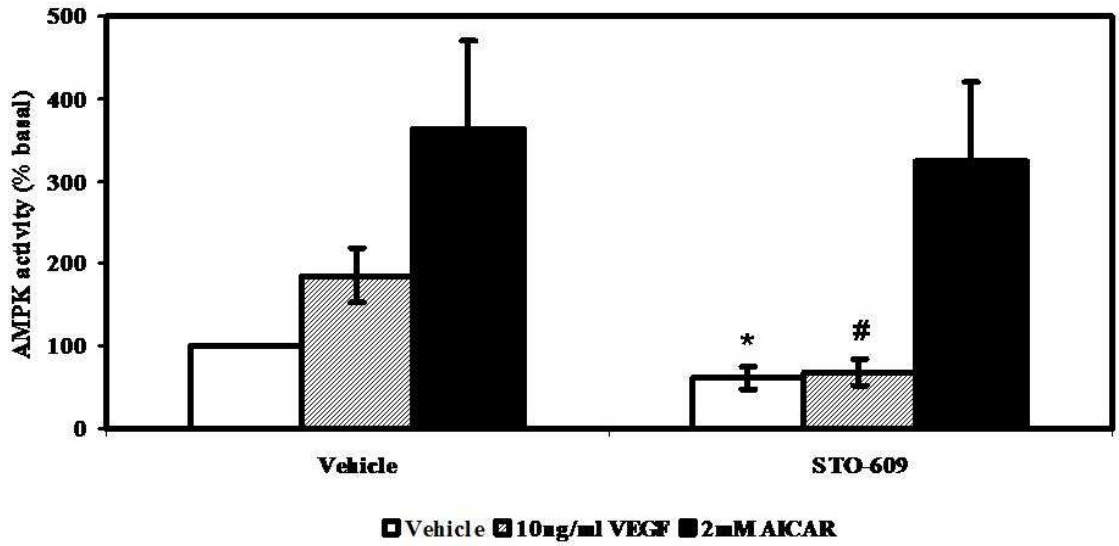
**Figure 4-4 Activation of AMPK in HeLa and HAEC**

(A) HAEC were treated with 2 mM AICAR for 45 minutes or 5  $\mu$ M A23187 for 10 minutes and lysates prepared. (B) HeLa cells were treated in the presence or absence of 10 ng/ml VEGF for 5 minutes, 2 mM AICAR for 45 minutes or 5  $\mu$ M A23187 for 30 minutes and lysates prepared. Total AMPK was immunoprecipitated from HAEC lysates and assayed for AMPK activity in both cases. Data shown in A & B are representative of two independent experiments in each case.



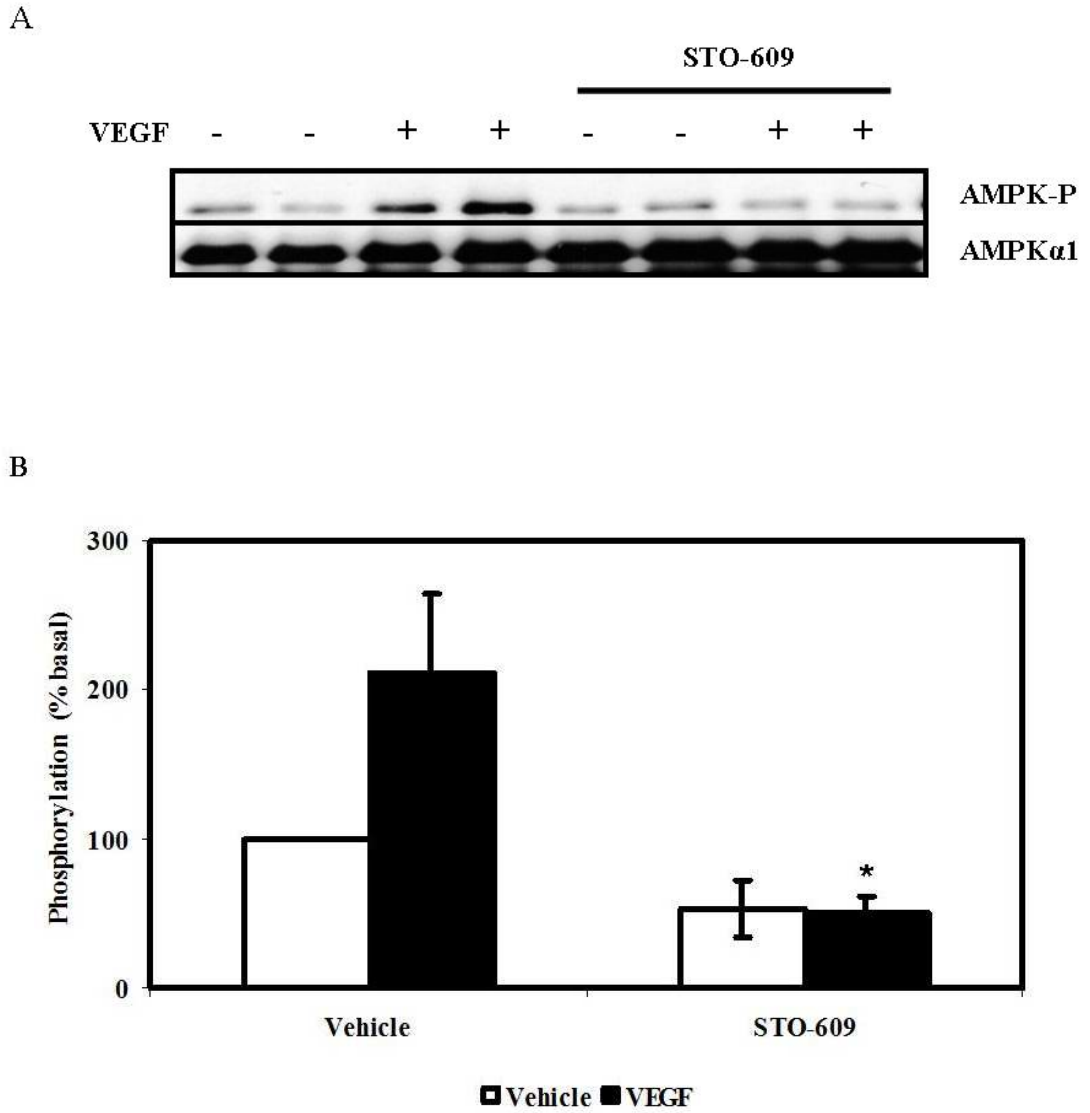
**Figure 4-5 Effect of BAPTA-AM on VEGF-stimulated AMPK activity**

HAEC were incubated in the presence or absence of 10 ng/ml VEGF for 5 minutes after pre-incubation with 25  $\mu$ m BAPTA-AM for 25 minutes and lysates prepared. Total AMPK was immunoprecipitated from HAEC lysates and assayed for AMPK activity. The results are expressed as the mean  $\pm$  SEM % basal AMPK activity for five independent experiments. \* $p$  < 0.01 relative to value in the absence of VEGF.



**Figure 4-6 Effect of STO-609 on VEGF-stimulated AMPK activity**

HAEC were incubated in the presence or absence of 10 ng/ml VEGF for 5 minutes, 2 mM AICAR for 30 minutes or 25  $\mu$ M STO-609 for 45 minutes and lysates prepared. Total AMPK was immunoprecipitated from HAEC lysates and assayed for AMPK activity. The results are expressed as the mean  $\pm$  SEM % basal AMPK activity for seven independent experiments. \* $p$  < 0.05 relative to value of vehicle treated control. # $p$  < 0.01 relative to value in the absence of inhibitor.



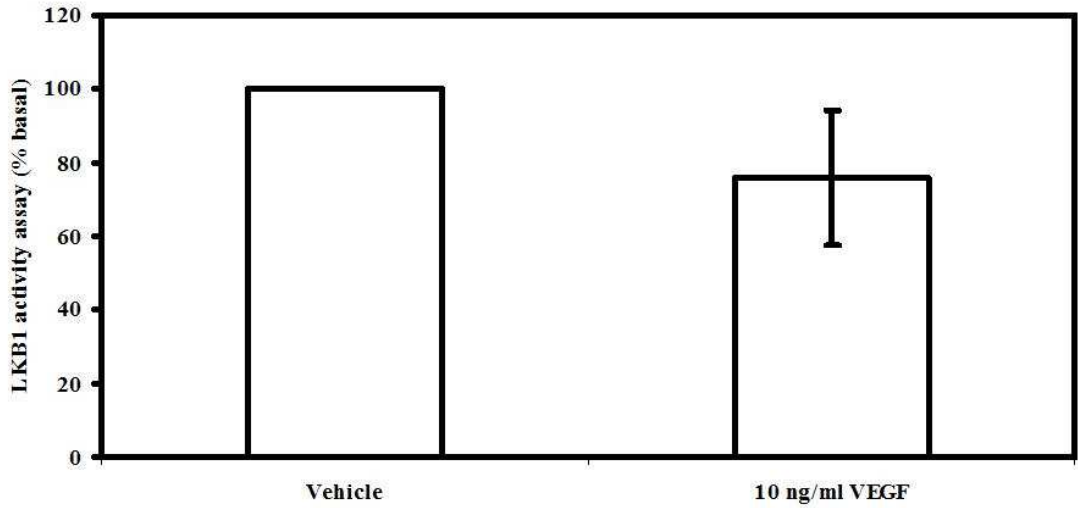
**Figure 4-7 Effect of STO-609 on VEGF-stimulated phosphorylation of AMPK at Thr-172**

(A) AMPK immunoprecipitates were resolved by SDS-PAGE, transferred to nitrocellulose and probed with the antibodies indicated. Representative immunoblots are shown, repeated with similar results on four different samples of lysates. Probing of lysates with  $\alpha$ -AMPK and  $\alpha$ -AMPK-Thr-172 resulted in a single band at ~62 kDa in both instances. (B) Quantification of AMPK phosphorylation from four independent experiments using ImageJ software. Results are expressed as the mean  $\pm$  SEM % basal AMPK Thr-172 phosphorylation. \* $p < 0.05$  relative to the value in absence of inhibitor.

The role of LKB1 in the regulation of VEGF-stimulated AMPK activity was also assessed. LKB1, immunoprecipitated from lysates made from VEGF-treated HAEC, was incubated with recombinant AMPK and assayed for activity. VEGF treatment was found to have no effect on LKB1 activity in HAEC (Figure 4.8).

As the action of LKB1 on AMPK activation is manifested through an increase in the cellular AMP:ATP ratio the effect of VEGF on intracellular adenine nucleotide concentrations in HAEC was determined. Stimulation of HAEC with concentrations of VEGF up to 50 ng/ml has no effect on the ADP:ATP ratio, whereas treatment of cells with phenformin and azide, LKB1-mediated activators of AMPK, both resulted in an increase in this ratio (Figure 4.9). The levels of intracellular AMP proved too low to measure accurately in these cells.

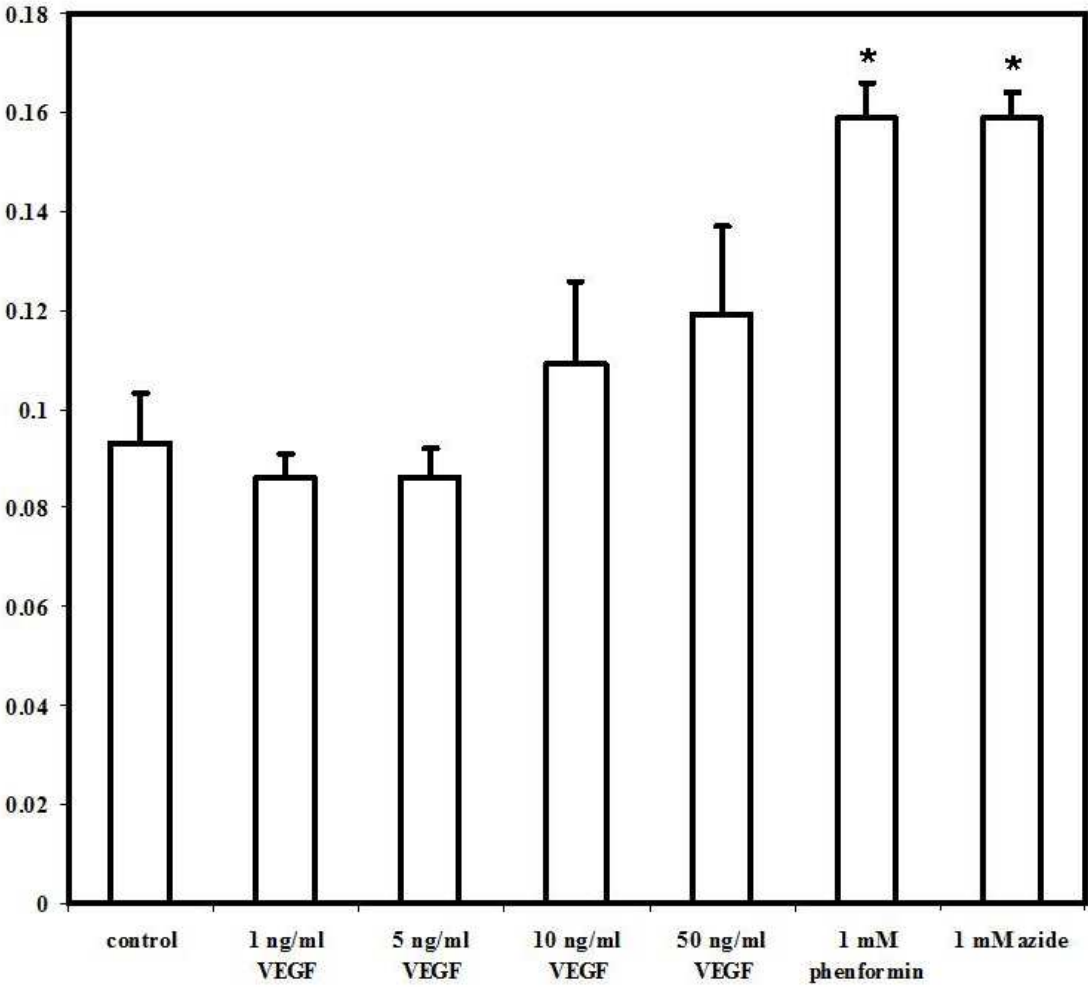
To further investigate the role of LKB1, the effect of VEGF on AMPK activity in HeLa cells stably expressing HeLa<sub>LKB1-KD</sub> or HeLa<sub>LKB1-WT</sub> (as described in 2.4.5) was determined (Sapkota et al., 2002). The expression of FLAG-epitope tagged HeLa<sub>LKB1-KD</sub> or HeLa<sub>LKB1-WT</sub> was successfully induced by the addition of tetracycline for 24h (Figure 4.10A). In both HeLa<sub>LKB1-KD</sub> and HeLa<sub>LKB1-WT</sub> cells AMPK activity was completely unaltered by VEGF treatment, yet seemed responsive to AICAR stimulation (Figure 4.10B). In addition there was a ~3.9-fold increase in basal AMPK activity in HeLa<sub>LKB1-WT</sub> cells ( $0.17 \pm 0.0175$  nmol <sup>32</sup>P-incorporated/min/mg protein, n=3, p<0.001) in comparison with their HeLa<sub>LKB1-KD</sub> counterparts ( $0.043 \pm 0.018$  nmol <sup>32</sup>P-incorporated/min/mg protein, n=3) (Figure 4.10B).



**Figure 4-8 Effect of VEGF treatment on LKB1 activity in HAEC**

HAEC were incubated with the indicated concentration of VEGF for 5 minutes and lysates prepared. Total LKB1 was immunoprecipitated from HAEC lysates (200  $\mu$ g), incubated with recombinant AMPK and assayed for AMPK activity. The results are expressed as the mean  $\pm$  SEM % basal AMPK activity for three independent experiments performed in triplicate.

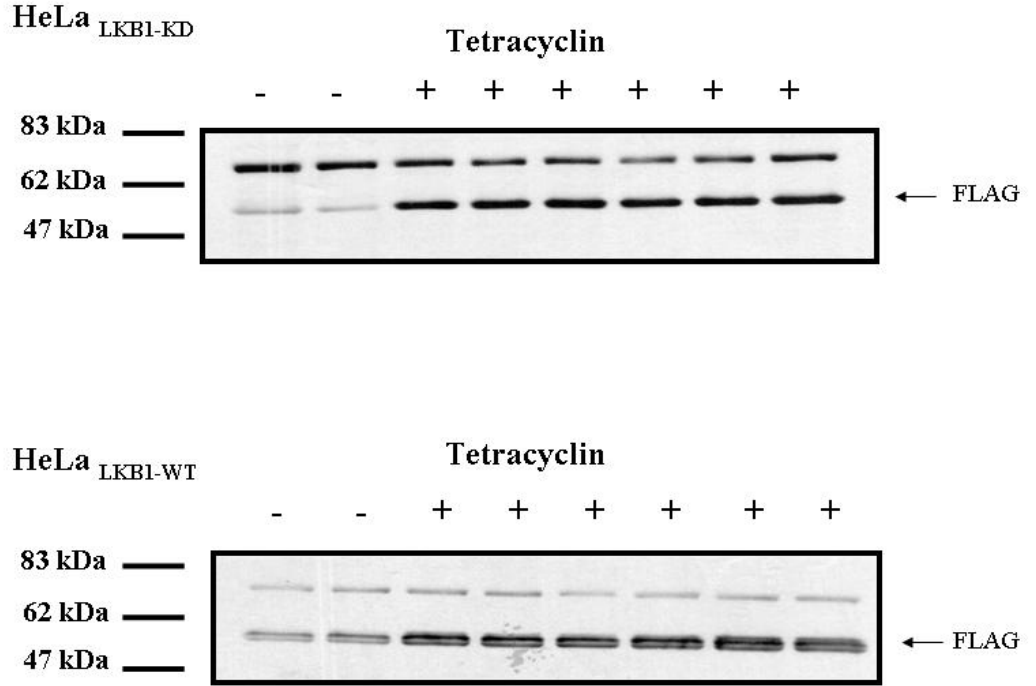




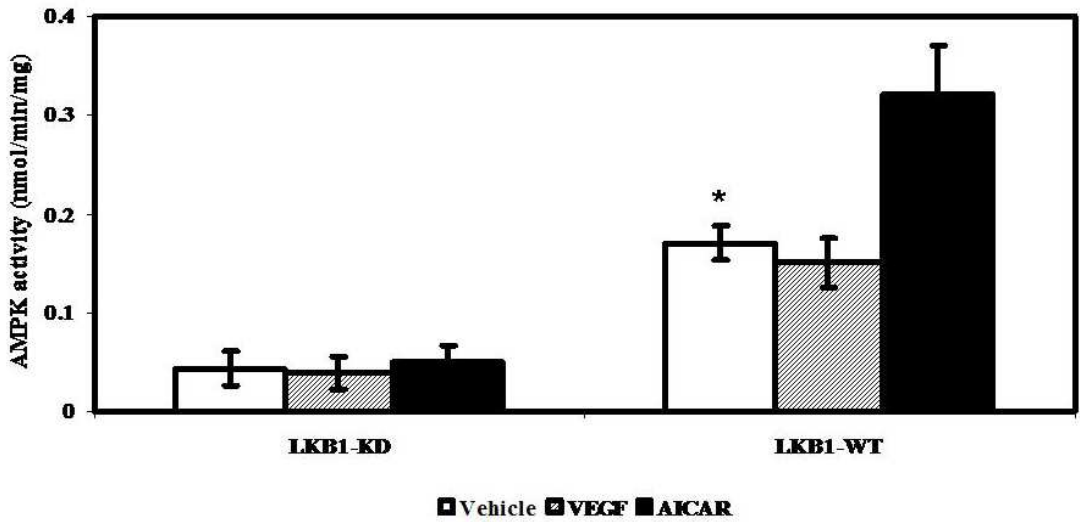
**Figure 4-9 ADP:ATP ratio in VEGF-treated HAEC**

HAEC were pre-incubated for 2h at 37 °C in KRH buffer containing test substances and incubated for various durations at 37°C. Neutralised perchloric acid extracts were prepared by Dr I. Salt as described previously (Boyle et al., 2008). Nucleotides were subsequently separated by HPLC. This work is representative of 4 independent experiments performed by Prof. D. G. Hardie’s laboratory, University of Dundee. \*p<0.01 relative to control value.

A



B



**Figure 4-10 Effect of VEGF in HeLa<sub>LKB1-KD</sub> and HeLa<sub>LKB1-WT</sub> expressing cells**

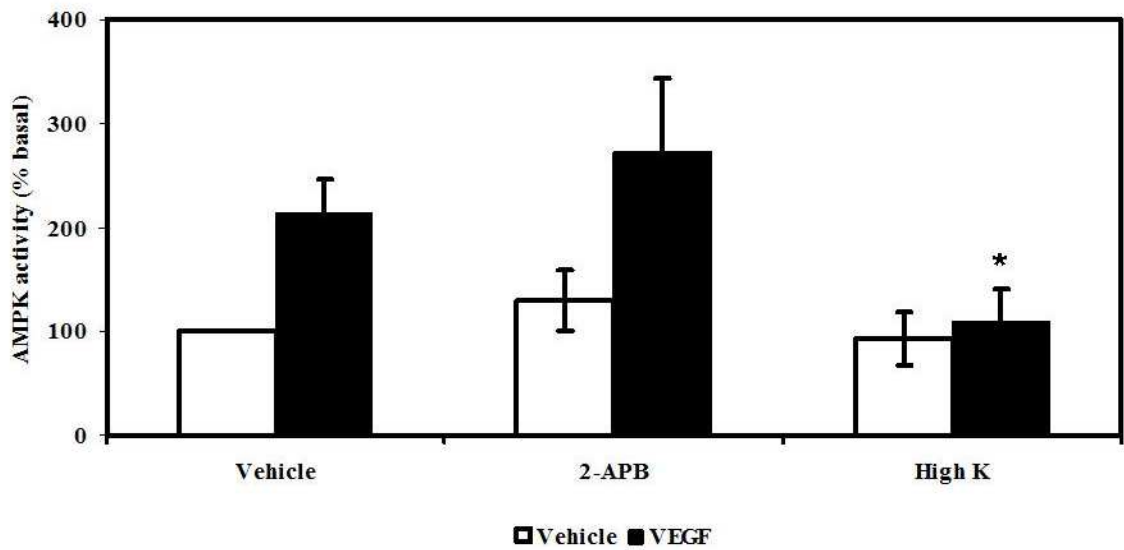
1µg/ml tetracycline was added to cells for 24h to induce the expression of LKB1-KD or LKB1-WT in HeLa cells. (A) Lysates were subsequently made from these cells, subjected to SDS PAGE, transferred to nitrocellulose then probed with the antibodies indicated. (B) LKB1-KD and LKB1-WT expressing HeLa cells were treated with the indicated concentrations of VEGF for 5 minutes or AICAR for 45 minutes. Total AMPK was immunoprecipitated from these cells and AMPK activity assessed. The results are expressed as the mean ± SEM nmol <sup>32</sup>P-incorporated/min/mg protein AMPK activity for three independent experiments. \*p<0.001 relative to vehicle-treated control cells.

Together the data presented in Figures 4.1-4.10) support a role for CaMKK, but not LKB1, in the regulation of VEGF-stimulated AMPK activity. As a result of VEGF-mediated activation, PLC $\gamma$  stimulates hydrolysis of PIP<sub>2</sub> thus generating the second messengers IP<sub>3</sub> (inositol triphosphate) and DAG (diacylglycerol). IP<sub>3</sub> diffuses into the cytosol whereas DAG remains within the plasma membrane, due to its hydrophobic properties. IP<sub>3</sub> stimulates the release of calcium ions from the smooth endoplasmic reticulum (Berridge, 1995a), whereas DAG is a physiological activator of PKC. In addition DAG may also affect intracellular Ca<sup>2+</sup> concentration through a direct action on a number of non-selective cation channels from the TRPC channel family which promotes Ca<sup>2+</sup> influx across the plasma membrane (Hofmann et al., 1999).

Whether either mechanism (IP<sub>3</sub> or DAG- mediated Ca<sup>2+</sup> mobilisation/influx) is involved in VEGF-stimulated AMPK activation was subsequently assessed. VEGF-stimulated AMPK activity remained intact (~2.7-fold) in HAEC which had been pre-treated with the IP<sub>3</sub> receptor blocker 2-APB (Figure 4.11). 2-APB treatment alone had no effect on AMPK activity (Figure 4.11). Conversely, in HAEC treated with a high K<sup>+</sup>-containing extracellular solution, which prevents Ca<sup>2+</sup> influx through non-selective cation channels in endothelial cells (He and Curry, 1991), VEGF-stimulated AMPK activity was reduced to basal levels (Figure 4.11). The addition of the high K<sup>+</sup>-containing solution alone had no effect on AMPK activity (Figure 4.11). These data suggest Ca<sup>2+</sup> influx, and not Ca<sup>2+</sup> mobilisation from IP<sub>3</sub>-sensitive intracellular stores, is essential for VEGF-stimulated AMPK activity. In agreement with this finding the removal of extracellular Ca<sup>2+</sup> abrogated VEGF-stimulated AMPK activity, without affecting AICAR-stimulated AMPK activity in HAEC (Figure 4.12).

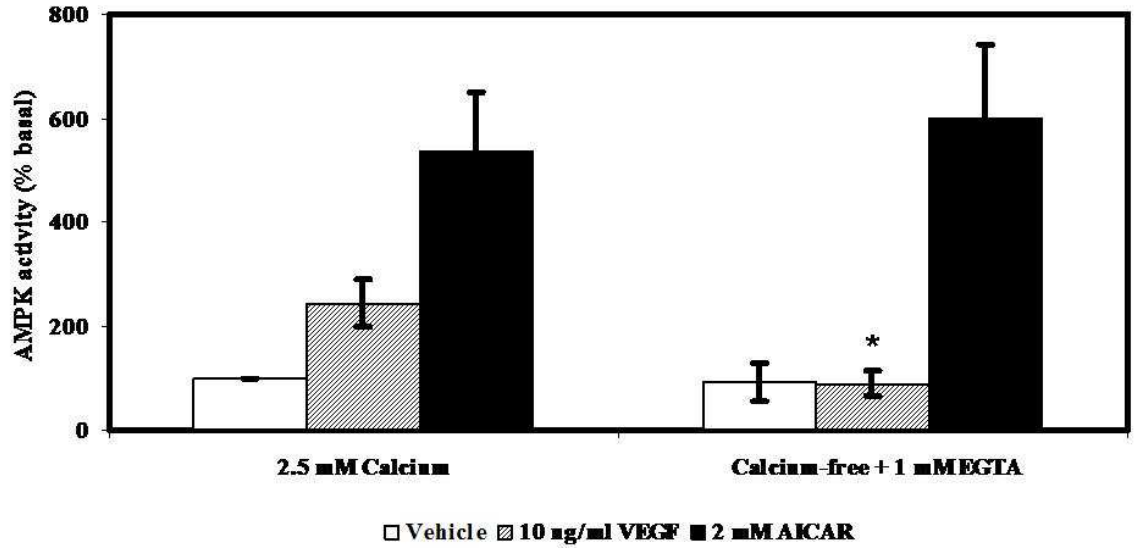
Of the seven members of the TRPC channel family (TRPC1-7), TRPC3/6/7 are known to be activated by DAG thus promoting Ca<sup>2+</sup> influx across the plasma membrane (Pedersen et al., 2005). RT-PCR was used to assess the expression of TRPC3/6/7 in HAEC. Figure 4.13 shows that a cDNA fragment of an appropriate size (435bp) was amplified using primers designed against TRPC6. TRPC3 (400bp) or TRPC7 (458bp) were not detected in this experiment. HAEC were then treated with a cell permeable mimetic of DAG. This agent, OAG (1-oleoyl 2-acetyl-sn glycerol), which stimulates TRPC3/6/7 (Pedersen et al., 2005; Venkatachalam et al., 2004), elicited a ~2.2-fold stimulation of AMPK activity, an effect sensitive to both STO-609 pre-treatment and high K<sup>+</sup>-induced depolarisation (Figure 4.14). STO-609 treatment alone was found to reduce AMPK activity (Figure 4.14).

Gadolinium ( $Gd^{3+}$ ) is an agent used to block of  $Ca^{2+}$  entry through non-selective cation channels. In HAEC  $Gd^{3+}$  elicited a variable effect on AMPK activity with an overall non-significant ~2.6-fold increase in kinase activity, an effect that was not increased further by VEGF application (Figure 4.15).



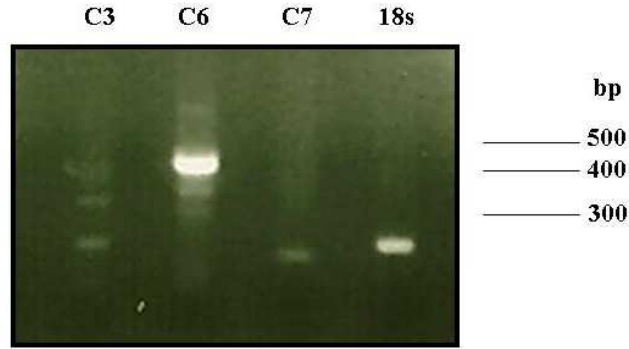
**Figure 4-11 Effect of high  $K^+$ -containing KRH on VEGF-stimulated AMPK activity**

HAEC were treated in the presence or absence of VEGF (10 ng/ml, 5 minutes) after pre-treatment with the  $IP_3$  receptor antagonist 2-APB (100  $\mu$ M, 20 minutes) or with a high- $K^+$  solution to depolarise endothelial cells (made as described in section 2.2.1) and lysates prepared. Total AMPK was immunoprecipitated from HAEC lysates and assayed for AMPK activity. The results are expressed as the means  $\pm$  SEM % basal AMPK activity for five independent experiments. \* $p < 0.05$  relative to the value in the absence of high  $K^+$ -containing extracellular solution.



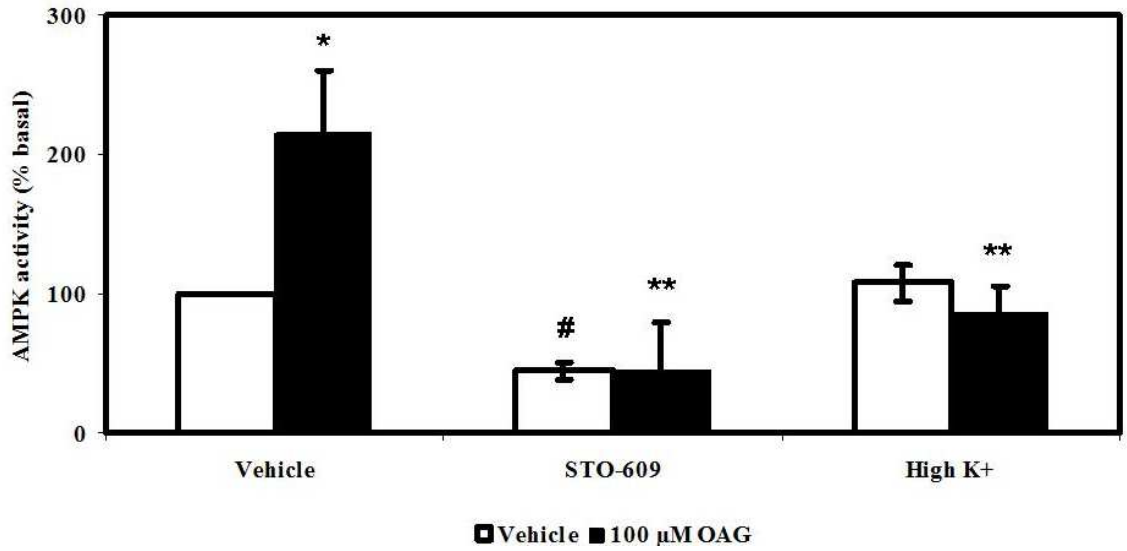
**Figure 4-12 Effect of extracellular  $\text{Ca}^{2+}$  on VEGF-stimulated AMPK activity**

HAEC were bathed in either normal KRH (2.5 mM  $\text{Ca}^{2+}$ ) or KRH without  $\text{Ca}^{2+}$  + 1 mM EGTA for 30 minutes then treated in the presence or absence of the indicated concentrations of VEGF for 5 minutes or AICAR for 45 minutes. Lysates were subsequently prepared, total AMPK immunoprecipitated from these lysates and assayed for AMPK activity. The results are expressed as the mean  $\pm$  SEM % basal AMPK activity for three independent experiments. \* $p < 0.05$  relative to value in KRH containing 2.5 mM  $\text{Ca}^{2+}$ .



**Figure 4-13 RT-PCR of TRPC channels**

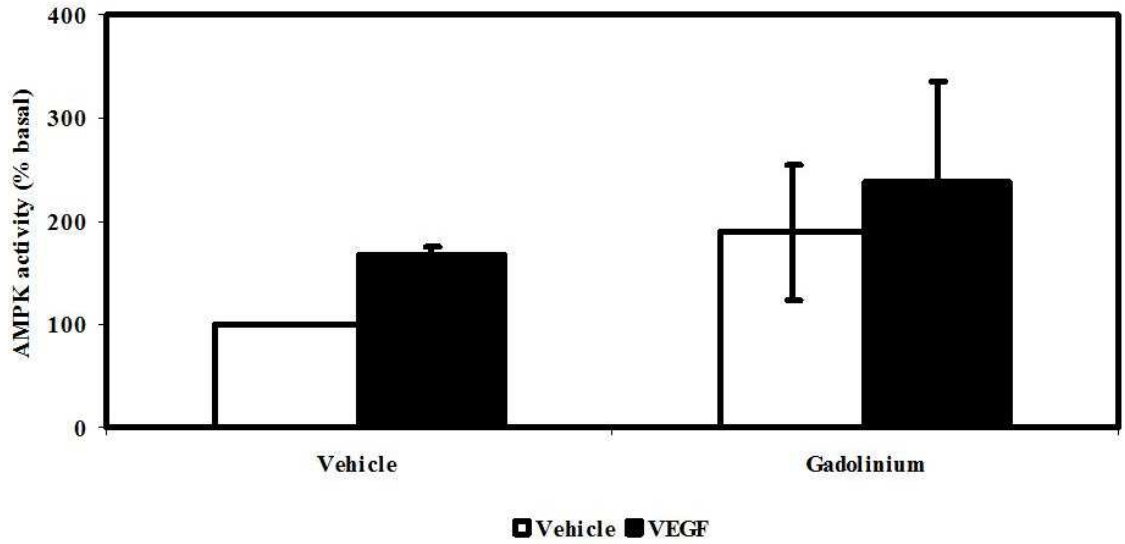
Equal volumes of HAEC cDNA (as described in section 2.3.5.3) were mixed with a PCR mastermix (as described in section 2.3.5.2), and gene-specific primers to the transcript of interest were added. PCR reactions were carried out in a gradient thermocycler under the conditions described in section (2.3.5.3). PCR products were resolved in ethidium bromide-stained agarose gel and visualised on a UVT-28 MP transilluminator. Targeted products were as follows: 400bp for TRPC3 (C3), 435bp for TRPC6 (C6), 458bp for TRPC7 (C7), 250bp for 18s RNA (18s).



**Figure 4-14 OAG-stimulated AMPK activity**

HAEC were treated in the presence or absence of OAG (100  $\mu$ M, 20 minutes) after pre-treatment with STO-609 (25  $\mu$ M, 15 minutes) or with a high  $K^+$ -containing KRH solution and lysates prepared. Total AMPK was immunoprecipitated from HAEC lysates and assayed for AMPK activity. The results are expressed as the mean  $\pm$  SEM % basal AMPK activity for five independent experiments. \* $p$  < 0.05 relative to value in absence of OAG. \*\* $p$  < 0.05 relative to value in absence of STO-609 or high  $K^+$ -induced depolarisation. # $p$  < 0.001 relative to value in absence of inhibitor.





**Figure 4-15 Effect of  $Gd^{3+}$  on VEGF-stimulated AMPK activity**

HAEC were incubated in the presence or absence of 10 ng/ml VEGF for 5 minutes after pre-incubation with 100  $\mu M$   $Gd^{3+}$  for 15 minutes and lysates prepared. Total AMPK was immunoprecipitated from HAEC lysates and assayed for AMPK activity. The results are expressed as the mean  $\pm$  SEM % basal AMPK activity for four independent experiments.

### 4.3 Discussion

Previous studies have suggested that AMPK stimulation is downstream of PI3K and Src activation in peroxynitrite- and metformin-treated BAEC (Zou et al., 2004; Zou et al., 2003). Another study, also conducted in BAEC, has reported that inhibition of Src prevents VEGF-induced AMPK and ACC phosphorylation (Levine et al., 2007), whereas it has also been proposed that VEGF stimulates complex formation of VEGF-R2 with Src and that Src activation is required for VEGF induction of PLC $\gamma$ 1 activation and subsequent eNOS stimulation (He et al., 1999). As eNOS has been shown to be activated downstream of VEGF-stimulated AMPK activation (Figures 3.3 and 3.4B) these mediators were of particular interest in the regulation of this process. However, in the current investigation, inhibition of Src had no effect on VEGF-stimulated AMPK activity (Figure 4.1), suggesting neither Src nor PLC-dependent Src activation mediates VEGF-stimulated AMPK activation in HAEC. Additionally, pre-treatment of HAEC with wortmannin, at a concentration shown to completely inhibit VEGF-stimulated PKB phosphorylation (Figure 3.6) was without effect on VEGF-stimulated AMPK activity suggesting PI3K is not an essential component of this signalling cascade. In contrast, pre-treatment of HAEC with the PLC inhibitor U73122 reduced VEGF-stimulated AMPK activity to basal levels. Interestingly, histamine has previously been reported to activate AMPK in HUVEC in a PI3K-independent, PLC dependent manner (Thors et al., 2004), suggesting histamine and VEGF may share a common mechanism by which they stimulate AMPK.

However, a number of additional experiments (Figure 4.2) conducted using the ‘specific’ PLC inhibitor U73122 (Thompson et al., 1991; Yule and Williams, 1992; Stam et al., 1998) indicate this agent may have a number of off-target effects. VEGF stimulates PKB via PI3K (Papapetropoulos et al., 1997; Xia et al., 1996), whereas AICAR directly activates AMPK through the action of the upstream kinase LKB1. In HAEC pre-treated with U73122 VEGF-stimulated phosphorylation of PKB at Ser-473 (Figure 4.2C) and AICAR-stimulated AMPK activity (Figure 4.2B) were completely abolished. The TRPC family of non-selective cation channels act downstream of PLC $\beta$  or PLC $\gamma$  when activated by GPCRs or RTKs respectively (Xia et al., 1996). TRP channels allow the flux of cations down their electrochemical gradient, raising intracellular Ca<sup>2+</sup> and Na<sup>+</sup>. It is widely accepted that of the seven members of the ‘canonical’ family (TRPC1-7), TRPC3/6/7 are DAG- (or the mimetic OAG)- activated channels (Pedersen et al., 2005). OAG was found to stimulate AMPK activity in HAEC (Figure 4.14), an effect also unexpectedly inhibited by U73122 pre-treatment (Figure 4.2A). Despite these observations it has recently been

shown that VEGF-mediated elevated intracellular  $\text{Ca}^{2+}$  and angiogenesis are inhibited by dominant negative TRPC6 in HMEC (Hamdollah Zadeh et al., 2008), and  $\text{Ca}^{2+}$  entry via TRPC channels is necessary for thrombin-induced AMPK activation in endothelial cells (Bair et al., 2008). These data, and the finding that OAG can stimulate AMPK activity in HAEC (Figure 4.14), suggest PLC-TRPC signalling may be an important component of the VEGF-AMPK signalling cascade in these cells.

A key finding of the current investigation is the identification of CaMKK as the responsible upstream kinase which mediates VEGF-stimulated AMPK activity (Figure 4.6). In agreement with this observation, a more recent study has similarly identified CaMKK as the AMPKK necessary for VEGF-stimulated AMPK activity in BAEC (Levine et al., 2007). An increasing number of studies have confirmed that CaMKK, especially the  $\beta$  isoform, can act as an alternate upstream kinase to LKB1 that activates AMPK in a  $\text{Ca}^{2+}$ -dependent and AMP-independent manner in a range of tissues and cell types (Hardie et al., 2006; Hawley et al., 2005; Woods et al., 2005; Birnbaum, 2005). Although initially characterised and isolated from neuronal tissue, both CaMKK $\alpha$  and CaMKK $\beta$  have a wide expression in rodent tissues (Edelman et al., 1996; Anderson et al., 1998; Vinet et al., 2003). Using isoform-specific anti-CaMKK antibodies, expression of both CaMKK $\alpha$  and CaMKK $\beta$  was confirmed in HAEC for the first time (Figure 4.3). Moreover, pre-incubation of HAEC with the CaMKK inhibitor STO-609 completely abolished VEGF-stimulated AMPK Thr-172 phosphorylation and AMPK activity (Figures 4.6 and 4.5 respectively). STO-609 is a selective, but not specific inhibitor, of both CaMKK isoforms, which at sufficient concentrations can inhibit other kinases including AMPK (Bain et al., 2007). Importantly, in the present investigation, CaMKK-independent stimulation of AMPK, induced by AICAR, was unaffected by STO-609 pre-treatment (Figure 4.6). Additional lines of evidence also support CaMKK as the responsible upstream kinase in the regulation of VEGF-stimulated AMPK activity. Treatment of HeLa cells, which express CaMKK but are LKB1-deficient (Hurley et al., 2005), with VEGF resulted in an increase in AMPK activity (Figure 4.4B). Incubation of HAEC with the  $\text{Ca}^{2+}$  ionophore A23187 also elicited a large stimulation of AMPK activity in both HAEC and HeLa cells (Figures 4.4A and B respectively), whilst  $\text{Ca}^{2+}$  influx across the plasma membrane in response to VEGF was shown to be essential in the maintenance of VEGF-stimulated AMPK activity in HAEC (Figures 4.11 and 4.12, discussed in detail below). In HAEC pre-treated with the  $\text{Ca}^{2+}$  chelator BAPTA-AM, VEGF treatment failed to increase AMPK activity in comparison to BAPTA-AM-treated controls (Figure 4.5). Of note however, although of no statistical significance, BAPTA-AM treatment alone did increase AMPK

activity. Additional work such as siRNA targeted knockdown of CaMKK in HAEC to further corroborate the finding CaMKK is the upstream kinase which mediates VEGF-stimulated AMPK may be of benefit. Moreover, this would also allow specific targeting of the individual CaMKK $\alpha$  and CaMKK $\beta$  isoforms, both of which are expressed in HAEC (Figure 4.3). CaMKK $\beta$  is has been shown to play a role in the regulation of AMPK activity in both neuronal tissue (Hawley et al., 2005) and T cells (Tamas et al., 2006). In addition, the aforementioned kinase appears to be the major isoform in endothelial cells regulating AMPK activity in response to a range of agonists (Stahmann et al., 2006; Levine et al., 2007; Xu et al., 2008). Conversely, CaMKK $\alpha$  appears to be the major isoform in skeletal muscle cells (Witczak et al., 2007; Jensen et al., 2007), where the expression of CaMKK $\beta$  is controversial (Kitani et al., 1997; Jensen et al., 2007; Shen et al., 2007). CaMKK $\alpha$  activation is responsible for AMPK activation by  $\alpha$ -lipoic acid in C2C12 myotubes (Shen et al., 2007), and has also been implicated in the regulation of skeletal muscle glucose uptake, though this effect occurred independently of AMPK and PKB activation (Witczak et al., 2007). Although CaMKK $\beta$  is likely the principal isoform which regulates endothelial AMPK activation in response to VEGF in HAEC it may be of additional interest to determine the role of CaMKK $\alpha$ .

The current investigation also sought to determine the contribution of LKB1 to VEGF-stimulated AMPK signalling. LKB1 activity is unchanged under many conditions that stimulate AMPK phosphorylation, including phenformin, AICAR, muscle contraction and extreme ischemia in a variety of tissues (Lizcano et al., 2004; Sakamoto et al., 2004). Consistent with these findings, in the present study, there was no alteration in recombinant AMPK activity upon incubation with LKB1 immunoprecipitated from VEGF-treated HAEC (Figure 4.8). *In vitro*, purified recombinant LKB1 directly phosphorylates AMPK at Thr-172, leading to activation. In LKB1-deficient cells, such as HeLa, activation of AMPK in response to stimuli that increase the AMP:ATP ratio or with AICAR, is abolished (Hawley et al., 2003). This suggests that LKB1 is required for the activation of AMPK in response to elevated AMP (or the AMP mimetic, ZMP). However, AMP does not directly activate LKB1. Indeed, the kinase is constitutively active and does not therefore require stimulation (Hawley et al., 2003). Current evidence suggests the action of AMP is manifested through an inhibition of AMPK dephosphorylation by protein phosphatases (Sanders et al., 2007b; Suter et al., 2006; Davies et al., 1995). Indeed the majority of AMPK stimuli have been shown to raise the intracellular AMP:ATP ratio. However, in the present investigation treatment of HAEC with VEGF had no effect on the ADP:ATP ratio which is indicative of the AMP:ATP ratio (Figure 4.9). Both phenformin and azide which

are known to stimulate AMPK through an increase in the AMP:ATP ratio resulted in a significantly increased ADP:ATP ratio in these experiments (Figure 4.9).

Interestingly, it has been reported that there is reduced vascular structure and angiogenesis in  $LKB1^{-/-}$  mouse embryos (Ylikorkala et al., 2001). These phenotypes were associated with a tissue-specific deregulation of VEGF expression, including a marked increase in the amount of VEGF mRNA. These findings provide an intriguing link between the LKB1 and VEGF signalling pathways. Other studies have assessed the role of LKB1 in endothelial cells using siRNA targeted to the kinase. However complete knockdown of LKB1 is difficult to achieve (Zhang et al., 2008; Zou et al., 2002; Hattori et al., 2008). In an attempt to definitively assess the role of LKB1, the effect of VEGF on AMPK activity in HeLa cells stably expressing HeLa<sub>LKB1-KD</sub> or HeLa<sub>LKB1-WT</sub> was determined (Sapkota et al., 2002). HeLa<sub>LKB1-KD</sub> or HeLa<sub>LKB1-WT</sub> FLAG-epitope tagged LKB1 was induced in these cell lines by the addition of tetracycline (Figure 4.10A). In both HeLa<sub>LKB1-KD</sub> and HeLa<sub>LKB1-WT</sub> cells AMPK activity was completely unaltered by VEGF treatment. This complete lack of effect was somewhat surprising as in earlier work VEGF had been shown to increase AMPK activity in HeLa cells (Figure 4.4). A number of observations suggest that the HeLa<sub>LKB1-KD</sub> and HeLa<sub>LKB1-WT</sub> cells are indeed functional. Consistent with the view that LKB1 is a constitutively active kinase, induction of HeLa<sub>LKB1-WT</sub> resulted in an increase in the basal activity of AMPK compared to that found in HeLa<sub>LKB1-KD</sub> controls (Figure 4.10B). LKB1-mediated AMPK activation stimulated using AICAR caused a ~2-fold, albeit non-significant, increase in AMPK activity in HeLa<sub>LKB1-WT</sub> compared to HeLa<sub>LKB1-KD</sub> cells in which it was without effect (Figure 4.10B). Moreover, other work conducted in our laboratory using these cell lines demonstrated that rosiglitazone-stimulated AMPK activity is dependent on LKB1 (Boyle et al., 2008). As CaMKK-dependent VEGF-stimulated AMPK activity is seemingly compromised in the HeLa<sub>LKB1KD/WT</sub> cell lines, the contribution, if any, of LKB1 could not be determined. At present the underlying mechanism of these seemingly disparate effects elicited in the various HeLa cell lines remains unknown.

As initial experiments conducted in the present study (Figure 4.1) suggested PLC mediated VEGF-stimulated AMPK activation; and histamine and thrombin had previously been reported to activate AMPK in HUVEC in a PLC-dependent manner (Thors et al., 2004; Thors et al., 2004) the role of PLC signalling was further characterised. Indeed, as a  $Ca^{2+}$ -dependent AMPKK mediates VEGF-stimulated AMPK activation (Figures 4.6 and 4.7), and PLC increases intracellular  $Ca^{2+}$  levels in response to VEGF in endothelial cells (Xia

et al., 1996; McLaughlin and De Vries, 2001), experiments were conducted to determine whether PLC-mediated elevated intracellular  $\text{Ca}^{2+}$  levels are responsible for the stimulation of CaMKK-AMPK signalling.  $\text{IP}_3$  is produced subsequent to  $\text{PLC}\gamma$  activation by VEGF.  $\text{IP}_3$  mobilises  $\text{Ca}^{2+}$  from intracellular stores (Berridge, 1995b), which may then stimulate  $\text{Ca}^{2+}$  influx by a capacitative entry pathway (Maruyama et al., 1997). However in HAEC pre-treated with the  $\text{IP}_3$  receptor blocker 2-APB VEGF-stimulated AMPK activity remained completely intact (Figure 4.11).

Since its discovery 2-APB has been a widely used tool to inhibit  $\text{IP}_3$ -mediated  $\text{Ca}^{2+}$  release (Maruyama et al., 1997; Gysembergh et al., 1999; Ascher-Landsberg et al., 1999; Sergeant et al., 2001; Dyachok and Gylfe, 2001; Bishara et al., 2002), and is an effective modulator of the  $\text{IP}_3$  receptor in many cell types including endothelial cells where the agent has been shown to inhibit  $\text{Ca}^{2+}$  release and subsequent capacitative  $\text{Ca}^{2+}$  entry induced by the agonists ATP, bradykinin, VEGF and receptor-independent  $\text{Ca}^{2+}$  mobilisation via ionomycin or thapsigargin (Bishara et al., 2002; Erdogan et al., 2005). However, emerging data suggest that in some cell types the specific action of 2-APB is on SOC entry rather than initial  $\text{Ca}^{2+}$  release (Ma et al., 2001; Broad et al., 2001). The best evidence for 2-APB inhibiting SOC through a mechanism not involving  $\text{IP}_3$  is that it is still effective in some cell types that do not express  $\text{IP}_3$  receptors (Gafni et al., 1997). In the present study 2-APB had no effect on VEGF-stimulated AMPK activity suggesting  $\text{IP}_3$ -mediated store release may not be involved. Indeed, if 2-APB had any effect on the subsequent entry of  $\text{Ca}^{2+}$  through SOC then this would additionally imply this mechanism may not play a role either.

Although 2-APB was used at a concentration demonstrated to inhibit  $\text{IP}_3$ -mediated  $\text{Ca}^{2+}$  release in endothelial and other cell types (Bair et al., 2008; Bishara et al., 2002; Peppiatt et al., 2003), the present investigation provides no direct evidence demonstrating  $\text{Ca}^{2+}$  release was effectively blocked upon 2-APB addition. The membrane permeable fluorescent calcium probe FLUO-3-AM was utilised in an attempt to directly determine whether 2-APB sufficiently inhibits  $\text{IP}_3$ -mediated  $\text{Ca}^{2+}$  release from intracellular stores under the conditions of the present study. Unexpectedly however, addition of this indicator proved toxic when added to HAEC, even when used at concentrations below that successfully utilised in other studies also conducted in endothelial cells (data not shown). Additional experiments are therefore required to fully determine the role, or lack thereof, of  $\text{IP}_3$ -mediated  $\text{Ca}^{2+}$  release in the regulation of VEGF-stimulated AMPK activity in HAEC. In addition to 2-APB there are a limited number of alternative  $\text{IP}_3$  modulators which may be used to assess the involvement of  $\text{IP}_3$ -mediated  $\text{Ca}^{2+}$  release. Xestospongins were first

described as membrane-permeant IP<sub>3</sub> receptor antagonists (Bootman et al., 2002). However xestospongins are expensive, slow to act and have exhibited variable results between laboratories (Taylor and Broad, 1998). Alternatively heparin is another commonly used IP<sub>3</sub> receptor antagonist. However this agent is not cell permeable and has been shown to have multiple actions such as the activation of ryanodine receptors (Adams et al., 1989; Cannell and Sage, 1989; Takeda and Klepper, 1990).

DAG, also produced upon PLC activation, increases intracellular Ca<sup>2+</sup> levels through non-selective cation channels in the endothelium (Pocock et al., 2004). Experiments on cultured endothelial cells show a relative lack of functional voltage-gated Ca<sup>2+</sup> channels (Colden-Stanfield et al., 1987; Brauneis et al., 1992; Groschner et al., 1994; Popp and Gogelein, 1992; Zhang et al., 1994). Indeed evidence suggests non-selective cation channels are the principal mechanism which account for agonist-induced Ca<sup>2+</sup> entry in the endothelium (Tran et al., 2000). Thrombin, bradykinin, serotonin, ATP and endothelin-1 have all been shown to activate endothelial non-selective cation channels (Adams et al., 1989; Bregestovski et al., 1988; Jacob, 1990; Sauve et al., 1988; Schilling, 1989). Unlike excitable cells, where depolarisation stimulates Ca<sup>2+</sup> influx, depolarisation reduces the driving force for Ca<sup>2+</sup> entry through non-selective cation channels in the endothelium (He and Curry, 1991; Curry, 1992). High potassium solutions have been shown to depolarise the membranes of endothelial cells in a number of studies (Adams et al., 1989; Bregestovski et al., 1988; Jacob, 1990; Sauve et al., 1988; Schilling, 1989). In the present investigation a high K<sup>+</sup>-containing KRH solution was prepared by replacing 59.5 mM Na<sup>+</sup> with KCl to give a final K<sup>+</sup> concentration of 64.3mM (described in detail in section 2.2.1). Similar K<sup>+</sup>-containing solutions have previously been shown to inhibit elevated intracellular Ca<sup>2+</sup> levels induced by Ca<sup>2+</sup> ionophores in endothelial cells and microvessels (He and Curry, 1991; Curry, 1992). The removal of extracellular Ca<sup>2+</sup> (Figure 4.12) and high K<sup>+</sup>-induced depolarisation (Figure 4.11) completely prevented VEGF-stimulated AMPK activity, indicating Ca<sup>2+</sup> entry through non-selective cation channels may underlie this process. There are now numerous reports which show the non-selective cation channels TRPC3/6/7 can be activated by DAG, and their role as DAG-activated channels is widely accepted. HAEC were subsequent treated with the DAG mimetic, OAG, which was found to significantly increase AMPK activity, an effect sensitive to STO-609 pre-treatment and high K<sup>+</sup>-induced depolarisation (Figure 4.14).

DAG kinase catalyses the conversion of DAG to phosphatic acid, whereas DAG lipase catalyses the hydrolysis of DAG, releasing free fatty acid and monoacylglycerol. TRPC6

has been identified as an important channel which participates in bradykinin-induced non-capacitive  $\text{Ca}^{2+}$  entry in endothelial cells. In addition to OAG, the action of bradykinin has been shown to be mimicked by the DAG lipase inhibitor RHC-80267 (Leung et al., 2006). RHC-80267 and the DAG kinase inhibitor R-59949 have also been shown to cause a transient increase in a TRPC-like channel cation channel activity in rabbit ear artery myocytes (Albert et al., 2005). RHC-80267 and R-59949 were used in HAEC to investigate whether inhibition of DAG metabolism could activate AMPK via an increase in endogenous DAG levels. However, neither RHC-80267 nor R-59949, when added alone to HAEC, had any effect on AMPK activity (data not shown). The effect of these agents in combination has, as yet, not been determined. This may be of value as sufficient metabolism of DAG may occur through the non-inhibited pathway, thus preventing a meaningful increase in endogenous DAG levels. In addition, the basal activity of DAG lipase and DAG kinase may be of importance. For example, it is known that in non-stimulated cells DAG kinase activity is low enabling DAG to be used in glycerophospholipid biosynthesis. Therefore, inhibition of this DAG kinase may have little effect on overall DAG levels. However, it may be the case that inhibition of DAG metabolism would greatly augment VEGF-stimulated AMPK activity. Further work is required to test this hypothesis.

$\text{Gd}^{3+}$ , a small lanthanide, is a well recognized blocker of many types of mechanosensitive cation channels. At submillimolar concentrations,  $\text{Gd}^{3+}$  blocks the non-selective mechano-gated channels (Hamill and McBride, Jr., 1996) and various  $\text{Ca}^{2+}$  permeable channels (Beedle et al., 2002; Biagi and Enyeart, 1990; Lansman, 1990; Mlinar and Enyeart, 1993). In addition  $\text{Gd}^{3+}$  has been used to inhibit members of the TRPC channel subfamily (Minke and Cook, 2002; Cheng et al., 2006). In experiments conducted in the present investigation  $\text{Gd}^{3+}$  elicited a highly variable effect on AMPK activity (Figure 4.15). In hindsight, this may not be of great surprise as dual effects of lanthanides, potentiating and blocking, have been reported in members of the mammalian TRP channel superfamily. The currents carried through TRPC4 and TRPC5 have been shown to be potentiated by submillimolar concentrations of  $\text{La}^{3+}$  or  $\text{Gd}^{3+}$ , whereas millimolar concentrations were inhibitory. In addition  $\text{Gd}^{3+}$  has previously been shown to inhibit TRPC3 and TRPC6 without affecting TRPC7 (Cheng et al., 2006).

Other modulators have been used to investigate the involvement of non-selective cation channels such as nickel and flufenamic acid. Nickel ions have previously been shown to attenuate  $\text{Ca}^{2+}$  influx in endothelial cells (He and Curry, 1991; Jacob, 1990). However, in



the present study the addition of nickel to endothelial cells proved highly toxic (data not shown). Flufenamic is another molecule often used to investigate the role of TRPC channels. However again this agent is not ideal as it is known to enhance TRPC6 whilst inhibiting TRPC3 currents (Pocock et al., 2004).

In general TRPC channels are almost ubiquitously expressed and most have splice variants. Primary endothelial cells in culture express multiple TRPC isoforms with different expression patterns occurring between endothelial cells from different species and vascular beds (Tiruppathi et al., 2006). For example, HUVEC express all of the DAG-activated TRPC isoforms, whereas RPAEC do not express any (Tiruppathi et al., 2006). Of the DAG-activated TRPC channels RT-PCR confirmed TRPC6 expression in HAEC (Figure 4.13). Neither TRPC3 nor TRPC7 were detected. However, a positive control such as HUVEC or any other TRPC3/7 expressing tissue would need to be included in order to rule out these isoforms. In addition isoform specific antibodies against TRPC channels are now commercially available and may be utilised to ascertain the TRPC expression profile in HAEC.

A number of other studies have implicated TRPC channels as components of various VEGF-signalling pathways. VEGF was initially shown to increase microvascular permeability *in vivo* through a mechanism that is dependent on PLC (Pocock et al., 2004). Subsequent work has shown DAG-mediated  $\text{Ca}^{2+}$  entry through TRPCs is essential for this increase in permeability (Pocock et al., 2004). A very recent report has implicated TRPC6 in the VEGF-mediated angiogenic pathway in human endothelial cells (Hamdollah Zadeh et al., 2008), and it has previously been shown that co-transfection of HEK cells with VEGF-R2 and TRPC6 enables the cells to respond to VEGF by increasing intracellular  $\text{Ca}^{2+}$  (Pocock et al., 2004). Also of particular interest is the recent identification of EETs as a component of the VEGF-AMPK signalling cascade leading to angiogenesis (Webler et al., 2008). It has been proposed that EETs regulate intracellular  $\text{Ca}^{2+}$  by inducing the translocation of TRPC6 channel proteins to caveolin-1 rich areas of the endothelial cell membrane (Fleming et al., 2007). Together, these reports and the findings of the present study suggest TRPCs (likely TRPC6) may be involved in the regulation of VEGF-stimulated AMPK activity in HAEC. In endothelial cells  $\text{K}^+$  channel activation results in a hyperpolarisation of the cell membrane building an electrochemical gradient for prolonged  $\text{Ca}^{2+}$  influx (Vaca et al., 1996; Luckhoff and Busse, 1990). In HUVEC VEGF has been demonstrated to cause a dose-dependent hyperpolarisation of the endothelial cell membrane resulting in SOC and subsequent endothelial cell proliferation and NO

production (Erdogan et al., 2005). These effects are sensitive to the potassium channel (Kv1.3) blocker margotoxin and 100  $\mu$ M 2-APB. It would be interesting to determine whether this mechanism is involved in the regulation of VEGF-stimulated AMPK activity. The sensitivity of this signalling mechanism to the same concentration of 2-APB found to be without effect on VEGF-stimulated AMPK activity in the present study suggests this may not be the case. Even so, it is important to note high  $K^+$ -induced depolarisation and the removal of extracellular  $Ca^{2+}$ , which were found to prevent VEGF stimulated AMPK activity in the current investigation, (Figures 4.11 and 4.12 respectively) would also likely prevent increased  $Ca^{2+}$  influx resultant from hyperpolarisation of the endothelial cell membrane. Indeed although OAG-stimulated AMPK activity is suggestive of a role for DAG in the regulation of AMPK it does not directly implicate DAG, nor the TRPC channels upon which it acts. Therefore further work is necessary to directly determine whether TRPCs are a component of the VEGF-AMPK signalling cascade in HAEC. Notably, TRPC1 was recently shown to be a necessary component of thrombin-induced AMPK activation through  $CaMKK\beta$  in endothelial cells (Bair et al., 2008), demonstrating these channels can modulate AMPK signalling.

PKC represents a family of serine/threonine kinases activated by signals which increase DAG concentration. As the mimetic OAG stimulates AMPK in a manner similar to that of VEGF the role of PKC is also of interest. Indeed, work by Zou's group has suggested that  $PKC\zeta$  may regulate AMPK by promoting the association of LKB1 with AMPK (Xie et al., 2006). Furthermore, it has been reported that stimulation of BAEC with the PKC activator PMA activates AMPK (Xie et al., 2006). Whether PKC contributes to VEGF-AMPK signalling remains unknown. VEGF-induced vascular permeability has been demonstrated to be dependent on DAG-mediated  $Ca^{2+}$  influx, but is unaffected by inhibition of PKC (Pocock and Bates, 2001; Pocock et al., 2004). Therefore, it may be the case that a similar DAG-dependent, PKC-independent mechanism mediates VEGF-stimulated AMPK activity in HAEC. However, further work is necessary to determine whether this is the case.

## **5 AMPK: A COMPONENT OF THE VEGF-STIMULATED ANGIOGENIC RESPONSE**

## 5.1 Introduction

Angiogenesis, the physiological process involving the growth of blood vessels from pre-existing ones, is essential for embryonic development, growth, formation of the corpus luteum and endometrium, regeneration and wound healing (Ferrara, 2002b; Munoz-Chapuli et al., 2004). Abnormal angiogenesis is associated with a number of pathologies; including cancer, diabetic retinopathy and arthritis (Ferrara, 2004a; Munoz-Chapuli et al., 2004).

One of the earliest intracellular signalling events described for VEGF was an increase in the intracellular  $\text{Ca}^{2+}$  concentration of endothelial cells (Criscuolo et al., 1989; Brock et al., 1991). It is now clear that an increase in intracellular  $\text{Ca}^{2+}$  plays a key role in the mitogenic and secretory effects of growth factors (Soltoff and Cantley, 1988; Nilius et al., 1997), and is required for most of the downstream functions of VEGF including angiogenesis (Kohn et al., 1995; Erdogan et al., 2005; Hamdollah Zadeh et al., 2008; Faehling et al., 2002). In addition NO is a key regulator of the angiogenesis (Papapetropoulos et al., 1997; Yu et al., 2005). As VEGF-stimulates the activation of AMPK, an effect mediated by a  $\text{Ca}^{2+}$ -dependent upstream kinase, leading to an increase in NO production the intriguing possibility that AMPK may also be involved in the angiogenic process was highlighted.

Interestingly some previous studies have demonstrated that AMPK may mediate angiogenesis in response to both adiponectin (Ouchi et al., 2004; Shibata et al., 2004) and hypoxia (Nagata et al., 2003). These studies, like many others, used a range of *in vitro* and *in vivo* models to assess the angiogenic response in their systems. Angiogenesis involves the escape of endothelial cells from their stable location through the basement membrane, with subsequent migration toward an angiogenic stimulus such as VEGF. Behind this migrating front endothelial cells must proliferate thus providing the requisite number of cells for a new blood vessel. Subsequent to this proliferation the new outgrowth of endothelial cells needs to reorganise into a 3D structure capable of carrying blood. Each of these elements, basement membrane disruption, cell migration, cell proliferation and tube formation are processes routinely assessed by investigators using various *in vitro* models designed to assess these individual angiogenic processes. In addition to these *in vitro* methods there are also a number of organ culture assays, namely the aortic ring assay and the chick aortic arch assay, which in addition to endothelial cells, include the surrounding mural cells also regarded as important in angiogenesis (Karamysheva, 2008). However, perhaps the best approach to determine angiogenesis is an *in vivo* model, of which the

chick chorioallantoic membrane (CaM) assay or the *in vivo* Matrigel plug assay are the most widely used.

The present study utilised an *in vitro* approach to initially determine whether AMPK may be involved in the VEGF-stimulated angiogenic response in HAEC. As endothelial cell migration and proliferation are key processes of the angiogenic response both were assessed as described in the following chapter.

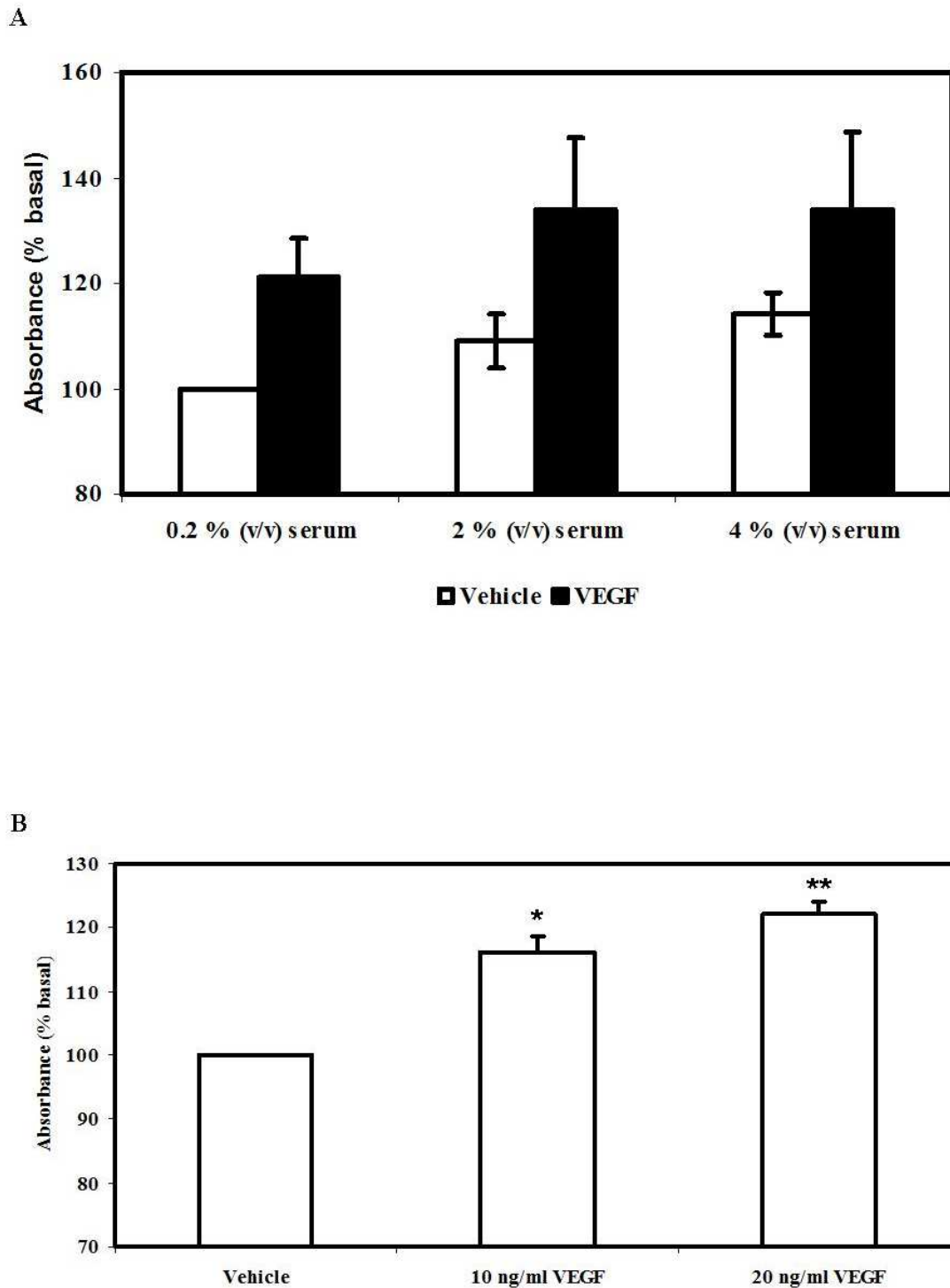
## 5.2 Results

HAEC proliferation was determined using a colorimetric method for determining the number of viable cells in proliferation assays (described in detail in section 2.6.6). Treatment of HAEC with serum (Figure 5.1A) or VEGF (Figure 5.1B) elicited a dose-dependent increase in HAEC proliferation. Throughout the course of the current investigation HAEC were routinely cultured in endothelial cell media containing 2 % (v/v) serum (formulation described in detail in section 2.1.6). However, serum contains a range of mitogens therefore, in order to assess the specific role of a given physiological concentration of VEGF on HAEC proliferation and migration, a low-serum-containing media formulation (0.2 % (v/v) serum) was utilised.

In HAEC incubated in this low-serum formulation, cells remained healthy and responsive to VEGF which was found to stimulate HAEC proliferation in a dose-dependent manner (10 ng/ml VEGF,  $116 \pm 3$  %,  $n=5$ ,  $p<0.01$ ; 20 ng/ml VEGF,  $122 \pm 2$  %,  $n=5$ ,  $p<0.001$  versus control cells) (Figure 5.1B).

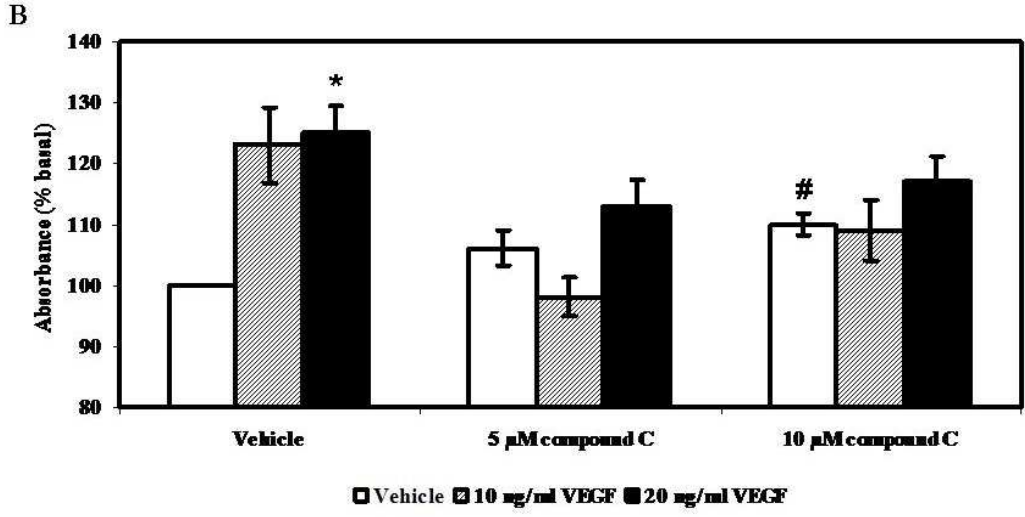
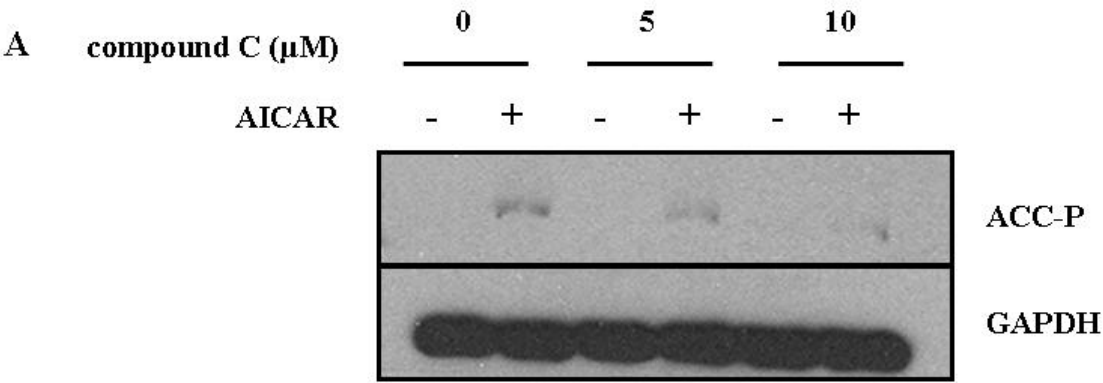
The AMPK inhibitor compound C was used to determine whether AMPK is involved in VEGF-stimulated proliferation. Pre-treatment of HAEC with this agent prevented AICAR-stimulated ACC-Ser-80 phosphorylation (Figure 5.2A), and moreover was found to attenuate VEGF-stimulated proliferation (Figure 5.2B). Treatment of HAEC with 10  $\mu$ M compound C resulted in an increase in proliferation even in the absence of VEGF treatment suggesting a role for AMPK in the regulation of basal proliferation.

The effect of the CaMKK inhibitor STO-609 on HAEC proliferation was also determined. VEGF (20 ng/ml) treatment was found to stimulate HAEC proliferation ( $125 \pm 5$  %,  $n=3$ ,  $p<0.05$  versus vehicle-treated cells), an effect abolished in cells treated with STO-609 (5  $\mu$ M STO-609,  $108 \pm 3$  %,  $n=3$ ,  $p<0.05$ ; 10  $\mu$ M STO-609,  $106 \pm 2$  %,  $n=3$ ,  $p<0.05$  in comparison to 20 ng/ml VEGF-treated cells) (Figure 5.3).



**Figure 5-1 VEGF- and serum-mediated HAEC proliferation**

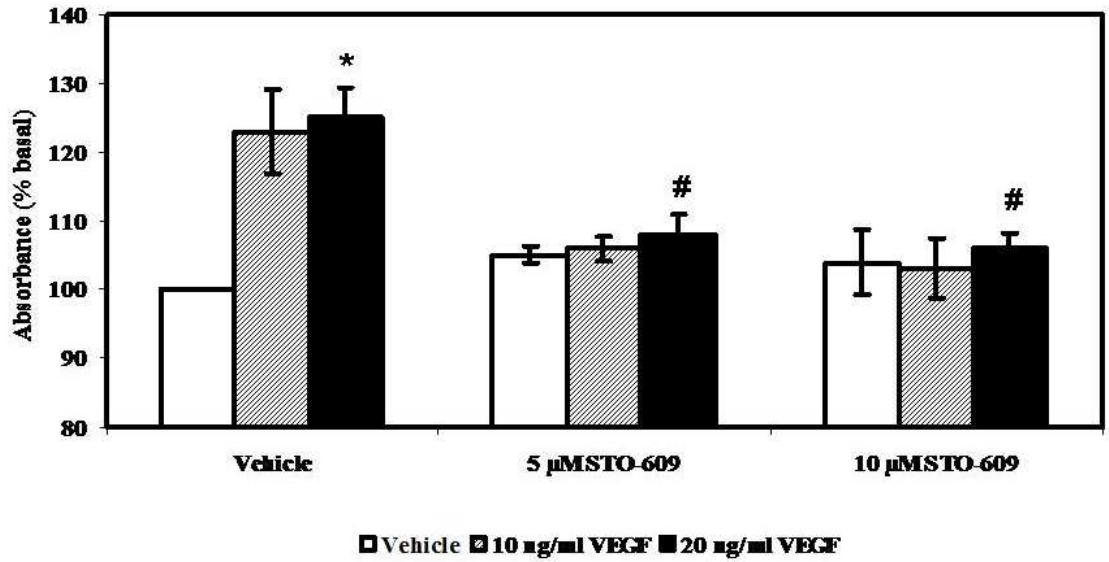
(A) HAEC were serum starved for 2h then incubated in basal endothelial cell media containing 0.2-4 % (v/v) serum in the presence or absence of 20 ng/ml VEGF and proliferation assessed. The results are expressed as the mean  $\pm$  SEM % basal absorbance measured at 488 nm for two independent experiments conducted in triplicate. (B) HAEC were serum starved for 2h then treated with the indicated concentrations of VEGF for 24h in basal endothelial cell media containing 0.2 % (v/v) serum (a formulation used in all subsequent experiments unless otherwise indicated) and proliferation assessed. The results are expressed as the mean  $\pm$  SEM % basal absorbance measured at 488 nm for five independent experiments conducted on 3-6 replicates in each case. \* $p < 0.01$  or \*\* $p < 0.001$  relative to the value in the absence of VEGF treatment.



**Figure 5-2 Effect of compound C on VEGF-stimulated HAEC proliferation**

(A) HAEC were incubated with the indicated concentrations of compound C for 24h, treated in the presence or absence of AICAR (2 mM, 45 minutes) and lysates prepared. Lysates were resolved by SDS PAGE, transferred to nitrocellulose and probed with the antibodies indicated. Probing of lysates with  $\alpha$ -ACC-Ser-80 resulted in a single band at ~280 kDa whereas as probing with  $\alpha$ -GAPDH resulted in a band at ~ 35 kDa. (B) HAEC were treated with the indicated concentrations of compound C and/or VEGF for 24h and proliferation assessed. The results are expressed as a mean  $\pm$  SEM % basal absorbance at 488 nm for three independent experiments conducted in triplicate. \* $p$ <0.05 relative to value in the absence of VEGF. # $p$ <0.05 relative to value in the absence of compound C.





**Figure 5-3 Effect of STO-609 on VEGF-stimulated HAEC proliferation**

HAEC were treated with the indicated concentrations of STO-609 and/or VEGF for 24h and proliferation assessed. The results are expressed as a mean  $\pm$  SEM % basal absorbance at 488 nm for three independent experiments conducted in triplicate. \* $p < 0.05$  relative to the value in the absence of VEGF treatment, # $p < 0.05$  relative to the value in the absence of STO-609.

Ad.DN was also used to further characterise the involvement of AMPK in the VEGF-mediated proliferative response. The Western blots shown in Figures 5.4A and B show Ad.DN potently inhibited AICAR-induced ACC Ser-80 phosphorylation; an effect which remained intact in control-infected HAEC. VEGF-treatment (20 ng/ml) was found to increase HAEC proliferation in control-infected cells (Figure 5.4C). However, in HAEC infected with Ad.DN cell proliferation evoked by 20 ng/ml VEGF treatment was abolished (Figure 5.4C). Incubation of HAEC with 10 ng/ml VEGF, a concentration previously shown to stimulate proliferation (Figure 5.1B), was found to be without effect in this set of experiments (Figure 5.4C). This observation may suggest virally infected HAEC have a reduced sensitivity to VEGF treatment. Infection of HAEC with Ad.DN also seemed to result in a reduction in the basal proliferative rate ( $86 \pm 6\%$ ,  $n=6$ ,  $p=0.053$ ) (Figure 5.4C). It may be the case that cell proliferation is altered in the absence of AMPK. Indeed, in HAEC treated with 10 ng/ml VEGF, a concentration which did not stimulate cell proliferation, Ad.DN infection reduced proliferation in comparison to control-infected cells (Figure 5.4C).

The use of Ad.DN was also employed to determine whether AMPK mediates VEGF-stimulated migration. Migration of HAEC toward VEGF was increased ~3-fold in control-infected HAEC (Figure 5.5). In contrast, when HAEC were infected with Ad.DN VEGF-stimulated migration was abolished (Figure 5.5).

A constitutively active mutant AMPK (Ad.CA) (Woods et al., 2000) was also expressed in HAEC, by adenovirus-mediated gene transfer, and the subsequent effect on HAEC migration assessed. This constitutively active mutant consists of a truncated AMPK $\alpha$ 1 (residues 1-312) where Thr-172 is mutated to an aspartic acid. Truncation of AMPK $\alpha$ 1 at residue 312 yields a polypeptide that no longer associates with the  $\beta$  and  $\gamma$  subunits, yet retains significant kinase activity (Crute et al., 1998). Mutation of Thr-172, the major site of phosphorylation by AMPK kinase (Hawley et al., 1996), to an aspartic acid, prevents its inactivation by protein phosphatases (Stein et al., 2000). As demonstrated in Figure 5.5 infection of cells with Ad.CA, which potently stimulates phosphorylation of ACC at Ser-80 (Figure 5.6), increased HAEC migration even in the absence of VEGF treatment (~7-fold). There was no additional increase in migration of Ad.CA-infected HAEC in response to VEGF (Figure 5.5).

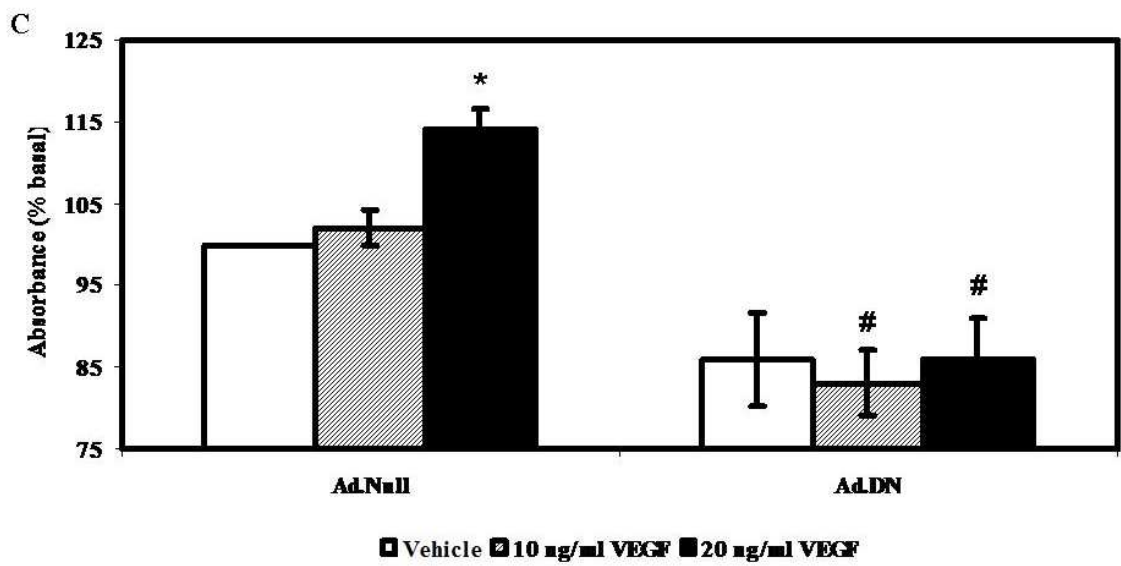
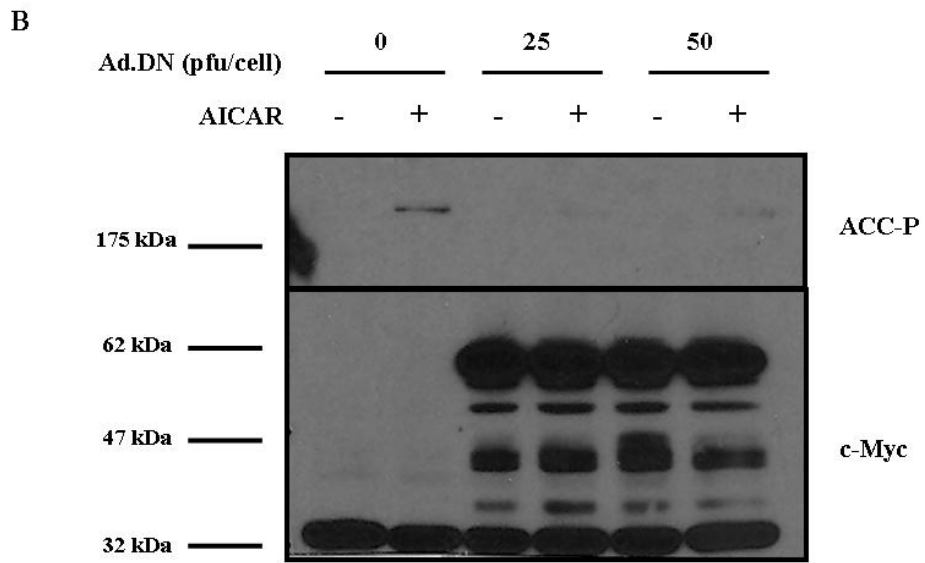
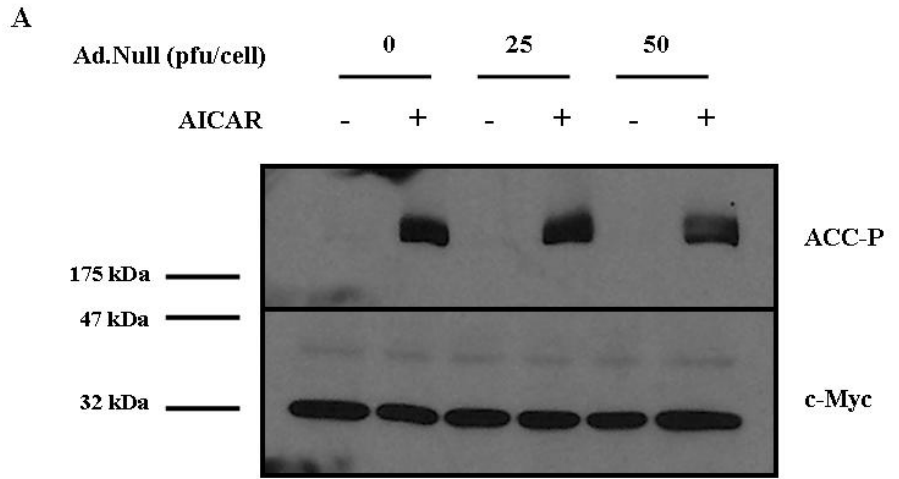
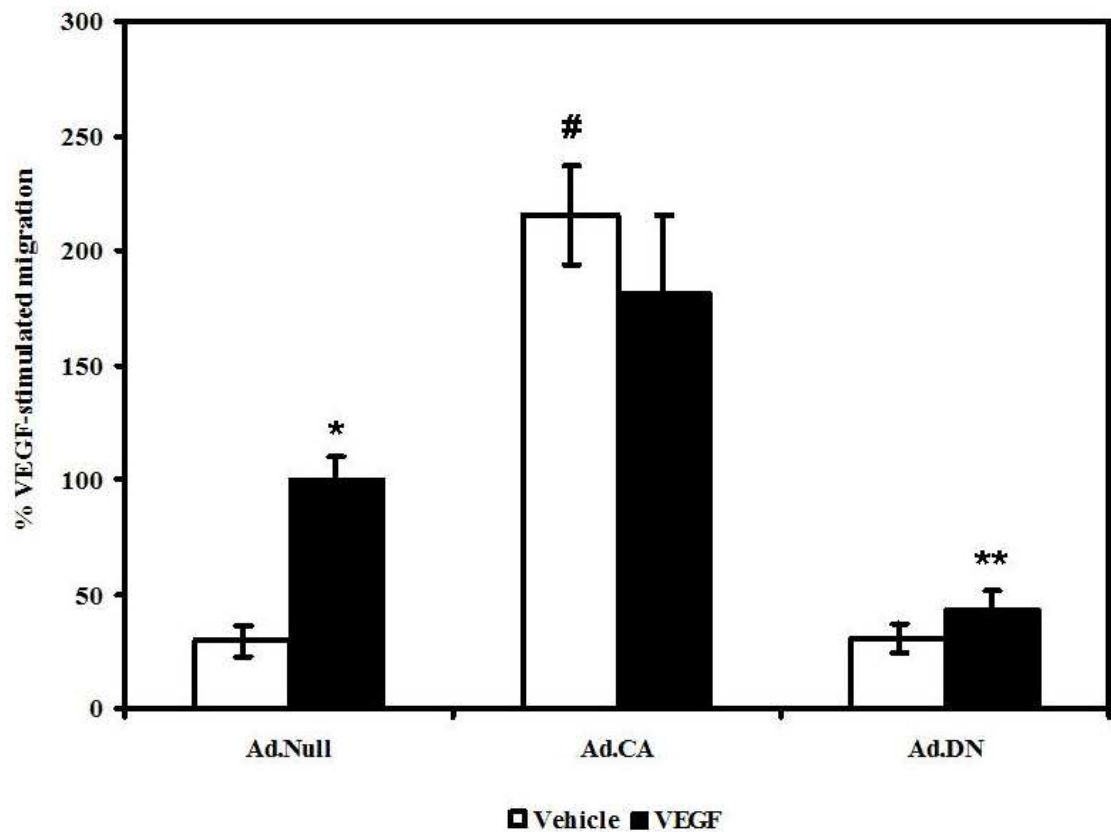


Figure legend overleaf

**Figure 5-4 Effect of Ad.DN on VEGF-stimulated HAEC proliferation**

(A and B) HAEC were infected with 0-50 pfu/cell Ad.Null or Ad.DN as indicated for 24h then incubated in the presence or absence AICAR (2 mM, 45 minutes). Lysates were prepared, resolved by SDS PAGE, transferred to nitrocellulose and probed with the antibodies indicated. (C) HAEC were infected with 25 pfu/cell adenovirus as indicated for 24h and subsequently treated in the presence or absence of the indicated concentrations of VEGF and proliferation assessed. The results are expressed as the mean  $\pm$  SEM % basal absorbance at 488 nm for six independent experiments conducted in triplicate. \* $p < 0.01$  relative to the value in the absence of VEGF, # $p < 0.01$  relative to the value in control-infected cells treated with the same concentration of VEGF.



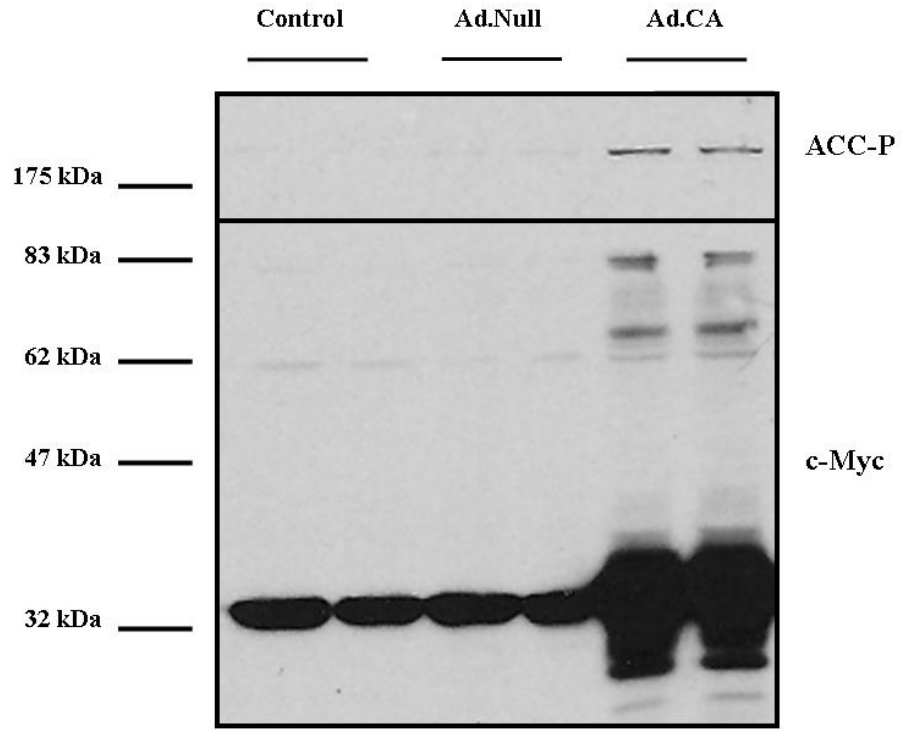
**Figure 5-5 Effect of Ad.DN on VEGF-stimulated HAEC migration**

HAEC were infected with Ad.Null, Ad.DN or Ad.CA adenovirus for 24h. HAEC migration in response to 10 ng/ml VEGF was subsequently assessed using a Boyden chamber (described in detail in section 2.6.7). The results are expressed as the mean  $\pm$  SEM % VEGF-stimulated migration based on 12-24 replicates for each condition. \* $p < 0.001$  relative to value in absence of VEGF. # $p < 0.001$  relative to value in control-infected cells. \*\* $p < 0.001$  relative to value in control-infected cells treated with VEGF. These experiments were performed by Dr. Marie-Ann Ewart.

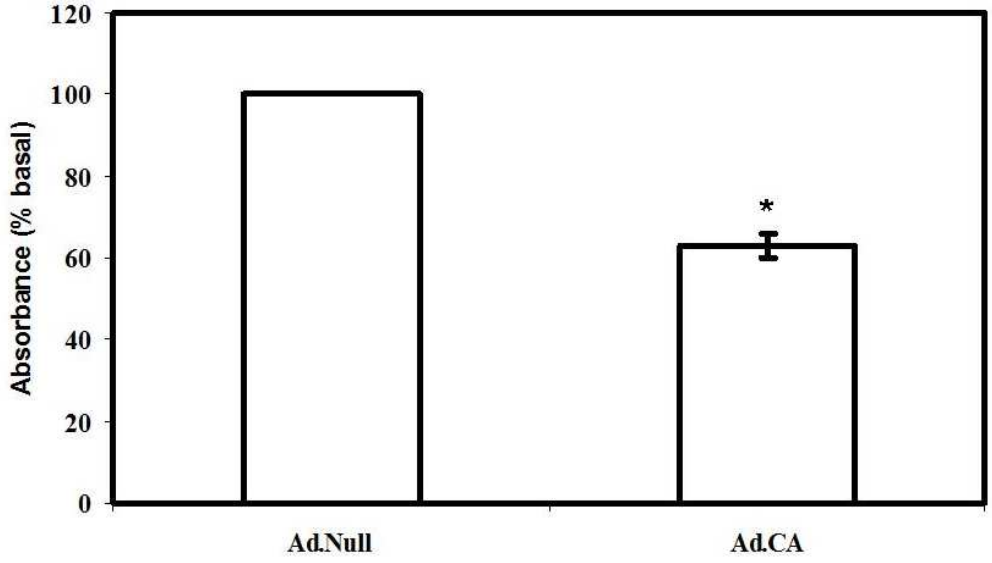
Experiments were also conducted in order to determine the effect of AMPK activation on HAEC proliferation. Treatment of HAEC with Ad.CA resulted in an increase in phosphorylation of ACC at Ser-80 (Figure 5.6A), and furthermore elicited a decrease in cell proliferation (Figure 5.6B). Similarly, treatment of HAEC with AICAR resulted in a dose-dependent decrease in HAEC proliferation (Figure 5.7).

In addition the newly identified AMPK activator A-769662 (a generous gift provided by Professor D.G Hardie, University of Dundee) was also used to investigate the effect of AMPK activation on HAEC proliferation. Treatment of HAEC with this agent resulted in a dose-dependent increase in phosphorylation of ACC at Ser-80 (Figure 5.8A) and furthermore resulted in a trend whereby proliferation was decreased in a dose-dependent fashion (Figure 5.8B).

A

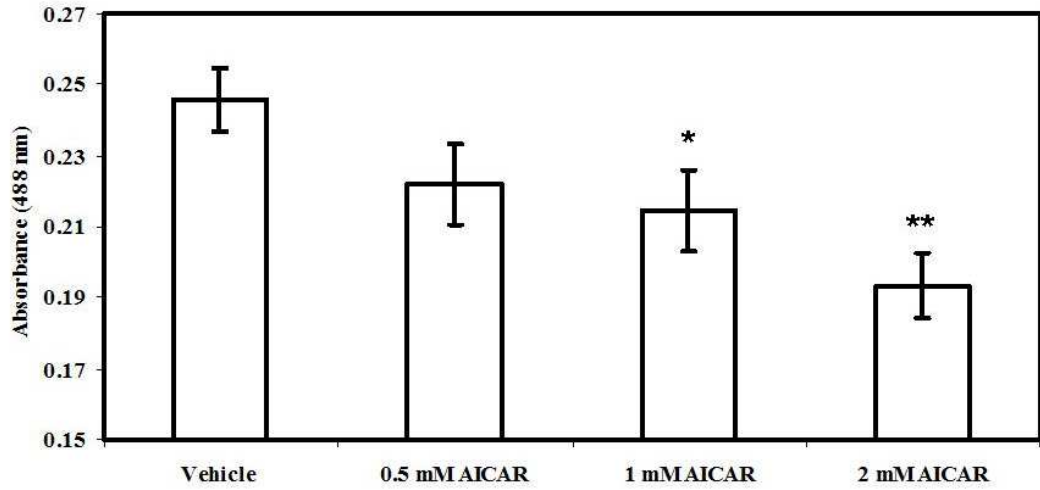


B



**Figure 5-6 Effect of Ad.CA on HAEC proliferation**

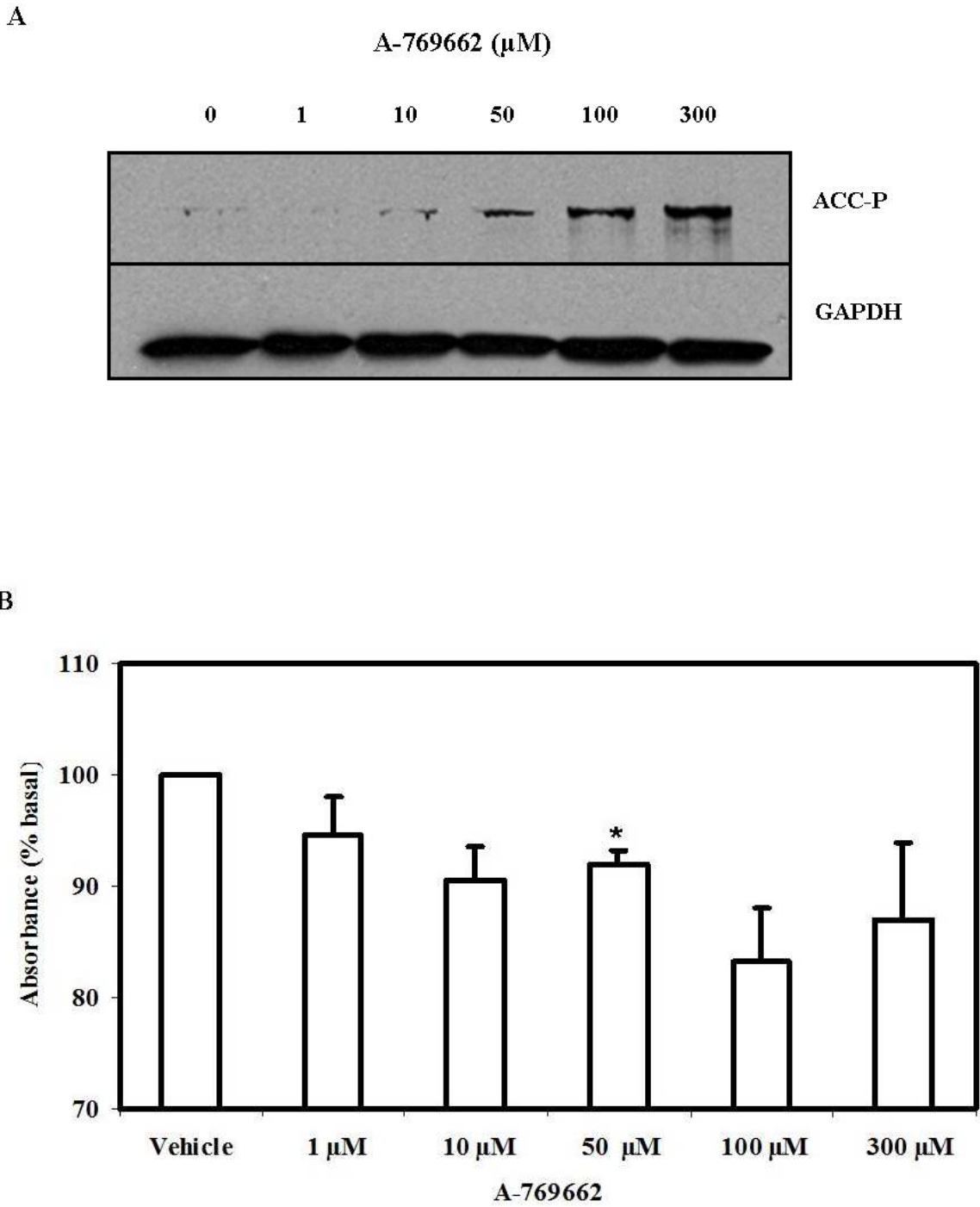
(A) HAEC were infected with Ad.Null or Ad.DN and lysates prepared. Lysates were also prepared from uninfected HAEC (control). Lysates were resolved by SDS PAGE, transferred to nitrocellulose and probed with the antibodies indicated. (B) HAEC were infected with Ad.Null or Ad.CA and proliferation assessed. The results are expressed as a mean  $\pm$  SEM % basal absorbance at 488 nm for three independent experiments conducted in triplicate. \* $p < 0.01$  relative to the value of control-infected cells.



**Figure 5-7 Effect of AICAR on HAEC proliferation**

HAEC were treated with the indicated concentrations of AICAR for 45 minutes, serum starved for 2h and proliferation assessed as normal. The results are expressed as a mean  $\pm$  SEM % basal absorbance at 488 nm based on 12 replicates. \* $p < 0.05$ , \*\* $p < 0.001$  relative to value in absence of AICAR. These experiments were performed by Dr Marie-Ann Ewart.





**Figure 5-8 Effect of A-769662 on HAEC proliferation**

(A) HAEC were incubated with the indicated concentrations of A-769662 for 24h and lysates prepared. Lysates were subsequently resolved by SDS PAGE, transferred to nitrocellulose and probed with the antibodies indicated. Probing of lysates with  $\alpha$ -ACC-Ser-80 resulted in a single band at  $\sim 280$  kDa whereas as probing with  $\alpha$ -GAPDH resulted in a band at  $\sim 35$  kDa (B) HAEC were treated with the indicated concentrations of A-769662 for 24h and proliferation assessed. The results are expressed as a mean  $\pm$  SEM % basal absorbance at 488 nm for three independent experiments conducted in triplicate. \* $p < 0.05$  relative to value in absence of A-769662.

### 5.3 Discussion

Compound C and STO-609 have been used in a number of studies on endothelial cells to inhibit AMPK and CaMKK respectively (Kim et al., 2007; Youn et al., 2008; Levine et al., 2007; Stahmann et al., 2006; Hattori et al., 2008; Levine et al., 2007). Treatment of HAEC with compound C attenuated VEGF-stimulated phosphorylation of ACC on Ser-80 (Figure 5.2A) and VEGF-stimulated proliferation (Figure 5.2B). Similarly STO-609 was found to abrogate VEGF-stimulated HAEC proliferation (Figure 5.3). Although these initial observations indicate a role for AMPK in the regulation of VEGF-mediated proliferation it is important to note that both STO-609 and compound C have multiple off-target effects. Indeed when tested against a panel of protein kinases STO-609, also inhibited, in addition to CaMKK $\alpha/\beta$ , ERK8, MAPK integrating protein kinase 1 (MNK1), casein kinase 2, AMPK, provirus integration site for Moloney murine leukaemia virus 2 and 3, dual-specificity tyrosine-phosphorylated and regulated kinase (DYRK) 2 and 3, and homeodomain interacting protein kinase 2 (HIPK2) (Bain et al., 2007). Similarly, in the same study, the AMPK inhibitor compound C proved to be a potent inhibitor of other kinases including ERK8, MNK1, phosphorylase kinase, maternal embryonic leucine zipper kinase, DYRK isoforms, HIPK2, Src, lymphocyte-cell specific protein tyrosine kinase, Yes, FGF receptor 1 and ephrin A2 receptor (Bain et al., 2007). Another report has also shown that compound C inhibits the hypoxic activation of HIF-1 independent of AMPK in MEFs (Emerling et al., 2007). A lack of effect in response to STO-609 or compound C treatment may provide some evidence precluding the involvement of CaMKK and AMPK respectively in a given physiological event. However, due to their relative lack of specificity, any attenuated response as a result of their use does not necessarily establish the involvement of CaMKK or AMPK. Therefore, in order to confirm AMPK as a mediator of VEGF-stimulated proliferation, and also migration, a dominant negative AMPK adenovirus was utilised. Both VEGF-stimulated HAEC proliferation and migration were abrogated in HAEC in which AMPK had been ablated by infection with Ad.DN (Figures 5.4C and 5.5 respectively). In contrast, VEGF evoked a significant stimulation of proliferation and migration in control-infected HAEC (Figures 5.4C and 5.5 respectively). It was additionally found that infection of HAEC with Ad.CA, even in the absence of chemoattractant i.e. VEGF, resulted in a significant increase in cell migration, indicative of the induction of chemokinesis in these cells (Figure 5.5).

Together these observations establish AMPK as a mediator of key physiological functions central to the formation of a new vessel in human endothelial cells. These findings are

consistent with a number of other reports implicating AMPK as a mediator of the angiogenic response. Calpain, a cytosolic cysteine protease that translocates to membrane when activated, is a key regulator of the cytoskeletal organisation of endothelial cells (Deroanne et al., 2001; Kulkarni et al., 2002); a process essential for angiogenesis. Calpain acts through a range of substrates to mediate effects on endothelial cell migration, differentiation and proliferation (Goll et al., 2003; Cuevas et al., 2003; Kulkarni et al., 1999; Hajimohammadreza et al., 1997). Calpain, shown to mediate VEGF-stimulated angiogenesis in human microvascular endothelial cells (Su et al., 2006), has recently been reported to be an upstream mediator of AMPK upon VEGF stimulation of BAEC (Youn et al., 2008). Calpain has additionally been shown to act upstream of Rac1 and RhoA to regulate the formation of focal adhesions and Rac- and Rho-induced cytoskeletal organisation in BAEC (Kulkarni et al., 1999). Rac1, acting downstream of the VEGF-AMPK signalling cascade in BAEC, has been identified as a mediator of both tube formation and BAEC migration (Levine et al., 2007). Other work conducted using transgenic mice overexpressing the calpain inhibitor calpastatin has suggested AMPK is a substrate for calpain in skeletal muscle (Otani et al., 2006).

A number of other AMPK activators have also been shown to participate in the regulation of the angiogenic response. Statins have been shown to regulate endothelial cell angiogenesis *in vitro* and *in vivo*, mediated through NO (Kureishi et al., 2000). Shyy and colleagues have demonstrated in HUVEC that statin-induced tube formation is significantly attenuated by infection of a dominant negative AMPK adenovirus or compound C treatment (Sun et al., 2006). Adiponectin has been shown to stimulate HUVEC migration and differentiation into capillary-like structures *in vitro* (Ouchi et al., 2004) and angiogenesis in response to tissue ischemia *in vivo* (Shibata et al., 2004), in an AMPK-mediated manner.

AMPK signalling has also been reported as essential for angiogenesis in response to hypoxic stress (Nagata et al., 2003). However, in contrast to data presented in the current investigation (Figure 5.5), Walsh and co-workers reported suppression of AMPK signalling was without effect on VEGF-stimulated migration under normoxic conditions (Nagata et al., 2003). Similar to the findings of the present study, in normoxic conditions, caffeine, at least in part, and adiponectin, have been shown to mediate endothelial cell migration in an AMPK-dependent manner (Spyridopoulos et al., 2008; Ouchi et al., 2004). Hypoxia has been reported to upregulate CYP epoxygenases (Michaelis et al., 2005), which metabolise arachidonic acid to EETs, known mediators of endothelial cell

proliferation, migration and angiogenesis (Chen et al., 1998; Fleming et al., 2001b; Medhora et al., 2003; Michaelis et al., 2005; Zhang and Harder, 2002) Whilst CYP 2C protein expression has been demonstrated in native endothelial cells; mRNA and protein levels rapidly decrease after cell isolation, so that in passaged cultured endothelial cells mRNA can only be detected using RT-PCR (Fisslthaler et al., 2000). Endothelial cell migration and tube formation are inhibited by a CYP 2C inhibitor and by downregulation of CYP 2C8/9 expression using antisense oligonucleotides (Michaelis et al., 2005). As it has been reported that CYP 2C-derived EETs act as second messengers in the angiogenic response evoked by VEGF in endothelial cells (Webler et al., 2008) it may be the case that the CYP isozyme expression level may play a role in VEGF-stimulated endothelial cell signalling. This may provide a possible explanation for the disparate results observed in the present study and by (Nagata et al., 2003) where sufficient levels of CYP may only have been present under hypoxic conditions in the endothelial cells used by Nagata and coworkers.

It is also worth noting that Nagata and colleagues used a dominant negative AMPK $\alpha$ 2 construct whereas the present study utilised a dominant negative AMPK $\alpha$ 1 version. Both  $\alpha$ 1- and  $\alpha$ 2-AMPK subunits are expressed in endothelial cells (Davis et al., 2006), though the predominant isoform is  $\alpha$ 1 (Zou et al., 2004; Davis et al., 2006), with AMPK $\alpha$ 2 barely detectable in these cells. Thus, it remains possible that AMPK $\alpha$ 1 (and not AMPK $\alpha$ 2) is responsible for mediating VEGF-stimulated angiogenesis under normoxic conditions, whereas the AMPK $\alpha$ 2 isoform may be of importance under hypoxic conditions. Further work is necessary to determine whether this is the case.

CaMKK is the upstream kinase for VEGF-stimulated AMPK activation (chapter 3) and (Levine et al., 2007). This is of particular interest considering the number of reports that have demonstrated the critical nature of transmembrane Ca<sup>2+</sup> entry in relation to processes associated with angiogenesis (Faehling et al., 2002; Erdogan et al., 2005; Hamdollah Zadeh et al., 2008). Preliminary work conducted in the present study (discussed in detail in chapter 4) and a recent report (Hamdollah Zadeh et al., 2008) suggest TRPC channels may underlie the Ca<sup>2+</sup> signal which is a key component of the VEGF-AMPK signalling cascade leading to angiogenesis. EETs, which have recently been implicated in VEGF-mediated AMPK signalling (Webler et al., 2008) and activate several signalling pathways to promote endothelial cell proliferation, migration and angiogenesis, have also been demonstrated to regulate Ca<sup>2+</sup> influx in response to bradykinin by a mechanism that involve translocation of TRP channels in endothelial cells (Fleming et al., 2007). If indeed

TRPC channels are involved in the regulation of VEGF-stimulated AMPK activation in HAEC it would be of additional interest to characterise their involvement, and that of CaMKK, in any subsequent downstream effects, such as proliferation and migration. In T lymphocytes triggering of the TCR has been shown to stimulate CaMKK-AMPK signalling (Tamas et al., 2006). It has been proposed that AMPK activation by TCR may be a mechanism to stimulate the conservation and production of ATP in anticipation of the demand for ATP initiated by  $\text{Ca}^{2+}$ -mediated signalling pathways; in this instance the energy demands of an immune response (Tamas et al., 2006). CaMKK-mediated activation of AMPK in the endothelium may underlie a similar mechanism to regulate events such as angiogenesis. It is also worth noting that in response to VEGF, a variety of parallel signalling pathways, in addition to AMPK, are also stimulated which are necessary for a proliferative response. For example, VEGF activated MAPKs, which are key components of the VEGF-induced signalling cascade leading to endothelial cell proliferation (Waltenberger et al., 1996; Ziche et al., 1997; Kroll and Waltenberger, 1997), are necessary for VEGF-induced proliferation, but are not sufficient to induce proliferation when activated alone (Faehling et al., 2002). Thus it is clear a multitude of pathways are necessary for a proliferative response. NO, a key regulator of this response (Papapetropoulos et al., 1997; Yu et al., 2005), which is produced, at least in part, downstream of VEGF-mediated AMPK activation may be responsible for the proliferative effects mediated by AMPK in HAEC; which acts in concert with  $\text{Ca}^{2+}$ -independent pathways such as MAPK to regulate proliferation.

Whilst the data presented in the current study demonstrate that AMPK is required for VEGF-mediated proliferation a wide range of experimental evidence has additionally shown activation of AMPK mediates a number of pathways (summarised in Figure 5.9) which result in the suppression of proliferation. These pathways, briefly described below, are reviewed in (Motoshima et al., 2006).

Up-regulation of the p53-p21 axis by AMPK has been shown to cause G1 cell cycle arrest due to the accumulation of the tumour suppressor p53, which in turn up regulates the CDKI protein p21<sup>CIP</sup> (Imamura et al., 2001; Jones et al., 2005; Igata et al., 2005). AMPK-mediated activation of mTOR, a serine/threonine kinase known to regulate protein translation and synthesis, suppresses mTOR signalling by growth factors and amino acids (Kimura et al., 2003; Krause et al., 2002; Bolster et al., 2002). FAS, ACC and HMGR are AMPK substrates for AMPK which regulate fatty acid and cholesterol synthesis. Many cancer cells exhibit an increased rate of *de novo* fatty acid synthesis. AICAR- or

rosiglitazone-induced AMPK activation has been demonstrated to reduce the expression of FAS and ACC resulting in the suppression of prostate cancer cell proliferation (Xiang et al., 2004).

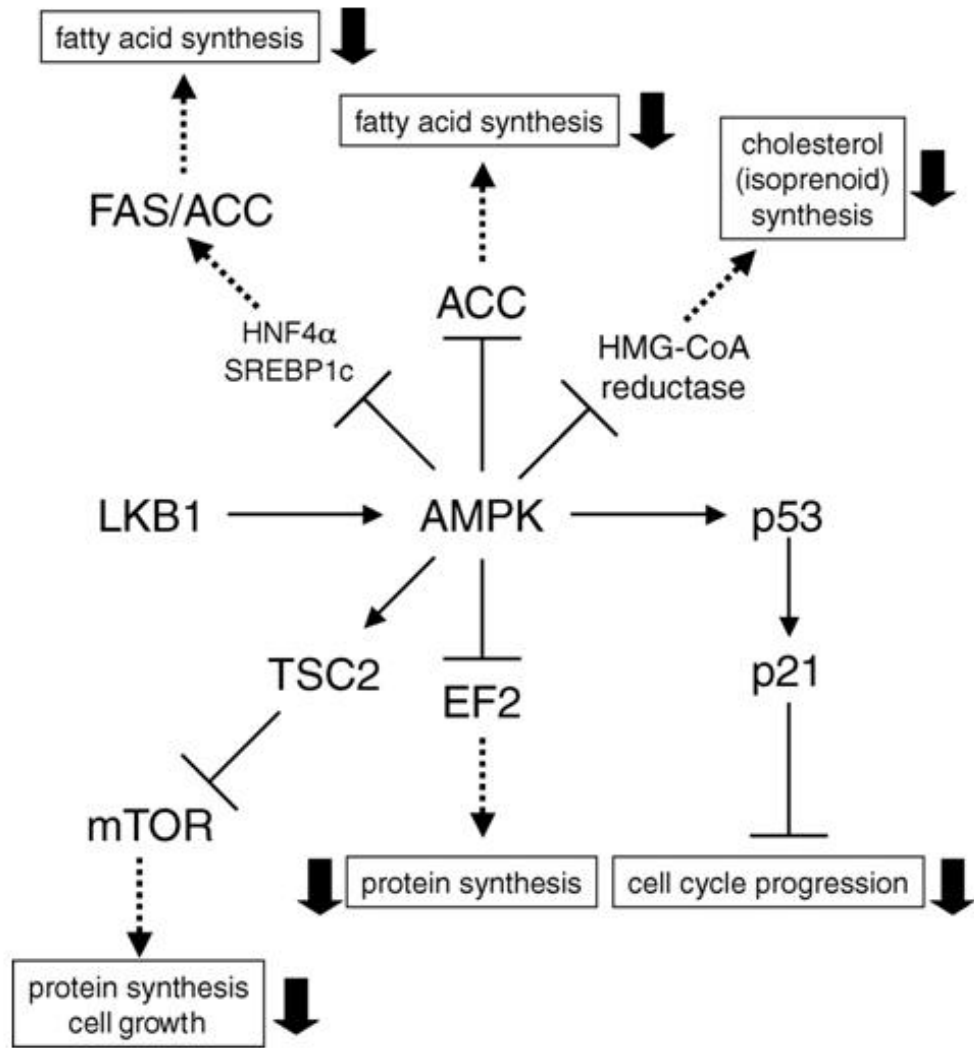
In the current study, activation of AMPK, using AICAR, was also found to result in a suppression of proliferation (Figure 5.7). This is consistent with the findings of several other studies. AICAR-induced AMPK activation causes cycle arrest in hepatoma HepG2 cells (Imamura et al., 2001), MEFs (Jones et al., 2005), human aortic smooth muscle cells and rabbit aortic strips (Igata et al., 2005). Activation of AMPK has also been shown to facilitate apoptosis of lung cancer cells, gastric cancer cells, pancreatic cancer cells, hepatic carcinoma cells, and prostate cancer cells (Rattan et al., 2005). However, although widely used as an activator of AMPK AICAR does not directly activate the kinase in cell-free assays. AICAR, a pro-drug taken up into cells by adenosine transporters (Gadalla et al., 2004), is converted to its active component (ZMP, an AMP analogue) inside the cell, which acts as a mimetic of AMP thus activating AMPK (Corton et al., 1995). ZMP, in addition to AMPK, is known to regulate other AMP-sensitive enzymes such as fructose-1,6-bisphosphatase (Vincent et al., 1996) and glycogen phosphorylase (Longnus et al., 2003). Accumulation of adenosine outside cells, with consequent binding to adenosine receptors can have effects in some *in vitro* systems (Gadalla et al., 2004). Notably AICAR has also been shown to inhibit proliferation in embryonic fibroblasts from both wild-type and LKB1 knock-out mice, suggesting AICAR itself may have a role in the inhibition of cancer cell growth (Rattan et al., 2005). It is therefore clear AICAR is not a model tool to determine the effect of AMPK activation on proliferation.

A-769662, which is described in detail in section 1.2.6.4, is a newly identified direct activator of AMPK (Cool et al., 2006; Sanders et al., 2007a; Goransson et al., 2007). Figure 5.8A demonstrates that treatment of HAEC with A-769662 resulted in a dose-dependent stimulation of phosphorylation of the major AMPK substrate ACC Ser-80. Moreover, incubation of HAEC with A-769662 resulted in a dose-dependent decrease in proliferation (Figure 5.8B). In further agreement with this observation, infection of HAEC with Ad.CA similarly resulted in a reduction in HAEC proliferation (Figure 5.6B).

So, on one hand, while AMPK has been demonstrated to underlie the VEGF-stimulated proliferative response, activation of the kinase, in this study and others, has been demonstrated to suppress proliferation. *In vivo* angiogenic endothelial cells must not only proliferate but produce molecules able to degrade the extracellular matrix, change their

adhesive properties, migrate, avoid apoptosis and differentiate into vascular tubes in order to carry blood (Munoz-Chapuli et al., 2004). As stated above, in response to VEGF, a variety of parallel signalling pathways, in addition to AMPK, are also stimulated which are required for this complex angiogenic response. Thus, the selective stimulation of AMPK may suppress proliferation, as in the absence of the overall signalling network necessary for the angiogenic response; its activation may merely represent a waste of energy. It may be the case that in the absence of CaMKK-mediated AMPK activation, such as that evoked by VEGF, the tumour suppressor LKB1 is the dominant upstream kinase which may confer an anti-angiogenic signal on AMPK.

Indeed the degree to which CaMKK/LKB1 contributes to AMPK activation may confer a pro- or anti-angiogenic signal. Mammalian cell proliferation is wholly dependent on the cell cycle machinery. Cell cycle progression is positively regulated by CDKs and negatively regulated by CDKIs and tumour suppressor genes (Motoshima et al., 2006). The various pathways activated by growth factors such as VEGF ultimately converge on common downstream regulators of the cell cycle such as CDKIs, tumour suppressors and CDKs, the balance of which regulates the progression of the cell cycle (Grana and Reddy, 1995). AMPK may be an important mechanism which contributes to this overall balance in a pro- or anti-angiogenic manner dependent on its mode of activation.



**Figure 5-9 AMPK-regulated pathways which may modulate cell proliferation**

AMPK activation may regulate cell proliferation not only by activation of p53–p21 axis and inhibition of mTOR signalling, but also by suppression of the mevalonate synthesis pathway and *de novo* fatty acid synthesis. When AMPK is activated, various pathways indicated by dashed arrows are suppressed. (Figure adapted from (Motoshima et al., 2006)).



## **6 VEGF AND ENDOTHELIAL CELL FATTY ACID OXIDATION**

## 6.1 Introduction

The storage of body fat acts as an essential energy reserve for times of starvation, but also substantially increases the risk of diabetes and heart disease (Bays et al., 2008). Thus, a suitable balance between fat utilisation and storage is imperative. ACC1, ACC2 and CPT1 are important molecules which regulate this balance. ACC, of which there are two distinct isoforms, ACC1 and ACC2, are crucial enzymes which regulate this balance of fatty acid synthesis and oxidation through the catalysis of acetyl-CoA to malonyl-CoA (Brownsey et al., 2006). The malonyl-CoA generated within the cell by ACC1 and ACC2 is in distinct pools (Wakil and Abu-Elheiga, 2008). The ACC1 isoform, present in the cytosol, is primarily responsible for fatty acid synthesis (as discussed in section 1.3.1.3), and while generally expressed in most tissues is found in high abundance in lipogenic tissues such as liver, adipose and lactating mammary gland (Iverson et al., 1990). ACC2, localised to the mitochondria, is an important regulator of fatty acid oxidation (as discussed in section 1.3.1.3), and is the predominant isoform expressed in heart, muscle and to a lesser extent liver (Abu-Elheiga et al., 2000).

The excess accumulation of triglyceride in tissues such as liver and muscle is associated with insulin resistance (Petersen and Shulman, 2006). At the level of the endothelium, exposure to excessive free fatty acids is associated with endothelial dysfunction, characterised by increased superoxide production, impaired nitric oxide activity and a reduction in endothelium-dependent vasodilation (McCarty, 2005). An excess of fatty acids in endothelial cells leads to an increase in the production of DAG with consequent activation of PKC (Lee et al., 1989; Inoguchi et al., 2000). Activation of PKC results in enhanced endothelial superoxide production via NADPH oxidase (Inoguchi et al., 2000), reduced NO synthase activity (Michell et al., 2001), impaired endothelial responsiveness to insulin (Ruderman et al., 2003) and the promotion of a pro-inflammatory phenotype through the activation of NF- $\kappa$ B (Pieper and Riaz, 1997; Sugiyama et al., 1998; Park et al., 2003).

AMPK phosphorylates ACC resulting in a reduction in the activity of both isoforms with a consequent increase in fatty acid oxidation and reduction in fatty acid synthesis (Hardie and Pan, 2002; Hardie, 2008). A number of reports have demonstrated that AMPK activation in response to AICAR, glucose deprivation and bradykinin stimulates fatty acid oxidation in endothelial cells (Dagher et al., 2001; Dagher et al., 1999; Mount et al., 2008). Activation of the AMPK-ACC system is therefore an attractive therapeutic target which

may ameliorate the adverse effects of excessive free fatty acids on the endothelium. In addition to the increased oxidation of fatty acids activation of AMPK in the endothelium likely confers some additional effects on lipid metabolism. PKC activation inhibits glycerol-3-phosphate acyltransferase, required for *de novo* synthesis of DAG. AMPK-mediated activation of eNOS may oppose the adverse impact of PKC on the enzyme (Chen et al., 1999; Morrow et al., 2003) and furthermore, it has been reported that AICAR-induced activation of AMPK prevents against the adverse effects of palmitate or hyperglycemia on endothelial superoxide production and inflammatory NF- $\kappa$ B activation (Ruderman et al., 2003).

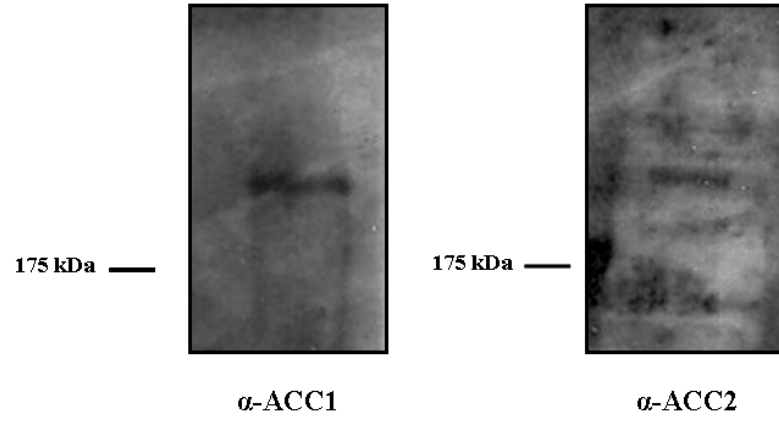
In light of the benefits associated with AMPK activation, the effect of the endothelial-specific AMPK activator, namely VEGF, on ACC phosphorylation and fatty acid oxidation was determined in HAEC. Intriguingly, increased fatty acid oxidation in response to bradykinin has recently been demonstrated in BAEC; an effect mediated by the same AMPKK (CaMKK) responsible for VEGF-stimulated AMPK activation (Mount et al., 2008).

## 6.2 Results

There are two major ACC isoforms expressed in mammals with potentially distinct functions. Using isoform-specific anti-ACC antibodies the expression of both isoforms of ACC, 1 and 2, was demonstrated in HAEC (Figure 6.1).

The Ser-80 site in ACC1 is conserved in ACC2 (Hardie and Pan, 2002). As well as the serine residue, some of the surrounding residues that are positive determinants for recognition by AMPK (Scott et al., 2002) are also conserved in ACC2 (Hardie and Pan, 2002). Because of the N-terminal extension in ACC2, the serine residue equivalent to Ser-80 on ACC1 is Ser-220 on ACC2. The effect of VEGF treatment on ACC phosphorylation was used using an antibody (section 2.1.7) that recognizes both phosphorylated species (referred to as Ser-80).

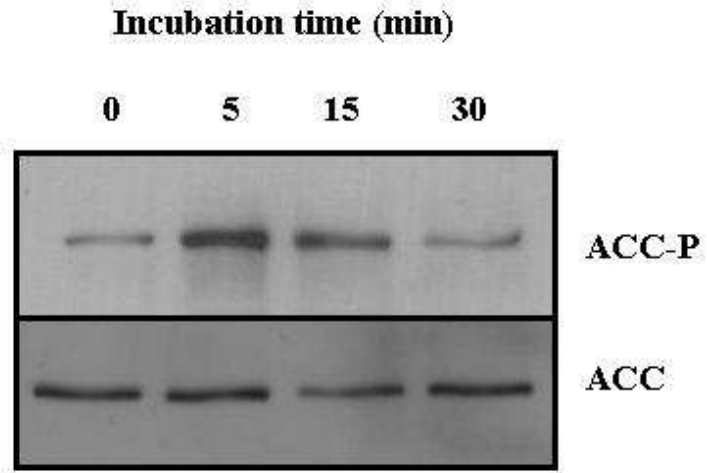
VEGF stimulated a transient phosphorylation of ACC (Figure 6.2). The effect reached a maximal ~2.2-fold stimulation of ACC at Ser-80 after 5 minutes and rapidly returned to basal levels within 30 minutes (Figure 6.2). Phosphorylation of ACC at Ser-80 was dose-dependent, such that phosphorylation of this residue was stimulated maximally (~5-fold) by 50 ng/ml VEGF (Figure 6.3).



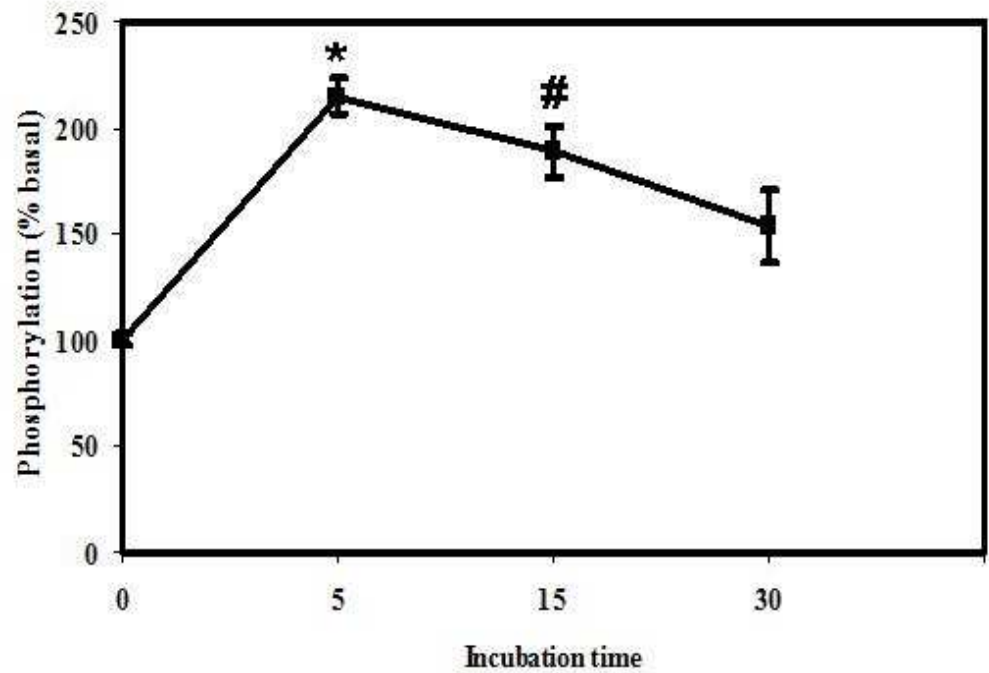
**Figure 6-1 ACC isoform expression in HAEC**

HAEC lysates (~200  $\mu$ g) were precipitated with streptavidin-immobilised agarose beads. Samples were resolved by SDS PAGE, transferred to nitrocellulose and probed with the antibodies indicated.

A

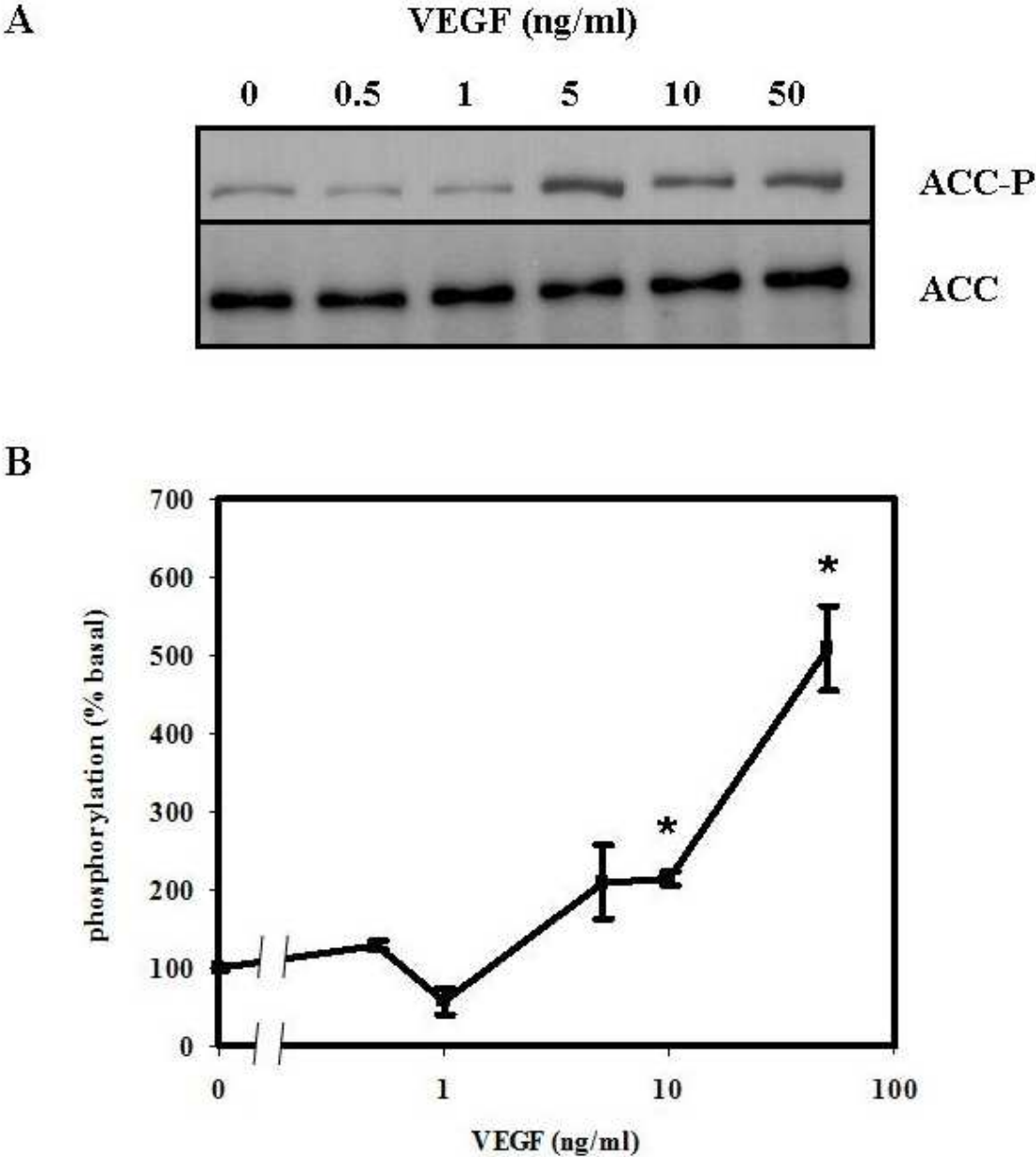


B



### Figure 6-2 Time course: VEGF-stimulated phosphorylation of ACC at Ser-80

HAEC lysates were prepared from cells incubated with 10 ng/ml VEGF for the indicated durations. Lysates were resolved by SDS PAGE, transferred to nitrocellulose and probed with the antibodies indicated. Probing of lysates with  $\alpha$ -ACC and  $\alpha$ -ACC Ser-80 resulted in a single band at ~280 kDa in both instances (A) Representative immunoblots are shown, repeated with similar results on 3 different samples of lysates. (B) Quantification of ACC Ser-80 phosphorylation from 3 independent experiments. \* $p < 0.01$  relative to value in absence of VEGF. # $p < 0.05$  relative to value in absence of VEGF.



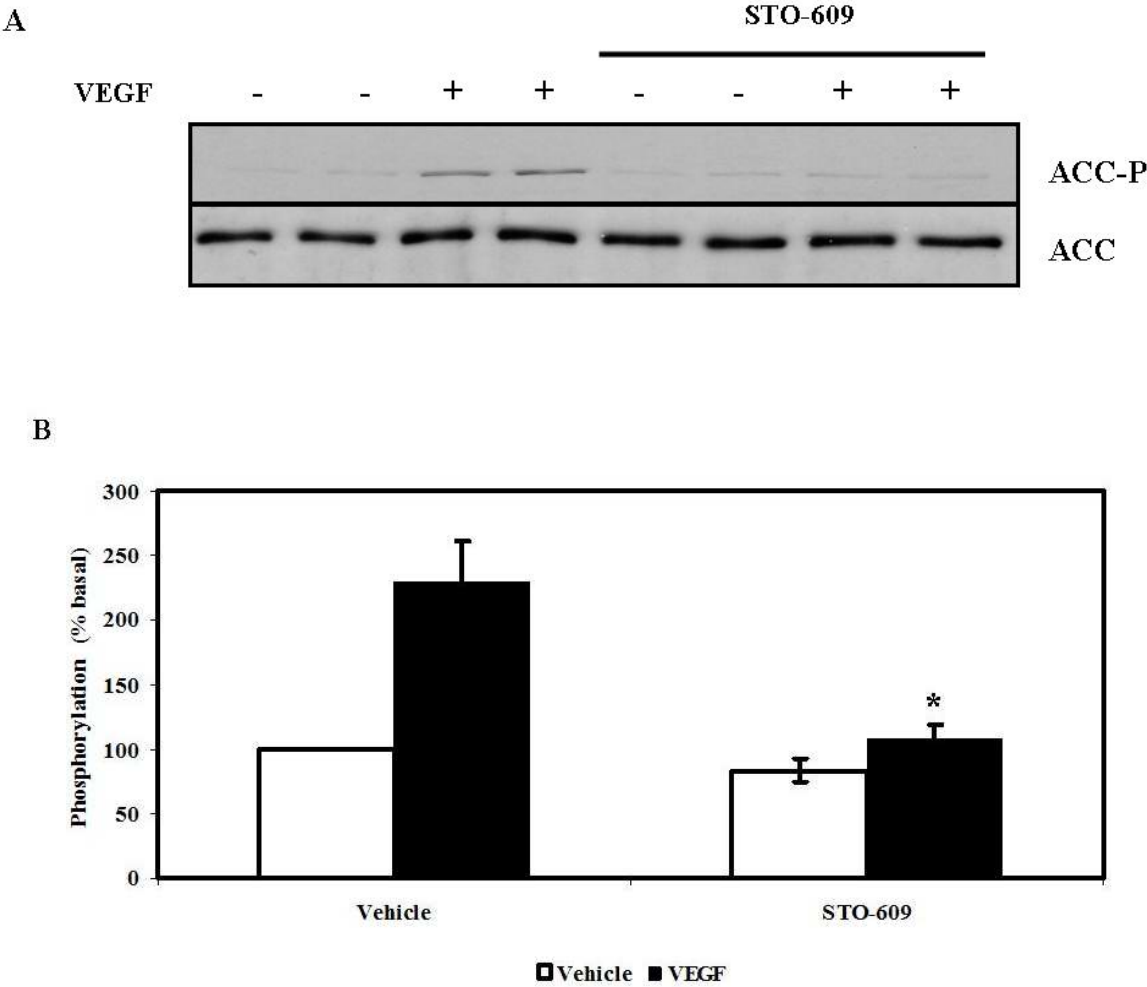
**Figure 6-3 Dose-response: VEGF-stimulated phosphorylation of ACC at Ser-80**

(A) HAEC lysates were prepared from cells incubated with the indicated concentrations of VEGF for 5 minutes and lysates prepared. Lysates were resolved by SDS PAGE, transferred to nitrocellulose and probed with the antibodies indicated. Probing of lysates with  $\alpha$ -ACC and  $\alpha$ -ACC Ser-80 resulted in a single band at ~280 kDa in both instances. (A) Representative immunoblots are shown, repeated with similar results on 3 different samples of lysates. (B) Quantification of ACC Ser-80 phosphorylation from 3 independent experiments. \*  $p < 0.01$  relative to value in absence of VEGF.

The involvement of AMPK in VEGF-stimulated ACC phosphorylation was subsequently determined. The CaMKK inhibitor STO-609 abrogated VEGF-stimulated ACC phosphorylation at Ser-80 (Figure 6.4) implicating a role for CaMKK-AMPK in this process.

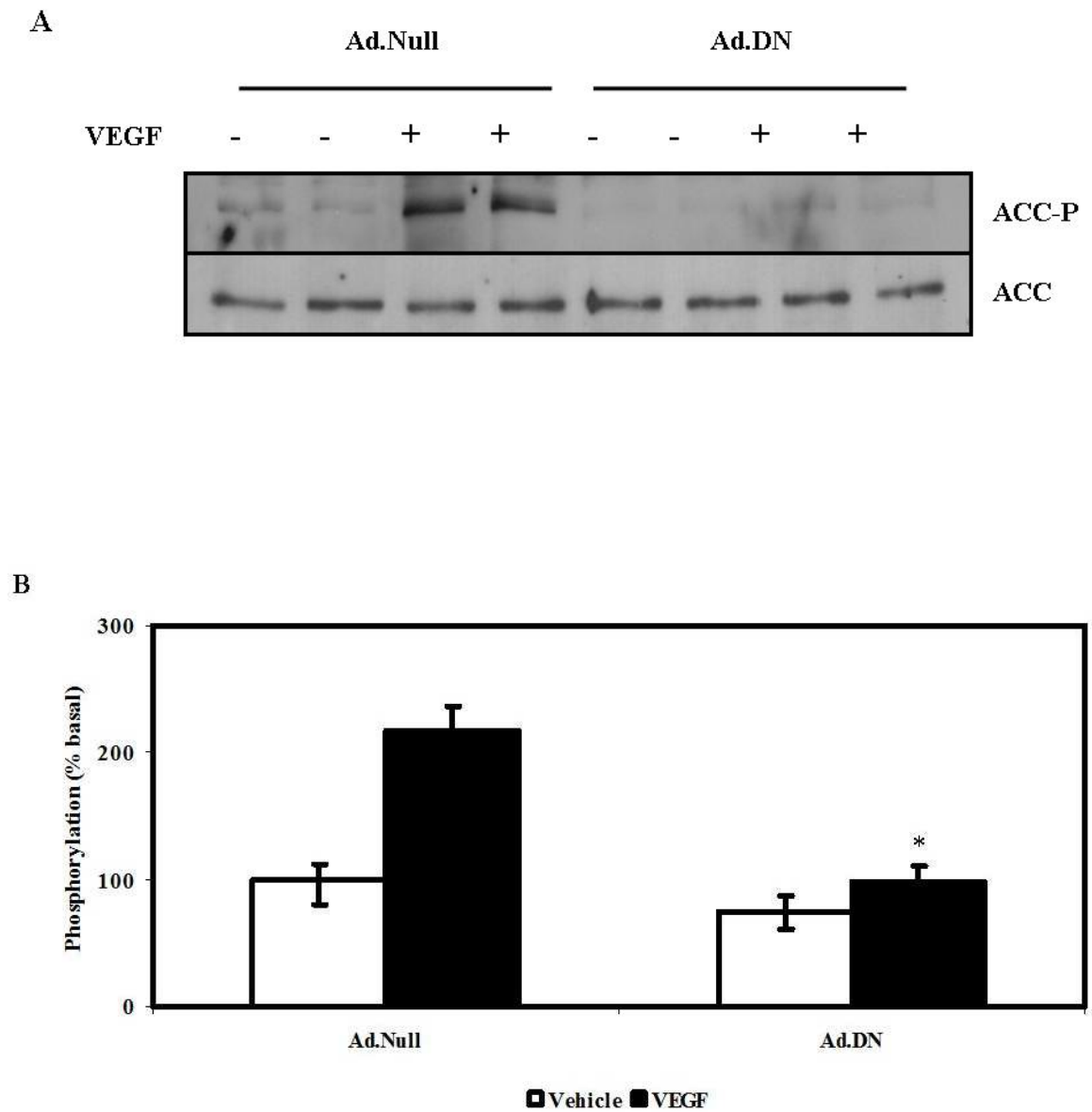
However, as discussed previously (section 5.3) STO-609 is not ideal candidate to determine the effect AMPK on downstream substrates. Consequently, HAEC were infected with Ad.DN and VEGF-stimulated ACC phosphorylation on Ser-80 phosphorylation determined. Ablation of AMPK by infection of HAEC with Ad.DN completely inhibited VEGF-stimulated ACC Ser-80 phosphorylation further indicating the involvement of AMPK in this process (Figure 6.5).





**Figure 6-4 Effect of STO-609 on VEGF-stimulated phosphorylation of ACC on Ser-80**

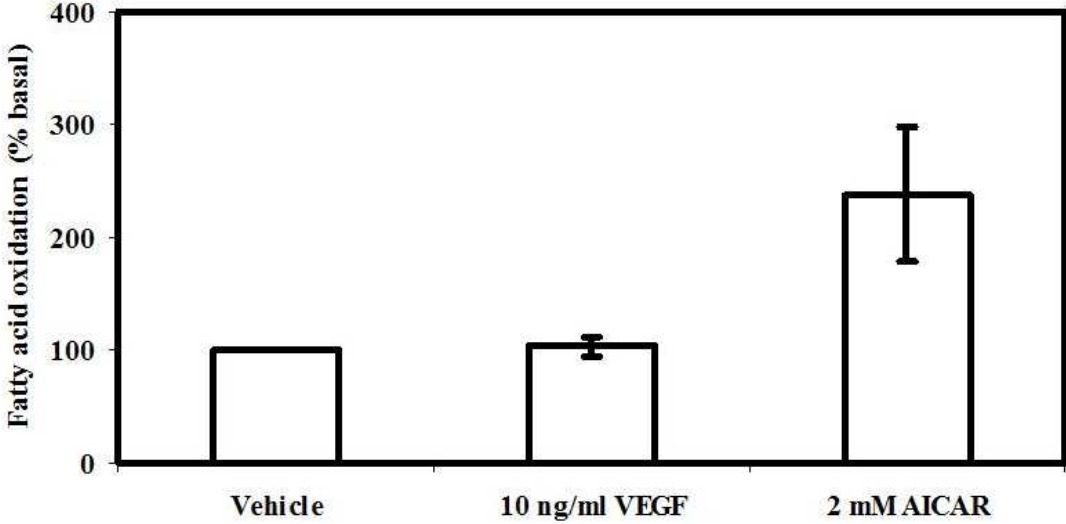
HAEC lysates were prepared from cells incubated with 10 ng/ml VEGF for 5 minutes after pre-treatment with 25  $\mu$ M STO-609 for 20 minutes. Lysates were resolved by SDS PAGE, transferred to nitrocellulose and probed with the antibodies indicated. Probing of lysates with  $\alpha$ -ACC and  $\alpha$ -ACC Ser-80 resulted in a single band at ~280 kDa in both instances. (A) Representative immunoblots are shown, repeated with similar results on 4 different samples of lysates. (B) Quantification of ACC Ser-80 phosphorylation from 4 independent experiments using ImageJ Software. \* $p < 0.05$  relative to value in absence of STO-609.



**Figure 6-5 Effect of Ad.DN on VEGF-stimulated phosphorylation of ACC on Ser-80**

HAEC were infected with Ad.DN or control-adenoviruses then lysates were subsequently prepared from cells incubated in the presence or absence of 10 ng/ml VEGF. Lysates were resolved by SDS PAGE, transferred to nitrocellulose and probed with the antibodies indicated. Probing of lysates with  $\alpha$ -ACC and  $\alpha$ -ACC-Ser-80 resulted in a single band at ~280 kDa in both instances. (A) Representative immunoblots are shown, repeated with similar results on 4 different samples of lysates. (B) Quantification of ACC-Ser-80 phosphorylation from 4 independent experiments. \* $p < 0.01$  relative to value in control virus-infected cells.

Inhibition of phosphorylation at Ser-80 of ACC by AMPK has been demonstrated to decrease fatty acid synthesis in adipose and liver whilst stimulating fatty acid oxidation in heart and skeletal muscle (Carling, 2004). In endothelial cells activation of AMPK has been reported to stimulate fatty acid oxidation (Dagher et al., 2001; Mount et al., 2008). This highlighted the possibility that VEGF stimulates the oxidation of fatty acids due to AMPK-mediated phosphorylation of ACC. Similar to the findings reported by (Dagher et al., 2001) incubation of HAEC with AICAR resulted in a ~2.3-fold increase in fatty acid oxidation in comparison to control cells ( $238 \pm 60$  %,  $n=3$ ,  $p=0.15$ ) (Figure 6.6). However, treatment of HAEC with VEGF did not alter fatty acid oxidation ( $103 \pm 10$  % versus control cells) (Figure 6.6).



**Figure 6-6 Effect of VEGF on HAEC fatty acid oxidation**

Fatty acid oxidation was measured in HAEC over 2h on the basis of <sup>3</sup>H<sub>2</sub>O production from <sup>3</sup>H-palmitate (described in detail in section 2.6.5) in HAEC treated with the indicated concentrations of VEGF or AICAR. Values are mean ± SEM % basal fatty acid oxidation for 3 independent experiments conducted in triplicate.

### 6.3 Discussion

VEGF stimulated ACC Ser-80 phosphorylation in a time- and dose dependent manner (Figures 6.2 and 6.3 respectively). Moreover, pre-treatment of HAEC with STO-609 or infection with Ad.DN abrogated VEGF-stimulated ACC Ser-80 phosphorylation (Figures 6.4 and 6.5 respectively), supporting a role for AMPK in this process. This is consistent with a number of studies which observed activation of AMPK causes inhibitory phosphorylation of ACC in endothelial cells (Zou et al., 2002; Zou et al., 2004; da Silva et al., 2006; Stahmann et al., 2006; Sun et al., 2006; Zou et al., 2003; Mount et al., 2008). In HUVEC and BAEC, activation of AMPK by AICAR and bradykinin respectively, has been shown to increase fatty acid oxidation (Dagher et al., 2001; Mount et al., 2008). In contrast however, in the present investigation, the AMPK activator VEGF was without effect on HAEC fatty acid oxidation. AICAR, similar to the effects reported by (Dagher et al., 2001), elicited a ~2.4-fold increase in fatty acid oxidation in comparison to control cells (Figure 6.6). Although not of statistical significance this observation indicates a suitable assay was used to assess fatty acid oxidation in this study.

In comparison to VEGF AICAR is a more potent (Figure 4.6) and sustained activator of AMPK. In the study by Dagher and colleagues AICAR mediated AMPK and ACC activity maximally after 30 minutes, an effect sustained for at least 2h (Dagher et al., 1999). VEGF stimulated AMPK and ACC maximally after 5 minutes; an effect which returned to basal levels within 30 minutes (Figures 3.1A and 6.2). This VEGF-induced response is consistent with the effects elicited by other CaMKK-dependent AMPK activators (Stahmann et al., 2006; Tamas et al., 2006). This raises the possibility that the activation of ACC by VEGF, and other transient activators, may not be sufficient, unlike AICAR, to measurably increase fatty acid oxidation over the time period measured. Interestingly, a recent report conducted in BAEC has demonstrated bradykinin stimulates fatty acid oxidation in a CaMKK-dependent manner (Mount et al., 2008). Bradykinin rapidly induced phosphorylation of ACC on Ser-80 within 30 seconds and reached a maximum after 5 minutes, yet unlike the response elicited by VEGF, the response to bradykinin still remained intact after 30 minutes (Mount et al., 2008). The effect of bradykinin after 30 minutes incubation was not reported in this study (Mount et al., 2008). Thus, it remains possible that VEGF elicited a transient increase in fatty acid oxidation which was not detected in the present study. Indeed any transient effect may have been masked by the basal oxidation of fatty acids occurring throughout the 2h time period measured. However it is also worth noting that in fat-fed rats a single injection of AICAR (Iglesias et al., 2002)

or prior exercise (Oakes et al., 1997), both of which transiently increase AMPK activity, cause sustained decreases in malonyl-CoA and increased insulin-stimulated glucose uptake in muscle 24h later. Thus a transient activation of AMPK may be sufficient to mediate fatty acid oxidation in the longer term. Therefore it may be appropriate to determine the effect of VEGF-treatment on malonyl-CoA levels and subsequently assess fatty acid oxidation over the relevant time period.

VEGF-stimulated fatty acid oxidation was assessed in HAEC incubated in Earle's balanced salt solution (for formulation see section 2.2.1) containing 5.5 mM glucose. Although recent reports have demonstrated that under certain conditions fatty acids may be a substantial fuel source for the endothelial cell (Dagher et al., 2001; Mount et al., 2008) it was originally believed these cells derive most of their metabolic energy from glycolysis (Spahr et al., 1989; Krutzfeldt et al., 1990). It may be the case that glycolytic breakdown of glucose is sufficient to cope with the energy requirements of HAEC under the conditions of this study. In times of necessity the oxidation of fatty acids may become more prominent.

The main actions of VEGF, as discussed in detail above, are to promote survival, induce proliferation and enhance migration and invasion of endothelial cells leading to angiogenesis. Contact inhibited endothelial cells have a reduced response to specific growth factor signals when they reach confluence (Fagotto and Gumbiner, 1996; Vinals and Pouyssegur, 1999), and intracellular contacts transfer a negative signal reducing the responsiveness of cells to proliferative signals with pro-survival signalling pathways becoming dominant. As confluent HAEC were used to assess VEGF-stimulated fatty acid oxidation it seems feasible that ATP-consuming pathways, mediating effects such as proliferation and migration, were not activated in response to VEGF, as would be the case in sub-confluent cells. Therefore it may also be worthwhile determining the effect of VEGF-treatment on fatty acid oxidation using sub-confluent (proliferative) HAEC.

Finally, whilst this study provides no evidence indicating that VEGF-stimulated AMPK activation mediates fatty acid oxidation in HAEC, isoform-specific ACC antibodies indicate the presence of both ACC1 and ACC2 in these cells (Figure 6.1). The phospho-ACC antibody used in this study detected ACC as a single band (Figures 6.2-6.5), and as such it is indistinguishable whether the band is the ACC1 or ACC2 isoform. Therefore it remains possible inhibition of ACC1 mediated by VEGF-stimulated AMPK activation may

inhibit fatty acid synthesis in these cells. Further work however is necessary to determine whether this is indeed the case.

## **7 FINAL DISCUSSION**



## 7.1 Summary of findings and future work

This project aimed to characterise the role of AMPK in human vascular endothelial cell function in response to VEGF exposure. VEGF and AMPK are important signalling molecules in the endothelium, both of which have been shown to promote NO production, a key molecule which maintains cardiovascular homeostasis and protects the endothelium against the development of atherosclerosis. At the beginning of this work VEGF had been demonstrated to stimulate NO production, proposed to be a result of phosphorylation and activation eNOS at Ser-1177 (Fulton et al., 1999), a residue also phosphorylated upon AMPK activation in cultured endothelial cells (Morrow et al., 2003). However, prior to the present study, the role of AMPK in VEGF-simulated NO production remained undetermined. As outlined in chapter 3 physiological concentrations of VEGF were found to transiently stimulate AMPK in a time- and dose-dependent manner, and furthermore AMPK was shown to partly mediate VEGF-stimulated eNOS phosphorylation at eNOS-Ser-1177 and NO production. More recent studies have further confirmed the finding that AMPK is a component of VEGF signalling in endothelial cells (Levine et al., 2007; Webler et al., 2008; Youn et al., 2008) that underlies eNOS activation (Levine et al., 2007; Youn et al., 2008). A similar mechanism has also been reported in endothelial progenitor cells (EPCs) (Li et al., 2008).

The present study then sought to determine the molecular mechanisms underlying VEGF-stimulated AMPK activation. As there is reduced vascular structure and angiogenesis in *LKB1*<sup>-/-</sup> mouse embryos, and increased concentrations of VEGF, it seemed possible there was a link between *LKB1* and VEGF/angiogenesis (Ylikorkala et al., 2001). However, a number of observations outlined in chapter 4 indicate *LKB1* is not involved in VEGF-mediated AMPK activation in HAEC. In contrast, the alternate upstream kinase for AMPK CaMKK was found to be essential for VEGF-stimulated AMPK activity. In a study by Levine and coworkers a genetic approach indicated the  $\beta$  isoform of CaMKK is responsible for VEGF-stimulated AMPK activation in bovine endothelial cells (Levine et al., 2007), again supporting the finding that CaMKK underlies VEGF-mediated AMPK activation. In addition to VEGF, an increasing number of groups have also identified that a range of other agonists including extracellular nucleotides, thrombin, bradykinin, ghrelin and NO also stimulate endothelial AMPK in a CaMKK-dependent manner (da Silva et al., 2006; Stahmann et al., 2006; Mount et al., 2008; Xu et al., 2008; Zhang et al., 2008).

CaMKK-mediated activation of AMPK is rapid and transient (Stahmann et al., 2006; Mount et al., 2008; Xu et al., 2008; da, X et al., 2000). It has been suggested that AMPK activation, mediated by CaMKK, may act as a mechanism to anticipate energy expenditure (Tamas et al., 2006). It makes sense that AMPK can anticipate energy expenditure, perhaps an inevitable outcome upon the initiation of  $\text{Ca}^{2+}$  signalling, in addition to acting retrospectively to compromised energy status (via LKB1). Together CaMKK and LKB1 may work to maintain energy balance in the short and longer term.

Chronic dysfunction of the endothelium, characterised by reduced NO bioavailability, is implicated in the pathophysiology of several cardiovascular disorders, including atherosclerosis, hypertension, diabetic vasculopathy and heart failure (Harrison et al., 2003). Therefore the role of AMPK in VEGF-stimulated NO production is of particular interest. However, VEGF only elicits a transient activation of eNOS at Ser-1177, thus one could reasonably argue that increased NO by this mechanism would need to be over a more sustained period for any beneficial effect in the context of cardiovascular disease. Interestingly, however, NO has been reported to act as an endogenous activator of AMPK in endothelial cells through a pathway involving CaMKK $\beta$  (Zhang et al., 2008). This observation, and the findings of the present study, would indicate that a positive feedback mechanism may exist whereby NO produced by VEGF-AMPK may in turn activate AMPK thus enabling an increase in NO production in the longer term. It has been further reported that NO up-regulates VEGF expression which may also contribute to this mechanism (Hood et al., 1998; Dembinska-Kiec et al., 1997).

Phosphorylation of eNOS at Ser-633 increases NO independent of increased intracellular  $\text{Ca}^{2+}$  concentration (Boo et al., 2003), and in response to several stimuli, including VEGF (Michell et al., 2002), the onset of eNOS Ser-633 phosphorylation is slower than that observed for Ser-1177 (Boo et al., 2002a). Thus, it may be the case that stimulation of eNOS Ser-633 phosphorylation is important in maintaining eNOS activation after initial activation by  $\text{Ca}^{2+}$  flux and/or Ser-1177 phosphorylation. A very recent report by Chen and coworkers has shown phosphorylation of eNOS Ser-633 in response to shear stress, atorvastatin, and adiponectin is AMPK-dependent. In light of this finding further work to characterise the role of AMPK in response to VEGF, at Ser-633, or the other known eNOS phosphorylation sites (Ser-114, Thr-495 and Ser-615), may be of interest.

Data outlined in chapter 4 suggest  $\text{Ca}^{2+}$  influx across the plasma membrane is essential for VEGF-stimulated CaMKK-AMPK activation. The TRPC family of non-selective cation

channels represents an attractive candidate which may underlie this observation. Very recent reports have shown that TRPC6 is required for VEGF-stimulated angiogenesis (Hamdollah Zadeh et al., 2008), and thrombin-induced AMPK activation (Bair et al., 2008) in endothelial cells. However, as yet, the role of these channels has not yet been directly determined in relation to VEGF-induced AMPK activation. Therefore the role of TRPC channels in relation to AMPK function represents an area of study which would advance the present investigation.

Another attractive candidate for future study is PLC and its role in VEGF-stimulated AMPK activation. PLC has been shown to underlie thrombin-induced AMPK activation (Stahmann et al., 2006), and moreover this signalling molecule represents a component of the RTK-induced activation of TRPC channels. Although attempts were made to determine the role of PLC in VEGF-mediated AMPK activity in this investigation, further work is necessary to elucidate its role.

VEGF is a critical regulator of angiogenesis, the physiological process involving the growth of new blood vessels from pre-existing ones. NO has been characterised as a critical regulator of VEGF-induced angiogenesis (Papapetropoulos et al., 1997; Yu et al., 2005; Fukumura et al., 2001). As the present study identified AMPK as a component of the VEGF-eNOS signalling cascade it seemed possible that AMPK may be involved in the VEGF-mediated angiogenic response. In addition, as  $\text{Ca}^{2+}$  signalling is a critical regulator of angiogenesis (Kohn et al., 1995; Faehling et al., 2002; Hamdollah Zadeh et al., 2008), it was interesting that the activation of AMPK was  $\text{Ca}^{2+}$  dependent. Angiogenesis is a complex process, two key features of which are endothelial cell migration and proliferation (Ferrara, 2004b). As outlined in chapter 5, ablation of AMPK prevented both VEGF-stimulated proliferation and migration, demonstrating a key role for the kinase. These observations are consistent with a number of other studies. In a Matrigel plug assay conducted in mice, endothelial cells were recruited into VEGF-impregnated plugs; an effect that was sensitive to the inclusion of siRNA directed against AMPK (Webler et al., 2008). Furthermore, in addition to VEGF; hypoxia, adiponectin and statins have also been shown to mediate angiogenesis in an AMPK-dependent manner (Ouchi et al., 2004; Shibata et al., 2004; Nagata et al., 2003; Sun et al., 2006). While it seems highly likely that AMPK-stimulated NO production underpins the importance of AMPK in the VEGF-mediated angiogenic response it is worth noting that this was not determined in the present study. Thus, further experiments to confirm this hypothesis may be worthwhile. For

example, does a NO donor rescue the effect of AMPK ablation on VEGF-stimulated proliferation and migration?

A large body of evidence indicates that VEGF-R2 is largely responsible for mediating the effects of VEGF including proliferation and migration, whereas it has often been suggested that VEGF-R1 signalling is of limited relevance in comparison. However, a number of reports suggest VEGF-R1 may also contribute to a number of important physiological processes. VEGF-R1-blocking antibodies have been demonstrated to prevent the migration, but not proliferation, of HUVEC in response to VEGF (Kanno et al., 2000) and one study has reported that VEGF-mediated NO synthesis is driven through VEGF-R1 in HUVEC, whereas specific activation of VEGF-R2 was unable to induce NO release (Bussolati et al., 2001). Furthermore, specific activation of VEGF-R1 using VEGF-B has been demonstrated to increase eNOS Ser-1177 phosphorylation, PKB Ser-473 phosphorylation and angiogenesis (Silvestre et al., 2003). VEGF-R1-mediated signalling appears to preferentially modulate the reorganization of actin via MAPK, whereas VEGF-R2 contributes to the re-organization of the cytoskeleton by phosphorylating FAK and paxillin, suggesting a different contribution of the two receptors to the chemotactic response. As initial studies in this study indicated VEGF-R1 and VEGF-R2 may potentially mediate AMPK activation in response to VEGF, it would be of interest to determine whether a kinase such as AMPK can mediate distinct functional effects depending on the activating VEGF receptor. For example does VEGF-R1 or VEGF-R2 have a particular importance in regards to the effects on proliferation and migration seen in the present investigation? Also, it may be of interest to determine whether the neuropilins, identified as VEGF co-receptors (Soker et al., 1998; Soker et al., 2002), are involved in VEGF-stimulated AMPK activation and any subsequent functional effects.

In addition to VEGF, a range of molecules serve as positive regulators of angiogenesis such as fibroblast growth factors, transforming growth factors, hepatocyte growth factor, TNF $\alpha$ , oestrogen, angiogenin, IL-8 and angiopoietins, often acting in synergistic fashion with VEGF (Goto et al., 1993; Yoshiji et al., 2005; Stannard et al., 2007; Xiao et al., 2004). In addition, cytokines, growth factors, and gonadotropins that do not stimulate angiogenesis directly can modulate angiogenesis by modulating VEGF expression (Deroanne et al., 1997; Finkenzeller et al., 1997; Ryuto et al., 1996; Pertovaara et al., 1994; Frank et al., 1995; Goad et al., 1996; Li et al., 1995; Cohen et al., 1996; Matsumoto et al., 1997). In the experiments outlined in chapter 5 endothelial cell proliferation was determined in response to VEGF which was added to a low serum-containing medium (0.2

% FCS). This was done in order to assess the role of a given concentration of VEGF on endothelial cell proliferation, and subsequently the role of AMPK in this process. However, the role of AMPK in regards to endothelial cell proliferation should also be determined in response to the physiological battery of growth factors.

While the proliferation and migration assays conducted in the present study provide valuable initial information it is also clear that they are limited by the fact that they are *in vitro* experiments, which differ from the *in vivo* setting in a number of ways. Importantly, for example, in the established vasculature of a normal adult it is estimated that only 0.01 % of cells are dividing at any given moment (Hobson and Denekamp, 1984). Clearly, the cultured endothelial cells used in a laboratory setting are different in nature. Additionally, it is also important to note that the angiogenic process, not only involves endothelial cells, but is also influenced by surrounding mural cells (Karamysheva, 2008). Short of an *in vivo* study, the rat aortic ring assay is a widely used *in vitro* study that more closely mirror the *in vivo* environment (West and Burbridge, 2009; Nicosia and Ottinetti, 1990). This assay includes the surrounding non-endothelial cells, and in addition the endothelial cells have not been pre-selected by passaging (and thus are not in a proliferative state at the time of explantation). However, it is also worth noting that angiogenesis is primarily a microvascular event, thus the aorta is not an ideal choice in this regard. This is relevant to the present study where aortic endothelial cells were used as a model. Therefore, it may be of additional benefit to determine the role of AMPK in response to VEGF in endothelial cells derived from the microvasculature. In addition, possible *in vivo* assay systems that may also be used to advance the current study include the chick chorioallantoic membrane assay, an *in vivo* Matrigel plug assay, and a group of assays that use implants of sponges containing test cells or substances.

## 7.2 Relevance of VEGF/AMPK signalling

As NO is pivotal in the regulation of vascular tone; and furthermore exerts anti-inflammatory and anti-thrombotic effects on the vascular wall, the finding that AMPK underlies its production in response to the endothelial-specific factor VEGF is an important finding. Further understanding of AMPK-signalling in the endothelium may provide some insight into possible therapeutic targets for the treatment of cardiovascular disorders. Proof of concept may be derived from the finding that the widely used treatment for type 2 diabetes, metformin, a known activator of AMPK, in contrast to other anti-diabetic therapies (e.g. insulin or sulphonylureas), diminishes the incidence of cardiovascular

disease when used as a monotherapy in obese patients with type 2 diabetes, despite the fact each of these modalities has similar effects on glycaemia control (UKPDS 1998). Metformin is proposed to indirectly activate AMPK by inhibiting complex I of the mitochondrial respiratory chain, a mechanism which may underlie some of the undesirable side effects of the drug. Thus, it seems likely that more specific activators of AMPK may act as better tolerated therapies. Intriguingly, the finding that the AMPK activator A-769662 only stimulates  $\beta$ 1-containing, and not  $\beta$ 2-containing, AMPK complexes, highlights the possibility that isoform specific AMPK activators may be obtainable enabling the tissue specific manipulation of AMPK.

AMPK has been described as the fuel gauge of the mammalian cell (Hardie and Carling, 1997), that also operates to regulate whole body energy balance (Hardie, 2008). As the delivery of nutrients and oxygen to the tissues requires an intact vasculature it makes sense that AMPK also affects vascular growth and function. AMPK acting as a component of VEGF-signalling may influence physiological processes including vasodilatation of the vasculature and mediate angiogenesis, thus regulating tissue perfusion and therefore metabolism. As VEGF signalling is largely regulated by hypoxia AMPK may be part of a mechanism that targets specific tissues which may otherwise become metabolically compromised. Clearly this action is of benefit to the metabolic status of the overall organism.

Therapeutic angiogenesis involves the promotion of new blood vessels to treat ischemic disorders. The angiogenic properties of VEGF have been exploited to stimulate *in vivo* angiogenesis as a potential treatment for ischemic disease (Baumgartner et al., 1998; Isner et al., 1996; Magovern et al., 1997; Tsurumi et al., 1997; Asahara et al., 1995). However, although promising results were initially reported, a number of placebo-controlled trials have been less than convincing (Lederman et al., 2002; Henry et al., 2003). Transplantation of stem and progenitor cells represents a new strategy for vascular regeneration (Rafii and Lyden, 2003). EPCs, first described by (Asahara et al., 1997) are one such candidate. While the role of EPCs remain incompletely understood, increasing evidence suggests they enhance re-endothelialisation in injured or diseased arteries (Shi et al., 1998; Xu et al., 2003) and improve endothelial function (Wassmann et al., 2006). In models of arterial injury EPCs have been shown to enhance the restoration of the endothelial monolayer and reduce neo-intimal hyperplasia (Werner et al., 2002; Griese et al., 2003; Kong et al., 2004). Interestingly, a VEGF-AMPK-NO signalling pathway, similar to that described in the present study, is required for EPC-endothelial cell differentiation; and statin-induced EPC

differentiation, is mediated at least in part by AMPK (Li et al., 2008). Thus, the mechanism underlying VEGF-AMPK signalling is important in regards to the generation of new vessels from EPCs and may be of relevance for the development of clinical therapies for ischemic heart disease.

VEGF-AMPK signalling may also be of importance in regards to the aetiology of diseases that are characterized by deregulated angiogenesis. Angiogenesis is required for the development of primary tumours and metastases (Folkman, 1995; Ferrara and Kerbel, 2005). Without the onset of angiogenesis, most tumours cannot grow beyond 1 to 2 mm because of diffusion limitations and thus may remain dormant (Gimbrone, Jr. et al., 1972). Therefore, the production of angiogenic factors, such as VEGF, by tumourigenic cells is essential for the development of solid tumours (Folkman, 1990). In addition to the finding of the present study that VEGF regulates AMPK activity, it has also been reported that AMPK regulates VEGF mRNA expression in response to exercise (Zwetsloot et al., 2008), and furthermore activation of AMPK stimulates VEGF expression and angiogenesis in skeletal muscle (Ouchi et al., 2005). It has been shown that prolonged hypoxia promotes the expression and functional activation of AMPK $\alpha$ 2 and VEGF production in glioma cell lines and glioblastoma multiform tumours, thus contributing to tumour survival and angiogenesis in high grade human gliomas (Neurath et al., 2006), and glucose deprivation leads to a significant increase in the mRNA level of a number of genes, including VEGF, in several cancer cells dependent on AMPK (Yun et al., 2005). In addition exposure of humans to both metals and metalloids exacerbates the risk of human diseases, particularly cancers. It has been suggested that AMPK is a critical regulatory component in metal-induced VEGF expression, implying a role for AMPK/VEGF in metal-induced carcinogenesis (Lee et al., 2006). Together, the finding that VEGF stimulates AMPK activation, and the observation that AMPK may increase VEGF expression in a number of pathophysiological settings, indicates the potential for a positive feedback mechanism between VEGF-AMPK, which would act to exacerbate the pathological angiogenesis seen in tumour development.

Efforts to inhibit VEGF-induced tumour angiogenesis include the development of humanized neutralizing anti-VEGF monoclonal antibodies (Presta et al., 1997), inhibitory soluble VEGF receptors (Ferrara et al., 1998; Kendall and Thomas, 1993; Lin et al., 1998), antisense VEGF mRNA expressing constructs (Cheng et al., 1996), VEGF-toxin conjugates (Ramakrishnan et al., 1996), antagonistic VEGF mutants (Siemeister et al., 1998) and inhibitors of VEGF receptor function (Skobe et al., 1997; Strawn et al., 1996).

While inhibition of VEGF via these strategies yields some promise it is likely that a better understanding of VEGF signalling may offer new therapeutic targets with a reduction in the undesirable side-effects. Bevacizumab, in combination with chemotherapy, has been shown to an effective treatment for patients with metastatic colorectal cancer (Hurwitz et al., 2004). However, hypertension requiring medical intervention developed in 11 % of bevacizumab-treated patients and is now recognized as a classic effect of VEGF blockers (Ferrara et al., 2007). It is tempting to speculate that this may be a result of reduced NO bioavailability. Thus in this context, the signalling molecules, such as AMPK, which underlie VEGF-stimulated NO production are of particular interest.

Retinal neovascularisation is a hallmark of proliferative diabetic retinopathy, an important risk factor for severe vision loss in patients with type 2 diabetes (Abdallah and Fawzi, 2009). VEGF, a key regulator of neovascularisation (Miller et al., 1994), is directly correlated with the severity of proliferative diabetic retinopathy (Aiello et al., 1994). Inhibition of VEGF can prevent iris neovascularisation in primates (Adamis et al., 1996), and studies in humans have indicated the potential for anti-VEGF therapy in retinal vascular disease (associated with pathological angiogenesis) (Adamis et al., 1994). The survival of endothelial cells is critical for the maintenance of microvascular integrity. It has been reported that retinal endothelial cells die prematurely and undergo apoptosis in both human and experimental diabetes (Mizutani et al., 1996). Kim and coworkers have reported that AMPK activation, in response to the hypolipidemic drug fenofibrate, prevents apoptotic cell death induced by serum deprivation in human retinal endothelial cells (Kim et al., 2007). Thus, AMPK activation may have a novel therapeutic property that can control unwanted cell death found in diabetic retinopathy. However, AMPK activation was also reported to increase VEGF mRNA (Kim et al., 2007). While VEGF may be of benefit in the early stage of diabetic retinopathy, maintaining vascular integrity, the excess production of VEGF is believed to be responsible for the proliferative and exudative stages of diabetic retinopathy (Witmer et al., 2003); and thus may be maladaptive with the progression of the disease. Therefore further characterisation of VEGF/AMPK signalling may be appropriate in relation to the development of treatments for vascular disease of the retina.

### 7.3 Summary

VEGF-stimulated NO production regulates a range of functional effects in the endothelium and is regarded as an important “protective” mechanism for the tissue which offsets the



development of atherosclerosis. Therefore, the finding of the present study that AMPK acts as an important intermediate in VEGF-stimulated NO production is of importance. The current investigation also provides evidence suggesting AMPK is necessary for the angiogenic response evoked by VEGF. As resistance to anti-angiogenic therapy is emerging (Kerbel and Folkman, 2002), a better understanding of pathways which mediate pathological angiogenesis in various circumstances is required. VEGF-AMPK signalling is of relevance in regards to tumour progression and diabetic retinopathy and furthermore may be of relevance in the context of therapeutic angiogenesis which is seen as a potential mechanism to treat ischemic disease. As the role of VEGF-AMPK signalling in relation to these pathologies remains incompletely understood the underlying molecular mechanisms warrant further investigation.

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