Endothelial Signal Transduction Pathways Mediating Thrombin and Histamine Stimulation of NO-production

Role of AMP-activated protein kinase

Brynhildur B. Thors

Thesis for the degree of Philosophiae Doctor

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Boðkerfi í æðaþeli tengd histamín og thrombín miðlaðri NO-myndun

Hlutverk AMP-örvaðs prótein kínasa

Brynhildur B. Thors

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Ágrip

Bakgrunnur: Heilbrigt æðaþel er forsenda þess að viðhalda eðlilegri starfsemi æðakerfisins. Á hinn bóginn er vanstarfsemi æðaþelsins lykilþáttur í tilurð ýmissa sjúkdóma sem tengjast hjarta- og æðakerfinu. Eiginleikar æðaþelsins, sem undir eðlilegum kringumstæðum varnar gegn segamyndun og bólgu, er æðavíkkandi og hamlar gegn fjölgun sléttra vöðvafrumna, geta við sérstakar aðstæður breyst og gert það að verkum að þekja æðanna veldur viðloðun við blóðflögur og bólgufrumur, þrengir æðar og verkar örvandi á fjölgun og skrið sléttra vöðvafrumna. Við sjúklegar aðstæður eins og æðakölkun, háþrýsting og sykursýki geta slíkar breytingar á æðaþelinu verið viðvarandi og þannig ýtt undir og verið lykilatriði í þróun þessara sjúkdóma. Síðustu ár hefur athygli beinst að þeim boðleiðum sem miðla viðbrögðum frumnanna við breytingum í umhverfinu. Eðlilegt ferli þessara boðleiða er forsenda réttra viðbragða frumunnar við áreitum og þannig geta vankantar á þessari starfsemi leitt til sjúklegs ástands. Eitt af lykilviðbrögðum æðaþelsins við breytingum í umhverfi frumnanna er framleiðsla nitur oxíðis (NO) úr argíníni sem á sér stað fyrir tilstuðlan ensímsins eNOS (endothelial NO-synthase).

Tilgangur: Aðalmarkmið verkefnisins var að auka skilning okkar á þeim líffræðilegu og lífeðlisfræðilegu eiginleikum æðaþelsins sem tengjast starfsemi eNOS. Við sóttumst sér í lagi eftir því að varpa ljósi á þær boðleiðir sem miðla örvun á æðaþelinu eftir meðhöndlun frumnanna með G-prótein tengdu boðefnunum þrombíni og histamíni með sérstakri áherslu á hlutverk AMP-örvaða prótein kínasans AMPK í að viðhalda eðlilegri framleiðslu nitur oxíðs.

Aðferðir: Notaðar voru æðaþelsfrumur úr bláæðum naflastrengja. Frumurnar voru meðhöndlaðar með Lipofectamine™ RNAiMAX til að slá sértækt út tjáningu próteinanna LKB1, AMPKα1 og/eða –α2 með siRNA. Luciferasa mæliaðferð var notuð til mælinga á ATP. Virkni eNOS var ákvörðuð með því að mæla myndun citrullíns úr argíníni eða með mælingum á innanfrumu cGMP með þar til gerðum aðferðum (enzyme immunoassay kit). Magn hvarfgjarnra súrefnissameinda (ROS) var mælt með DCF fluorescence aðferð. Western blott og ECL var notað til að sjá tilvist próteina í frumunum sem og fosfórun á próteinunum á ákveðnum amínósýrum.

Niðurstöður: Niðurstöðurnar hafa verið birtar í fjórum tímaritsgreinum. Í grein I sýndum við fram á tilvist boðleiðar í æðaþelsfrumum sem olli virkjun á eNOS eftir meðhöndlun frumnanna með G-prótein örvurunum histamíni eða

þrombíni og var óháð PI3K-Akt. Við sýndum að histamín og þrombín hindruðu fosfórun á Akt með virkjun á PKCδ og eins að þessi áverkunarefni höfðu örvandi áhrif á fosfórun eNOS á Ser1177 sem ekki var hindruð með PI3K-hindranum wortmannini. Eftir að hafa útilokað ýmsa kínasa, sem vitað var að gátu fosfórað eNOS, settum við fram þá tilgátu, byggða á frumniðurstöðum okkar, að AMPK miðlaði fosfórun á eNOS sem verður í æðaþelsfrumum eftir örvun þeirra með histamíni eða þrombíni. AMPK gegnir mikilvægu hlutverki í frumum, með því að ræsa ferla sem hækka orkustig frumunnar og slökkva jafnframt á orkukrefjandi ferlum. Á seinni árum hefur komið í ljós að AMPK hefur einnig víðtækt hlutverk í boðflutningi og stjórnun efnaskipta og fæðunáms, í oxunarvörnum ofl.

Í grein II var sýnt fram á hlutverk AMPK í þessari áður óþekktu boðleið frá G-prótein tengdum viðtökum að eNOS. AMPK var þar með skilgreindur sem mikilvægur hlekkur í að miðla örvun á eNOS eftir áverkunarefnin histamín og þrombín, óháð PI3K-Akt boðleiðinni. Bæði efnin ollu fosfórun á AMPK sem og virkjun sem var mæld með fosfórun á acetyl-CoA carboxylase (ACC) sem er þekkt og mikilvægt skotmark AMPK. eNOS fosfórun og virkjun (citrullínmyndun) af völdum histamíns og thrombíns var hindruð með H89, þekktum hindra á AMPK og PKA (sem við síðar útilokuðum með IBMX+forskólíni og RpcAMPS). Eins sýndum við fram á að áverkunarefni sem örva AMPK, þ.e. 5-aminoimidazole-4-carbozamide-1-β-4 ribofuranoside (AICAR) og carbonyl cyanide *m*-chlorophenylhydrazone (CCCP), ollu fosfórun á eNOS. Ca^{+2} klóbindirinn BAPTA kom í veg fyrir fosfórun AMPK og eNOS sem og NO-myndun sem sýnir að þessi boðleið er Ca^{+2} háð. Þar sem LKB1 var á þessum tímapunkti eini þekkti AMPKkínasinn í spendýrum lögðum við fram þá tilgátu að eNOS örvunin eftir meðhöndlun frumnanna með histamíni eða þrombíni ætti sér stað fyrir tilstilli LKB1-AMPK, hugsanlega vegna örvunar orkukræfra ferla sem virkjuðust við hækkun á innanfrumustyrk Ca^{+2}.

Í greinum III og IV er sýnt fram á að þessi boðleið virkjaðist aðeins við þær ætisaðstæður sem leyfðu snöggt fall í innanfrumustyrk ATP eftir meðhöndlun með þrombíni eða histamíni (ræktunaræti 199). Við aðstæður sem ekki „leyfðu“ slíka lækkun á ATP (ræktunaræti 1640) örvaðist AMPK í gegnum aðra boðleið (CaMKK-miðluð örvun á AMPK) og sú örvun olli ekki AMPK-miðlaðri örvun á eNOS. Þessar niðurstöður fengust með notkun á AMPK-hindrum og hindrum á tjáningu AMPK með siRNA. Við sýndum einnig með siRNA að AMPK-eNOS boðleiðin var háð virkjun AMPK gegnum LKB1. Útsláttur α2 ísóforms AMPK (en ekki α1) með siRNA olli minnkun frumnanna og því að þær misstu tengslin sín á milli sem bendir til mikilvægis α2 ísóforms

AMPK í viðhaldi samfelldrar æðaþelsþekju. SOD-hermirinn Tempol dró verulega úr þeim útlitsbreytingum frumnanna sem urðu við útslátt AMPKα2 og eins jókst við þessa meðhöndlun tjáning á próteininu GRP78, sem er mælikvarði á frymisnetsálag í frumunum. Þetta hvort tveggja bendir til mikilvægs hlutverks AMPKα2 í vörnum frumunnar gegn álagi af völdum hvarfgjarnra súrefnissameinda.

Samantekt: Í þessari ritgerð og greinunum fjórum sem hún byggist á er staðfest tilvist boðleiðar í æðaþelsfrumum sem aðrir rannsóknahópar höfðu fundið (Stahmann, Woods et al. 2006). Hún leiðir til fosfórunar á eNOS eftir þrombín eða histamín örvun og er háð CaMKK en óháð (LKB1-)AMPK boðleiðinni og virkjast við ætisaðstæður þar sem innanfrumustyrkur ATP breytist ekki við örvun. Að auki var sýnt fram á virkjun annarrar áður óþekktrar boðleiðar við þær ætisaðstæður þar sem lækkun verður á innanfrumustyrk ATP við meðferð frumnanna með histamíni eða þrombíni og leiðir til fosfórunar og virkjunar á eNOS í gegnum LKB1-AMPK. Við þessar aðstæður valda áverkunarefnin hækkun á hlutfalli AMP/ATP innan frumunnar og virkjun á þessari boðleið. Sú mikilvæga uppgötvun að umhverfisaðstæður ráði því hvaða boðleið virkjast í frumunni er áhugaverð og ekki síst lykilhlutverk orkumiðilsins ATP en lækkun á innanfrumu ATP er vel þekkt afleiðing í sjúklegum aðstæðum eins og blóðþurrð, súrefnisskorti og í sýkingum. Þannig er ljóst að þrátt fyrir að þrombín geti valdið örvun á eNOS óháð AMPK, veldur LKB1-AMPK boðleiðin mun meiri myndun á NO sem getur við ákveðnar aðstæður verið lykilatriði í starfsemi æðaþelsins og viðhaldi heilbrigðs æðakerfis.

Lykilorð:

Æðaþel, innri boðkerfi, thrombín, eNOS, AMPK.

Abstract

Background: Healthy, normally functioning endothelium is critical for maintenance of vascular homeostasis. Endothelial dysfunction, on the other hand, is a key early step in numerous vascular diseases. A vascular surface that normally is thromboresistant, antiinflammatory, vasodilatory and antiproliferative can turn into a surface that is thrombogenic, proinflammatory, vasoconstrictive and stimulatory of smooth muscle cell proliferation. In certain disease states such as atherosclerosis, hypertension and diabetes such changes may involve a chronically perturbed vascular behaviour critical for disease progression. The signaling that mediates the response to environmental changes has in recent years attracted attention. The transduction of these signals is critical for an appropriate cellular response while derailment of the signaling can be a key feature of a pathologic response or disease. One of the crucial responses of the vascular endothelium to a variety of signals is the production of nitric oxide (NO) from arginine through the activation of endothelial NO-synthase (eNOS).

Objective: The main goal of the work described in this thesis was to enhance our understanding of the biological and physiological properties of the healthy vascular endothelium with regards to the activity of eNOS. Specifically, we sought to elucidate the signaling pathway involved in mediating endothelial stimulation by the G-protein linked agonists thrombin and histamine with emphasis on the role of AMP-activated protein kinase (AMPK) in maintaining normal NO-production.

Methods: We used primary human umbilical vein endothelial cells (HUVEC). Cells were treated with Lipofectamine^{TM} RNAiMAX for selective downregulation of LKB1, AMPKα1 and/or –α2 by siRNA. Luciferase assay was used for ATP measurements. eNOS activity was determined by monitoring the conversion of arginine into citrullin or by measuring intracellular cGMP using an enzyme immunoassay kit. The level of reactive oxygen species (ROS) was measured by DCF fluorescence. Western blotting and ECL was used for the quantification of cellular proteins and for the detection and quantification of phosphorylated proteins.

Results: In paper I we demonstrated the presence of a PI3K-Akt independent pathway activating eNOS in HUVEC after treatment of cells with the G-protein agonists histamine or thrombin. We showed that thrombin and histamine

inhibited Akt phosphorylation by activation of PKCδ and that the phosphorylation of eNOS at Ser1177 by these agonists was unaffected by the PI3K-inhibitor wortmannin. After having ruled out several kinases known to phosphorylate eNOS we hypothesized a role for AMPK based on preliminary results showing AMPK phosphorylation by histamine and thrombin.

In paper II we were able to demonstrate a role for AMPK in this previously unrecognised pathway, making AMPK an important link in mediating thrombin and histamine induced activation of eNOS independent of PI3K-Akt. AMPK was phosphorylated by these agonists and activation was demonstrated by phosphorylation of acetyl-CoA carboxylase (ACC) a known target of AMPK. eNOS phosphorylation and activation (citrullin formation) after histamine or thrombin was inhibited by H89, an inhibitor of AMPK and PKA (whose involvement we ruled out using IBMX+forskolin and RpcAMPS). Also, 5-aminoimidazole-4-carbozamide-1-β-4 ribofuranoside (AICAR) and carbonyl cyanide *m*-chlorophenylhydrazone (CCCP), both known to activate AMPK, caused phosphorylation of eNOS. The Ca^{+2}-chelator BAPTA inhibited the phosphorylation of AMPK, eNOS and NO-formation, demonstrating the Ca^{+2}-dependency of this pathway. With LKB1 being the only kinase known to activate AMPK in mammals at this time, we suggested an LKB1-AMPK dependent pathway causing eNOS activation after treatment with histamine or thrombin, possibly explained by activation of energy-consuming pathways by the elevation of intracellular Ca^{+2}.

In papers III and IV we discovered that this pathway was only activated under culture conditions that allowed a sharp but brief fall in cellular ATP after thrombin or histamine stimulation. Under culture conditions that prevented or did not allow such a fall in the energy level of the endothelial cells AMPK was still activated but exclusively via a different upstream pathway (CaMKK-mediated activation of AMPK) and downstream this AMPK-activation played no role in the activation of eNOS. This we demonstrated by using both an inhibitor of AMPK and by gene silencing by siRNA. Also, we demonstrated, using siRNA, that the AMPK-eNOS pathway is dependent on the activation of AMPK through LKB1. Downregulation of the α2-isoform of AMPK but not α1 resulted in shrinkage of the cells and loss of contact between cells suggesting that the α2-isoform of AMPK is critical for endothelial integrity. The inhibiting effects of the SOD-mimetic Tempol on these morphologic changes seen in AMPKα2-downregulated cells and the increased expression of the ER stress marker GRP78 caused by this downregulation also suggested a role for AMPKα2 in oxidant defences.

Conclusions: We confirm a pathway dependent on CaMKK causing activation of eNOS under culture conditions where intracellular ATP is unchanged without the involvement of (LKB1-)AMPK, as also described by others (Stahmann, Woods et al. 2006). However, we also demonstrate a previously unknown pathway, activated by a rise in intracellular AMP/ATP ratio, causing LKB1-AMPK-dependent phosphorylation and activation of eNOS by thrombin or histamine. This pathway is activated under culture conditions where the intracellular AMP/ATP ratio is elevated by these agonists. Our fundamental discovery that environmental conditions dictate which pathway is activated is interesting but even more so the key role played by the ATP-level, a fall in the cellular energy level being a well known consequence of pathologic conditions such as ischemia, hypoxia and infection. Although thrombin can activate eNOS through an AMPK-independent pathway the activation of the LKB1-AMPK pathway greatly enhances the NO-production and may therefore be of major importance for endothelial function and endothelial health.

Keywords:

Endothelium, signal transduction, thrombin, eNOS, AMPK.

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"Develop a passion for learning.
If you do, you will never cease to grow."
- Anthony J. D´Angelo –

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Contents

List of abbreviations

2DG	2-deoxy-glucose
AA	Arachidonic acid
ACC	Acetyl-CoA carboxylase
AICAR	5-aminoimidazole-4-carbozamide-1-β-4 ribofuranoside
AMP	Adenosine 5´-monophosphate
AMPK	5´-AMP-activated protein kinase
ATP	Adenosine 5´-triphosphate
BH4	Tetrahydrobiopterin
CaM	Calmodulin
CaMKII	Ca^{+2}/calmodulin-dependent protein kinase II
CaMKK	Ca^{+2}/calmoduline kinase kinase
cAMP	Adenosine 3´5´-cyclic monophosphate
CCCP	Carbonyl cyanide m-chlorophenylhydrazone
EGF	Epidermal growth factor
EF2	Elongation factor 2
eNOS	Endothelial NO-synthase
ER stress	Endoplasmic reticular stress
FAD	Flavin adenine denucleotide
FMN	Flavin mononucleotide
GLUT	Glucose transporter
GS	Glycogen synthase
HMGR	HMG-CoA reductase
HSL	Hormone-sensitive lipase
HUVEC	Human umbilical vein endothelial cells
IBMX	1-isobutyl-3-methyl xanthine
IP	Inositol phosphates
LKB1	Liver kinase B 1
LPC	Lysophosphatidylcholine
mTOR	Mammalian target of rapamycin
NADPH	Nicotinamide adenine dinucleotide phosphate

NO	Nitric oxide
PCG1α	Peroxisome proliferator-activated receptor-γ co-activator 1α
PFK2	Phosphofructokinase 2
PFKFB	6-phosphofructo-2-kinase/fructose-2,6-biphosphatase
PI3K	Phosphoinositide 3-kinase
PK	Protein kinase
PLC	Phospholipase C
PP	Protein phosphatase
Ox-LDL	Oxidized LDL
ROCK	Rho-dependent protein kinase
ROS	Reactive oxygen species
S1P	Sphingosine 1-phosphate
SOD	Superoxide dismutase
Tak1	Transforming growth factor β-activated kinase-1
TGFβ	Transforming growht factor β
TIFIA	Transcription initiation factor IA
TPA	12-O-tetradecanoylphorbol-13-acetate
TSC2	Tuberous sclerosis complex
VEGF	Vascular endothelial growth factor
Wm	Wortmannin

List of inhibitors and activators

Below is a list of inhibitors and activators as used in Papers I-IV. It must be kept in mind that many of these substances are highly unspecific and cause more complex effects than stated in this list.

2-deoxy-glucose	Lowers intracellular ATP by inhibiting glycolysis
8-bromo-cAMP	Activates PKA by being an analogue of cAMP
A23187	Calcium ionophore
AICAR	Mimicks the activating effects of AMP on AMPK
Apyrase	Apyrase is used to hydrolyze nucleoside trisphosphates and diphosphates
BAPTA	Calcium chelator
CCCP	Activates AMPK by lowering the ATP/AMP ratio
Compound C	Inhibitor of AMPK
PD98059	Inhibitor of ERK1/2
GF109203X	Inhibitor of PKC
Gö6976	PKCα, β and γ inhibitor
H89	Inhibitor of AMPK and PKA
IMBX+foskolin	Activates PKA by increasing intracellular cAMP
KN-62/KN-93	Inhibitors of CaMKII
Rottlerin	PKCδ inhibitor
Rp-cAMPS:	Inhibitor of PKA
SB203580	Inhibitor of p38
STO-609	Inhibitor of CaMKK
Tempol	A mimetic of SOD
TPA	Short term treatment stimulates PKC, whereas long term treatment causes inhibition
Tween 80	Non-ionic detergent
U73122	Inhibitor of PLC
Wm	Inhibitor of PI3K
Y27632	Inhibitor of ROCK

List of figures

List of original papers

This thesis is based on the following original publications, which are referred to in the text by their Roman numerals:

I. **Inhibition of Akt phosphorylation by thrombin, histamine and lysophosphatidylcholine in endothelial cells. Differential role of protein kinase C.** Thors B, Halldórsson H, Clarke GD, Thorgeirsson G. Atherosclerosis. 2003 Jun;168(2):245-53.

II. **Thrombin and histamine stimulate endothelial nitric-oxide synthase phosphorylation at Ser1177 via an AMPK mediated pathway independent of PI3K-Akt.** Thors B, Halldórsson H, Thorgeirsson G. FEBS Lett. 2004 Aug 27;573(1-3):175-80.

III. **Mechanism of thrombin mediated eNOS phosphorylation in endothelial cells is dependent on ATP levels after stimulation.** Thors B, Halldórsson H, Jónsdóttir G, Thorgeirsson G. Biochim Biophys Acta. 2008 Oct;1783(10):1893-902.

IV. **eNOS activation mediated by AMPK after stimulation of endothelial cells with histamine or thrombin is dependent on LKB1.** Thors B, Halldórsson H, Thorgeirsson G. Biochim Biophys Acta. 2011 Feb;1813(2):322-31.

Declaration of contribution

Brynhildur Thors contributed to the planning of the experiments with Guðmundur Þorgeirsson and Haraldur Halldórsson. Culturing of the cells, work with siRNA and Western blotting/ECL was in the hands of Brynhildur Thors. Haraldur Halldórsson did the measurements of ATP. Other laboratory work was in the hands of Brynhildur Thors and Haraldur Halldórsson. Brynhildur Thors, Guðmundur Þorgeirsson and Haraldur Halldórsson all contributed to the interpretation of the results and writing of the papers.

1 Introduction

The capability of cells to sense their environment and react appropriately to various signals is crucial for development and survival of every multicellular organism. Exploring individual components of cell signaling pathways is the basis for understanding the network that coordinates the development of normal tissues and organs, comprehension of which is vital for detecting and understanding errors in cell-to-cell signaling that lead to abnormal development and diseases. Cellular signaling involves extracellular signaling molecules between cells, receptors on the cell´s membrane to receive the signal and intracellular signaling cascades to interpret the signal and give the appropriate "response". We use the term "signaling pathway" to describe a single cascade within the cell. However, if we add all the crosstalk between proteins of different cascades, the term "signaling network" is more appropriate (Dumont, Pecasse et al. 2001).

When Rudolph Virchow, in 1858, delivered his lecture series at the Pathologic Institute in Berlin that virtually established cellular pathology as a biological principle, he devoted a brief section to the vascular endothelium: „A capillary vessel is a simple tube, in which we have, with the aid of our present appliances, hitherto only been able to discover a simple membrane, beset at intervals with flattened nuclei.." He also referred to the endothelial layer as „a membrane as simple as any that is ever met with in the body" (Virchow 1989).

For almost a century this view did not change, but with the advent of new „appliances" new discoveries have followed. The last few decades have seen investigations of the vascular endothelium that have unraveled considerable structural complexity and variability between the endothelia of various segments of the circulatory system and between the vascular beds of various organs (Aird 2007a; Aird 2007b).

More importantly, we can no longer view the endothelium as a static physical barrier that simply separates blood from tissue. Healthy endothelium is vital for correct maintenance of vascular homeostasis. By secretion or surface expression of surface molecules the endothelium ensures appropriately regulated blood flow under normal conditions, counteracting intravascular activation of platelets and coagulation. Endothelial cells play an important role in controlling vascular tone, in blood coagulation and are important mediators of inflammation. In response to pathophysiolocical mediators endothelial cells

support vessel growth or repair and guide the resolution of inflammatory or infectious processes. In most instances, the change in phenotype (from thromborestant, antiinflammatory, vasodilatory and antiproliferative, towards thrombogenic, proinflammatory, vasoconstrictive and stimulatory of smooth muscle cell proliferation) is transient and reactive and may contribute to successful restoration of vascular homeostasis. However, certain disease states such as atherosclerosis, hypertension and diabetes may involve a chronically perturbed vascular behaviour critical for disease progression.

It can therefore be concluded that precise control of endothelial function is critical for the maintainance of vascular homeostasis. Efforts to understand cell biological and physiological properties of the vascular endothelium as well as strategies to alleviate endothelial dysfunction provide a promising pathway for deeper understanding and new treatment of some of the most common and devastating diseases affecting mankind.

1.1 Intracellular signaling

1.1.1 Phosphorylation/dephosphorylation

Various intracellular activities control the complex behavior of cells. There is highly restricted transport of particles in and out of the cell, accurate control of gene transcription, regulation of protein synthesis and posttranscriptional modification of intracellular proteins to control their subcellular location and activity. A very important modification to control the activity of proteins and the main focus of this thesis, involves the reversible addition of a phosphate group to certain amino acid residues of the protein or phosphorylation/ dephosphorylation. This event can affect the protein in two important ways (Alberts 2008). First, due to the negative charges of each phosphate group, the addition of the group to a protein can cause a major conformational change by attracting clusters of positively charged amino acid side chains. This could affect the binding of ligands to the dramatically changed protein and hence alter the protein´s activity. This would then be reversed by removal of the phosphate group. Second, the attached phosphate group can cause a binding of the protein to certain domains on other proteins, recognizing the phosphorylated amino acid on the modified protein. Thus, addition or removal of phosphate groups are important regulatory mechanisms of the function of protein complexes (Alberts 2008).

1.1.2 Kinases/phosphatases

Protein phosphorylation involves the enzyme-catalyzed transfer of the terminal phosphate group of an ATP molecule to the hydroxyl group on a

serine, threonine, or a tyrosine side chain of the protein, catalyzed by protein kinases. A protein phosphatase catalyzes the reverse reaction of phosphate removal, or dephosphorylation (Alberts 2008). The state of phosphorylation of a protein and thus its activity, depend on the relative activity of the protein kinases and phosphatases that modify it. There are hundreds of different kinases and phosphatases in a eukaryotic cell, affecting different proteins and causing protein kinase cascades upon activation. The cell strictly regulates the activity of its kinases and phosphatases, that are such important players in the complex behaviour of the cell/in cellular signaling. The aim of this study was to explore the role of the kinase AMP-activated protein kinase (AMPK) in the phosphorylation of endothelial nitric-oxide synthase (eNOS) on the amino acid serine 1177, which has been shown to be a strong indicator of the enzyme´s activity and hence NO-production both in vitro and most recently in vivo (Fleming and Busse 2003; Zhang, McMillin et al. 2009; Cacicedo, Gauthier et al. 2011).

1.1.3 G-protein coupled receptors (GPCR)

Signal molecules that are not small and hydrophobic, need specific receptor proteins on the surface of the target cell. These receptors convert an extracellular signal into intracellular signals by binding to the ligand or signaling molecule. This binding, in turn, changes the behavior of the target cell (Alberts 2008).

G-protein coupled receptors are the largest family of cell-surface receptors, binding ligands of various structure and function. GPCR are all similar in structure, spanning the lipid bilayer seven times and all use G-proteins attached to the cytoplasmic face of the plasma membrane to deliver the signal into the cell interior. Two principal signaling pathways are activated by GPCR, the pathway of cAMP production and the inositol phospholipid signaling pathway.

1.2 The endothelium

The vascular endothelium, the monolayer that forms the inner lining of blood vessels, plays a crucial role in many physiological processes. Because of its wide distribution throughout the body it communicates with each and every tissue in the human body and is involved in almost every disease (Aird 2007b). By virtue of their unique location, endothelial cells are constantly exposed to a variety of stimulatory interactions, physical, humoral and cellular. The signal transduction mechanisms involved in linking exposure to endothelial response have in recent years attracted considerable attention.

The transduction of these signals is critical for an appropriate cellular response while derailment of the signaling can be a key feature of a pathologic response or disease (Yang and Ming 2006). One of the crucial responses of the vascular endothelium to a variety of signals is the production of nitric oxide (NO) from arginine through the activation of endothelial NO-synthase (eNOS).

In 1980, Furchgott and Zawadski observed that endothelial cells produce a factor that causes relaxation of the surrounding vascular smooth muscle cells (Furchgott and Zawadzki 1980). Palmer *et al.* later demonstrated that it was the freely diffusible gas nitric oxide that accounted for the biological activity of the endothelium-derived relaxing factor (Palmer, Ferrige *et al.* 1987). Besides causing vasodilation, NO generated by eNOS inhibits platelet aggregation, leukocyte adherence and vascular smooth muscle cell proliferation. These effects exert a profound influence on blood flow, vascular remodeling and angiogenesis. Diminished endothelial NO production or availability is associated with several cardiovascular disorders, including atherosclerosis (Yang and Ming 2006). Detailed information regarding the signal transduction that regulates eNOS and NO-production is therefore crucial for the understanding of normal vascular endothelial function as well as in endothelial dysfunction, a condition in which reduced NO-bioavailability usually plays an important role.

1.3 Endothelial dysfunction

Endothelial cells play an important role in controlling vascular tone and homeostasis. They are important mediators of inflammation and by secretion or expression of surface molecules the endothelium ensures appropriately regulated blood flow under normal conditions, counteracting intravascular activation of platelets and coagulation. Under pathological conditions a critical change in phenotype can occur, from thromboresistant, antiinflammatory, vasodilatory and antiproliferative, towards thrombogenic, proinflammatory, vasoconstrictive and stimulatory of smooth muscle cell proliferation.

Many of the effects of the endothelium are mediated by NO. Reduction in endothelial release of NO is a major link in the chain of events that results in endothelial dysfunction, the first step in atherogenesis and other vascular diseases (Vanhoutte, Shimokawa et al. 2009). Reduction in the release of NO is caused by aging, certain lifestyle factors (e.g. lack of exercise, Western diet, pollution, smoking) or diseases (diabetes, hypertension) (Vanhoutte, Shimokawa et al. 2009). Attenuated formation of NO can be linked to

inhibition of the enzyme eNOS, uncoupling of the enzyme or less NO bioavailability due to reaction with other free radicals. Like the other NOS isoforms, eNOS can produce superoxide (O_2^-) and hydrogen peroxide when concentrations of the substrate L-arginine or the essential co-factor tetrahydrobiopterin (BH_4) are below optimal levels. Uncoupling of NOS results in the generation of reactive oxygen species (ROS) instead of NO, therefore reduction in NO bioavailability resulting in endothelial dysfunction. Also, pathological imbalance of ROS levels causing oxidative stress, a common feature of many pathological conditions, leads to endothelial dysfunction by inactivation of NO and eNOS uncoupling (Kolluru, Siamwala et al. 2010).

Endothelial dysfunction refers not only to abnormal endothelial vasodilator function due to reduction of NO. The endothelium has various other functions, as previously mentioned. Endothelial cells play an important role in blood coagulation and are important mediators of inflammation, via NO. With the endothelium being the key gatekeeper controlling the exchange of molecules between the blood and the vessel wall (Aird 2007c) disruption of the integrity of the endothelial barrier, due to shortage of NO, causes increased permeability and inflammation, eventually leading to tissue edema and organ dysfunction (Aird 2007d).

1.4 eNOS

1.4.1 The enzyme

The vascular endothelium regulates vascular tone by releasing numerous vasodilators and vasoconstrictors. The most important vasodilator is nitric oxide (NO) produced from arginine by endothelial nitric oxide synthase (eNOS), an enzyme regulated by a complex battery of regulatory mechanisms. These include intracellular Ca^{+2}, subcellular localization, protein-protein interactions and post-translational modifications of the enzyme. Dysregulation of these processes cause diminished eNOS activity and reduced production of NO (Yang and Ming 2006).

eNOS (or NOS3) is a member of the NOS family, expressed most abundantly in vascular endothelium but also in diverse tissues including neurons, epithelial cells and cardiac myocytes (Dudzinski and Michel 2007; Hill, Dranka et al. 2010). The enzyme functions as a dimer, with each monomere composed of an oxygenase domain (N-terminal), a reductase domain (C-terminal) and an interposed Ca^{+2}/calmodulin (CaM) binding domain. The C-terminal reductase domain of one monomere of eNOS binds

NADPH from which it transfers electrons through FAD and flavin mononucleotide (FMN) to the N-terminal oxygenase domain of the other monomere (Fleming and Busse 2003). The oxygenase domain contains a heme as well as binding sites for arginine, BH_4 and CaM. The enzyme uses molecular oxygen and the electrons from NADPH to oxidize its substrate, L-arginine in two cycles. First L-arginine is oxidized into OH-L-arginine which subsequently is oxidized into NO and L-citrulline (Fleming and Busse 2003; Sessa 2004; Balligand, Feron et al. 2009; Fleming 2009). NO produced by eNOS is an important factor in the regulation of vascular function. However, dysfunctional eNOS (caused by uncoupling of the enzyme) generates superoxide instead of NO and plays an important role in the pathology of various diseases such as diabetes, hypertension and atherosclerosis (Forstermann and Munzel 2006) as is discussed elsewhere.

The other known isoforms of the NOS family with similar structure as eNOS are neuronal NOS (nNOS or NOS1) and inducible NOS (iNOS or NOS2 expressed in multiple cell types) (Balligand, Feron et al. 2009; Hill, Dranka et al. 2010). The nomenclature of the NOS enzymes is historical and the tissue expression of the enzymes is much wider than implied by their names. eNOS is expressed in diverse tissues besides the vascular endothelium, as previously described, and nNOS is also present in various tissues including neurons, skeletal muscle, vascular smooth muscle, cardiac myocytes and epithelial cells (Hill, Dranka et al. 2010; Lima, Forrester et al. 2010). While the Ca^{+2}-sensitive eNOS and nNOS are “constitutively” expressed and strictly controlled with both transcriptional and posttranscriptional regulation, iNOS is mostly regulated transcriptionally (mainly with inflammatory stimuli) and is not sensitive to Ca^{+2} (Balligand, Feron et al. 2009).

1.4.2 Function of NO

NO is a highly reactive free radical, having a lifetime of a few seconds. Endothelial NOS generates the least amount of NO compared to the other NO-synthases (Hill, Dranka et al. 2010). NO is now known as a fundamental signaling molecule in the cardiovascular system, inhibiting platelet aggregation, leukocyte adherence and smooth muscle cell proliferation besides causing vasodilation (Vanhoutte, Shimokawa et al. 2009). It is uncharged and highly soluble in a hydrophobic environment, allowing free diffusion across biological membranes. Thus, NO has the potential to signal many cell diameters distant from its site of generation (Hill, Dranka et al. 2010). Therefore, both concentration and location are key determinants of its ability to activate

different signaling pathways in different cells. The main target of eNOS-derived NO and the first to be described is the soluble guanylyl cyclase (sGC) (Murad 1986). Binding of NO to the heme group of sGC causes formation of cGMP from GTP, activating PKG which in turn causes relaxation of the underlying vascular smooth muscle cell by inactivating myosin light-chain kinase. Under conditions where the level of reactive oxygen species (ROS) is elevated, NO reacts rapidly and forms oxidation products, the best studied example being the reaction of NO with superoxide (O_2^-) to form peroxynitrite (Forstermann 2010). Peroxynitrite has been proposed to be a major mediator of the pathological effects associated with NO (Beckman 2009). Unlike O_2^-, NO is highly diffusible. The location of the two radicals is an important factor in controlling peroxynitrite formation (Hill, Dranka et al. 2010).

Besides the well studied effects of NO on smooth muscle cell relaxation, NO has a wide range of cGMP-independent cellular effects in the cardio-vascular system. This is primarily due to NO-mediated modification of protein cysteine residues or S-nitrosylation, resulting in the generation of S-nitrosothiol or SNO. Several proteins are affected by S-nitrosylation, including sGC and eNOS, which are inhibited by S-nitrosylation, as well as eNOS-regulating proteins like heat shock protein 90 and Akt/PKB (Lima, Forrester et al. 2010). The amount of protein S-nitrosylation is not only determined by the activities of the NOS enzymes and rates of NO-synthesis but also a highly regulated equilibrium between S-nitrosylation and denitrosylation pathways including various proteins such as thioredoxin-1/-2 (Benhar, Forrester et al. 2009).

1.4.3 The regulation of eNOS

eNOS is constitutively expressed. Besides a highly complex transcriptional regulation of the eNOS gene expression and a regulation at the level of mRNA stability (Fleming and Busse 2003), the enzyme itself is regulated by a complex battery of regulatory mechanisms (**Figure 1**). Binding of CaM to eNOS is required for its activity and until recently, the model of eNOS activation was relatively simple, mostly based on Ca^{+2}/CaM interaction with the enzyme. Thorough studies for the past 10 years have given us a far more complex picture of the regulation of eNOS, where its activity is influenced by the subcellular location of the enzyme and the formation of complexes with regulatory proteins, by Ca^{+2} levels and phosphorylation at several sites by a variety of protein kinases (Sessa 2004). This enables endothelial cells to regulate precisely the time and place of NO production in response to a wide variety of humoral, metabolic, pharmacological or physical stimuli (Mount, Kemp et al. 2007).

1.4.3.1 Membrane targeting

The eNOS monomers are irreversibly, co-translationally myristoylated at the glycine residue in position 2 and reversibly, post-translationally palmitoylated at the cysteine residues at positions 15 and 26 (Shaul 2002). These three acyl anchors target the eNOS dimer specifically to the cytoplasmic aspect of the Golgi complex and to caveolae, small invaginations of the plasmalemma. They are invaginated membrane micro-domains rich in cholesterol and sphingolipids, also locating diverse receptors and signaling proteins, i.e. G-protein coupled receptors and G-proteins, growth factor receptors and calcium regulatory proteins (Dudzinski and Michel 2007).

Although eNOS deficient in myristoyl can produce NO from arginine, Sessa and coworkers demonstrated that the Golgi compartmentalization of eNOS based on this myristoylation was necessary for the enzyme to respond to intracellular signals and produce NO (Sessa, Garcia-Cardena et al. 1995). The location of eNOS to caveolae therefore affects the function of the enzyme by facilitating protein-protein and protein-lipid interactions necessary for cellular signaling (Shaul 2002) and conditions preventing eNOS localization in caveolae decrease the activity of the enzyme (Gonzalez, Kou et al. 2002).

As discussed later, eNOS is associated with caveolin-1 in resting cells, a protein present in plasma membrane and the Golgi complex. Upon activation, followed by increased intracellular Ca^{+2}, eNOS disassociates from membranes and becomes a diffused cytosolic enzyme, no longer attached to caveolin-1. This is an obligatory step to the overall activation process of eNOS and involves various protein-protein interactions (Rafikov, Fonseca et al. 2011).

The localization of eNOS within caveolae and interaction with caveolin-1 is dependent on both myristoylation and palmitoylation of the enzyme, at least *in vivo* (Sessa, Barber et al. 1993). The role of palmitoylation in eNOS translocation to the cytosol and hence activation is, however, controversial (Rafikov, Fonseca et al. 2011).

1.4.3.2 Nitrosylation

Post-translational modification of eNOS by S-nitrosylation at Cys94 or Cys99 inhibits the enzyme whereas de-nitrosylation increases its activity. The mechanism of inhibition is not known, although it has been suggested that S-nitrosylation might cause a dissociation of the eNOS homodimer to form inactive monomers (Ravi, Brennan et al. 2004), might modify substrate or cofactor binding or impede electron transfer within the enzyme (Erwin, Lin et al. 2005). Since eNOS appears to be the source of the NO required for its

own S-nitrosylation, this might be an example of a negative feedback regulation of the enzyme (Erwin, Lin et al. 2005).

1.4.3.3 BH_4

All NOS isoforms depend on BH_4 as a cofactor for catalytic activity. When levels of BH_4 are below optimal, eNOS is uncoupled leading to formation of superoxide anions and hydrogen peroxide (H_2O_2) instead of NO (Rafikov, Fonseca et al. 2011). Degradation of BH_4 in arteries can be caused by oxidative stress, explaining in part the role of oxidative stress in endothelial dysfunction.

1.4.3.4 Phosphorylation

Phosphorylation/dephosphorylation of eNOS is a major regulatory mechanism catalyzed by various kinases affecting the activity of the enzyme. These kinases activate or inactivate the enzyme, depending on the site phosphorylated, and dephosphorylation of the enzyme by protein phosphatases can, likewise, cause activation or inhibition. Subcellular localization of eNOS is crucial for agonist-induced activation of the enzyme. Key regulatory phosphorylation sites on eNOS (in the human sequence) are at Ser1177, Ser633, Ser615, Ser114, Tyr567, Tyr81 and Thr495.

1.4.3.5 Ser1177

Being a critical requirement for eNOS activation, serine 1177 (1177 for human/1179 for bovine eNOS) is the most thoroughly studied phosphorylation site of eNOS and most if not all factors activating the enzyme cause phosphorylation of this site (Mount, Kemp et al. 2007). Ser1177 is phosphorylated by various kinases depending on the extracellular stimuli, including Akt, PKA, AMPK, PKG, CaMKII (Sessa 2004). It has been suggested that phosphorylation at this site increases eNOS catalytic activity two to threefold above basal level by increasing electron flux at the reductase domain and by reducing calmodulin dissociation from activated eNOS when calcium levels are low (McCabe, Fulton et al. 2000). However, Dimmeler and coworkers, demonstrated that eNOS was activated by Akt in response to shear stress without changes in intracellular calcium (Dimmeler, Fleming et al. 1999).

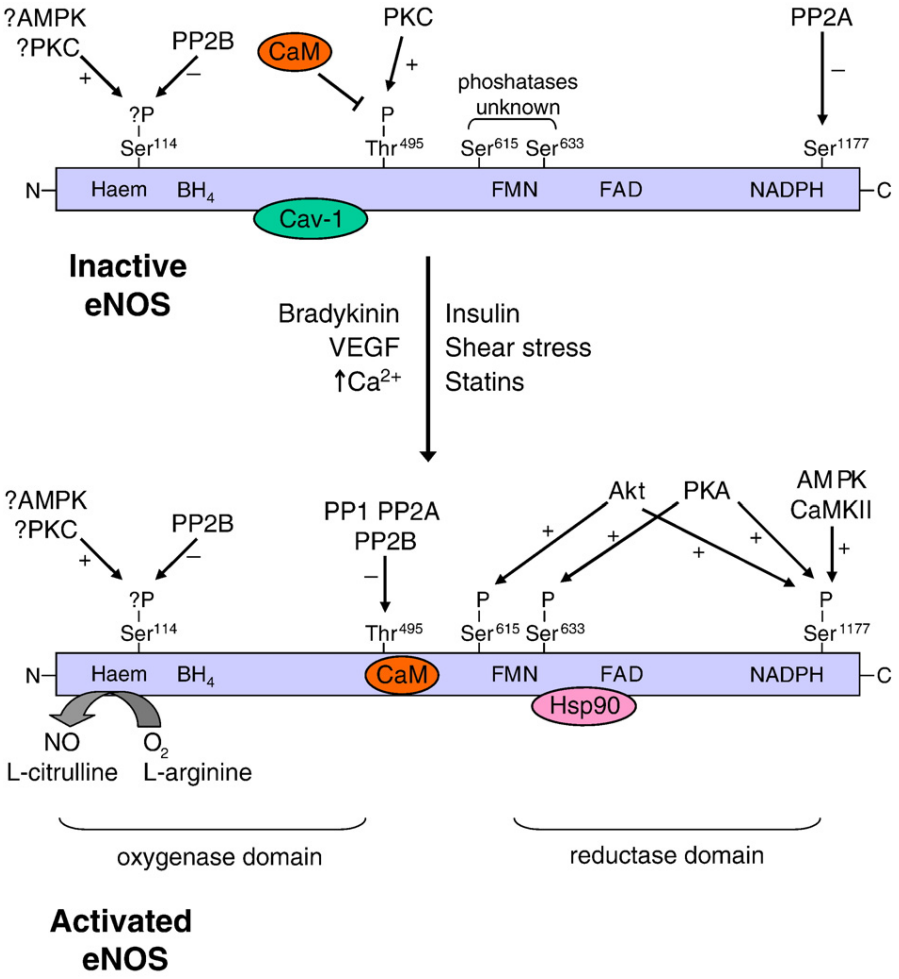

Figure 1 The complex regulation of eNOS. + indicates kinase-mediated phosphorylation, - indicates phosphatase-mediated dephosphorylation. BH4, tetrahydrobiopterin; Ca+2, calcium; CaM, calmodulin; Cav-1, caveolin-1; FAD, flavin adenine dinucleotide; FMN, flavin mononucleotide, NADPH, nicotinamide adenine dinucleotide phosphate; PK, protein kinase; PP, protein phosphatase. (Figure from (Mount, Kemp et al. 2007)).

1.4.3.5.1 Other phosphorylation sites

The other serine phosphorylation sites are either stimulatory or inhibitory:

- Ser633 – lowering the level of $[Ca^{+2}]_i$ necessary for eNOS activation (Boo, Sorescu et al. 2003), phosphorylated by PKA (Mount, Kemp et al. 2007) or AMPK (Chen, Peng et al. 2009).

- Ser615 – its role being controversial (Bauer, Fulton et al. 2003; Tran, Leonard et al. 2008), phosphorylated by PKA and Akt (Michell, Harris et al. 2002).
- Ser114 – by most investigators thought to cause decreased activity (Li, Ruan et al. 2007) although others have suggested enhanced activity (Gallis, Corthals et al. 1999).

The tyrosine residues on eNOS exert opposing effects on eNOS activity with Tyr81 phosphorylation causing activation of the enzyme (caused by Src kinase/pp60^{src}) but Tyr567 inhibiting the enzyme (phosphorylated by proline-rich tyrosine kinase (PYK2) (Fulton, Church et al. 2005; Fisslthaler, Loot et al. 2008). Tyrosine phosphorylation is possibly lost when endothelial cells are cultured and could explain why results have suggested no eNOS tyrosin phosphorylation (Rafikov, Fonseca et al. 2011).

Thr495 is localized within the CaM-binding domain and is constitutively phosphorylated (Fleming and Busse 2003). Phosphorylation at Thr495 results in inhibition of eNOS (Fleming, Fisslthaler et al. 2001) and some factors stimulating phosphorylation of eNOS at stimulatory sites also cause dephosphorylation at Thr495 (Boo, Sorescu et al. 2003). However, this is not obligatory for eNOS activation since phosphorylation at Ser1177 can cause NO-production without dephosphorylation at Thr495 or even with increased Thr495 phosphorylation (Li, Ruan et al. 2007).

1.4.3.6 Ca^{+2} levels

The activity of eNOS is to a large extent determined by intracellular calcium levels. Various agonists activate eNOS by elevating $[Ca^{+2}]_i$, via activation of PLC and the formation of DAG and IP_3. In resting cells, eNOS is mostly associated with caveolin-1, a protein present in both plasma membranes and the Golgi apparatus (Averna, Stifanese et al. 2008). Caveolin-1 is the major coat protein of the caveolae and its association with eNOS causes inhibition of electron transfer from the reductase domain of one monomer to the oxygenase domain of the other and, therefore, negatively regulates NO synthesis. In order to release the inhibitory caveolin-eNOS interaction, calmodulin must bind eNOS on the CaM-binding motif and thereby facilitate the electron flux from the reductase domain of the protein to the oxygenase domain. Michel et al. demonstrated that addition of calmodulin disrupted the heteromeric complex formed between eNOS and caveolin in a Ca^{+2}-dependent manner (Michel, Feron et al. 1997). Overexpression of caveolin attenuated eNOS enzyme activity, an effect reversed by addition of

exogenous calmodulin. Also, García-Cardena et al. (Garcia-Cardena, Martasek et al. 1997) demonstrated that incubation of pure eNOS with peptides derived from the scaffolding domains of caveolin-1 and 3 resulted in inhibition of eNOS activity.

eNOS stimulation in response to bradykinin or acetylcholine is inhibited by extracellular Ca^{+2} chelation or CaM antagonist (Luckhoff, Pohl et al. 1988; Busse and Mulsch 1990). Certain stimuli, like shear stress can, however, activate eNOS without an increase in intracellular Ca^{+2}, although these effects are inhibited by intracellular Ca^{+2} chelation. Under these conditions, NO production can be activated at resting Ca^{+2} levels since the kinase phosphorylating eNOS is not dependent on elevated Ca^{+2} (Dimmeler, Fleming et al. 1999).

1.4.3.7 Regulatory proteins and phosphatases

The activation of eNOS has long been known to be dependent on protein-protein interactions, especially between CaM and eNOS. Numerous additional proteins have been identified and shown to have an impact on the activity of the enzyme. In caveolae, eNOS is kept inactive by interaction with caveolin-1 which sterically blocks the calmodulin binding site in eNOS (Michel, Feron et al. 1997), as previously described. One of the most abundant cytosolic proteins, Hsp90, also plays a crucial role in the regulation of eNOS activity, its association with eNOS being critical for mediating vasorelaxation and vascular tone by various stimuli (Garcia-Cardena, Fan et al. 1998).

Several other proteins affect eNOS activity/location. Proteins shown to be associated with increased eNOS activity/NO release are for example dynamin (Dyn), porin and soluble guanylyl cyclase (sGC), endoglin (Toporsian, Gros et al. 2005) and eNOS interacting protein (NOSIP) (Schleicher, Brundin et al. 2005). eNOS trafficking inducer protein (NOSTRIN) has been shown to decrease eNOS activity and significantly inhibit NO release (Zimmermann, Opitz et al. 2002; Schilling, Opitz et al. 2006).

Protein phosphatases affect the activity of eNOS by dephosphorylation, the outcome depending on the site dephosphorylated. Phosphatases participating in eNOS regulation include PP1, PP2A and PP2B (calcineurin) (Fleming and Busse 2003; Dudzinski and Michel 2007; Mount, Kemp et al. 2007) although site-specific contribution of each phosphatase is still largely unknown (Rafikov, Fonseca et al. 2011).

From this brief review it should be obvious that the regulation of eNOS is complex and that phosphorylation of the enzyme is only one of many ways to

control its activity. One of the kinases mediating this phosphorylation leading to activation of the enzyme is the 5´-AMP-activated protein kinase, AMPK.

1.5 AMPK

1.5.1 Role of AMPK in cells

5´-AMP-activated protein kinase, AMPK, is the central component of a protein kinase cascade that plays an important role in the regulation of energy metabolism, often referred to as the cell´s metabolic master switch (Hardie 2003) (**Figure 2**). As reflected by its name, AMPK acts as a sensor of cellular energy status in many different cell types and responds to any stress that causes a fall in the cellular AMP/ATP ratio by activating catabolic pathways to generate ATP (e.g. fatty acid oxidation and glycolysis) and by turning off ATP-requiring processes that are not essential for short term survival (e.g. fatty acid synthesis and cholesterol synthesis). To achive this, AMPK causes direct phosphorylation of its downstream enzymes but also has long term effects on gene and protein expression. Recently, it has been demonstrated that AMPK not only senses and regulates energy balance at the cellular level but also plays a role in whole body-energy metabolism by responding to hormones and nutrient signals, leading to changes in energy homeostasis (controls both energy expenditure and appetite) (Kahn, Alquier et al. 2005; Dzamko and Steinberg 2009).

Besides its role as an energy sensor, AMPK is also known to be activated by phosphorylation in a nucleotide-independent way, e.g. by activators such as hypoxic stress (Marsin, Bouzin et al. 2002), oxidants (Zou, Hou et al. 2002; Zhang, Dong et al. 2008), adiponectin (Yamauchi, Kamon et al. 2002), leptin (Minokoshi, Kim et al. 2002), metformin (Hawley, Gadalla et al. 2002) and CaMKK (Hawley, Pan et al. 2005; Woods, Dickerson et al. 2005).

1.5.2 Role of AMPK in cardiovascular health

AMPK has been shown to mediate several protective effects in the endothelium by increasing NO-bioavailability through eNOS activation, suppressing ROS production and lipid accumulation and by anti-apoptotic and anti-inflammatory effects, making AMPK a potential drug target in prevention and treatment of atherosclerosis (Zou and Wu 2008). In addition, recent data suggest that activation and function of AMPK contributes to cardiovascular health, since the AMPK cascade triggers vascular protective mechanisms shown to reduce myocardial ischaemic or hypoxic injury to cardiac tissue, e.g. via its ability to improve glucose homeostasis, cholesterol concentrations and suppressing ER stress (Dong, Zhang et al. 2010; Ewart and Kennedy 2010). However, much

work remains to be done to answer important questions on whether AMPK is indeed a target for therapeutic development, considering the various effects of AMPK activation in different cell types/tissues (Young, Li et al. 2005).

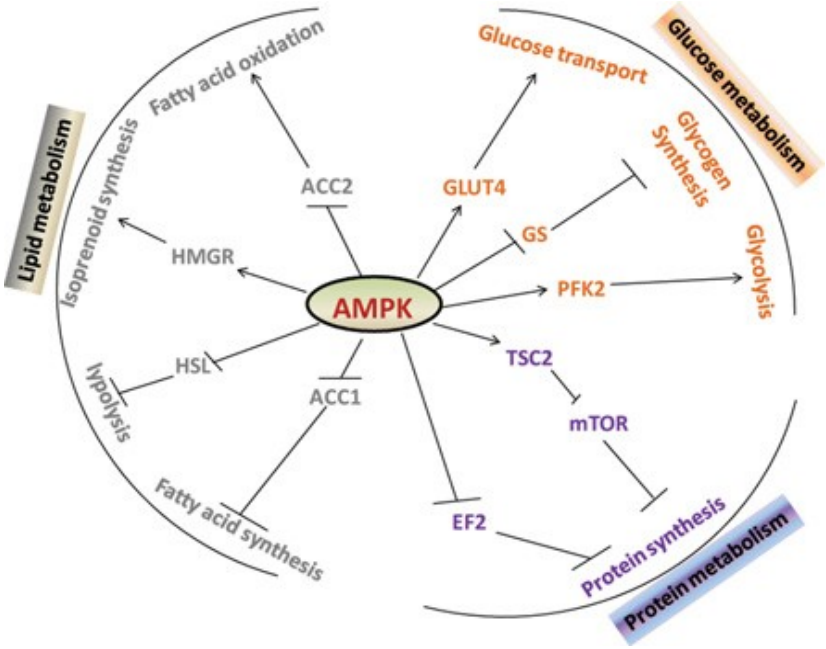

Figure 2 Regulation of downstream targets by AMPK. ACC, acetyl-CoA carboxylase; EF2, elongation factor 2; GLUT4, glucose transporter 4; GS, glycogen synthase; HMGR, HMG-CoA reductase; HSL, hormone-sensitive lipase; mTOR, mammalian target of rapamycin; PFK2, phosphofructokinase 2; TSC2, tuberous sclerosis complex. (Figure from (Wang, Song et al. 2012)).

1.5.3 Structure of AMPK

The highly conserved AMPK exists in heterotrimeric complexes comprising a catalytic α subunit and regulatory β and γ subunits. In humans, each subunit is encoded by two or three distinct genes (α1, α2, β1, β2, γ1, γ2, γ3) with homologues of these subunits found even in very primitive eukaryotes like Gardia lamblia (Hardie, Scott et al. 2003; Carling 2004).

1.5.3.1 The α subunit

The α subunit of AMPK comprises an N-terminal catalytic domain, containing the critical threonine residue Thr172, and a C-terminal regulatory domain involved in complex formation. Mutation of Thr172 to alanine totally abolishes kinase activity (Stein, Woods et al. 2000). The autoinhibitory domain in the C-

terminal regulatory domain hinders activation of AMPK in the absence of AMP (Crute, Seefeld et al. 1998). The two α subunits are differentially expressed in different tissues. While it has been shown that α1 and α2 are equally distributed in the liver (Woods, Salt et al. 1996), α2 is the predominant isoform in tissues with high energy demand like skeletal (Thornton, Snowden et al. 1998) and cardiac (Dyck, Kudo et al. 1999) muscle whereas the α1-subunit largely predominates in adipose tissue, pancreatic islets cells and vascular smooth muscle cells (Lihn, Jessen et al. 2004). Endothelial cells dominantly express the α1-isoform of AMPK (Morrow, Foufelle et al. 2003; Thors, Halldorsson et al. 2008; Dong, Zhang et al. 2010) and it has even been doubted whether they express the α2-isoform at all (Mount, Hill et al. 2005; Colombo and Moncada 2009). In HUVEC, the AMPKα subunit expression is even thought to vary from donor to donor (Fisslthaler and Fleming 2009).

1.5.3.2 The β subunit

The β subunit interacts with both α and γ subunits forming an active αβγ-complex (Woods, Cheung et al. 1996). This subunit also contains a region conserved across all eukaryotes referred to as the glycogen-binding domain (GBD). The GBD causes AMPK to associate with glycogen suggesting that AMPK may be able to sense the status of cellular energy reserves in the form of glycogen (McBride, Ghilagaber et al. 2009).

1.5.3.3 The γ subunit

The γ subunits contain the two Bateman domains, a structure each formed by two tandem CBS motifs (Kemp 2004). Each of the Bateman domains binds one molecule of AMP or ATP in a mutually exclusive manner, explaining the antagonizing effects of ATP on AMP-mediated AMPK activation (Scott, Hawley et al. 2004).

In mammalian cells, binding of AMP to this domain allosterically activates the complex as well as inhibiting dephosphorylation by protein phosphatases. These stimulatory effects of AMP are antagonized by high concentrations of ATP, making the kinase a monitor on cellular AMP/ATP ratio (Kahn, Alquier et al. 2005).

1.5.4 The activation of AMPK

The activation of AMPK requires phosphorylation of threonine 172 (Thr172) within the α catalytic subunit mediated by one or more upstream kinases (AMPKK) (**Figure 3**). In mammals, two kinases have been identified as

physiological kinases upstream of AMPK, liver kinase B1 (LKB1, also known as STK11) and Ca^{+2}/calmodulin-activated protein kinase kinase (CaMKK) (Hawley, Boudeau et al. 2003; Hawley, Pan et al. 2005; Hurley, Anderson et al. 2005; Woods, Dickerson et al. 2005). In addition, Tak1 (transforming growth factor-beta-activated kinase) has been shown to phosphorylate and activate AMPK *in vitro* (Momcilovic, Hong et al. 2006). The phosphorylation of AMPK Thr172 is reversible and the phosphatase PP2C is suggested to play a major role in the regulation of AMPK activity (Sanders, Grondin et al. 2007).

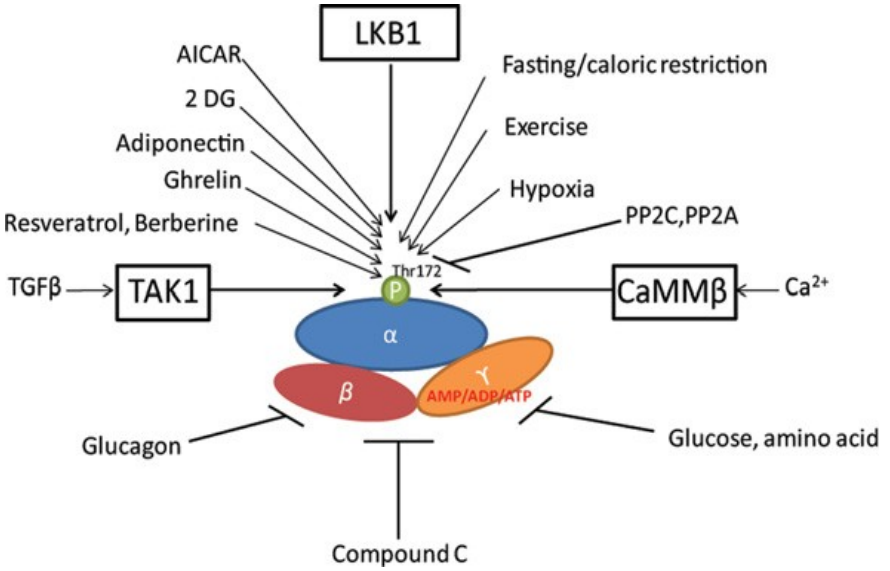

Figure 3 Regulation of AMPK by upstream kinases and various effectors. CaMMβ, Ca^{+2}/calmoduline kinase kinase; 2DG, 2-deoxy-glucose; PP, protein phosphatase; TAK1, TGFβ -activated protein kinae-1; TGFβ, transforming growh factor β. (Figure from (Wang, Song et al. 2012)).

It has been suggested that 5´-AMP activates AMPK by four distinct mechanism, involving i) direct activation of AMPK, ii) direct activation of AMPKK, iii) AMP binding to AMPK and thereby making it a better substrate for AMPKK and iv) a less attractive substrate for phosphatases (Davies, Helps et al. 1995; Hawley, Selbert et al. 1995). Recently, however, it has been demonstrated that AMP does not directly activate LKB1 or CaMKK or promote phophorylation of AMPK by these upstream kinases. A new model by Sanders *et al.* proposes two distinct mechanisms for AMP-mediated activation of AMPK, by direct allosteric activation and by protection from phosphatases (Sanders, Grondin et al. 2007). This model assumes that AMPK is phosphorylated by a Ca^{+2}-activated pathway dependent on CaMKK

and an AMP-dependent pathway, mediated by LKB1. The phosphorylating and activating effects of the constitutively active LKB1 on Thr172 would according to this model increase when the level of intracellular AMP rises and inhibits Thr172 dephosphorylation by phosphatases. Since CaMKK requires a signal that raises intracellular Ca^{+2} to be activated and consequently phosphorylates AMPK, this pathway is not dependent on AMP, as demonstrated by Stahmann *et al.* and others (Hawley, Pan et al. 2005; Stahmann, Woods et al. 2006). The work described in this thesis aims at elucidating the transduction pathways mediating the phosphorylation of AMPK after treatment with thrombin or histamine and at exploring the role of the two AMPK kinases in that pathway.

1.5.5 AMPKkinases

1.5.5.1 LKB1

The activity of AMPK is regulated by upstream kinases, one of which is LKB1 (Hawley, Boudeau et al. 2003). The 59 kDa serine/threonin protein kinase LKB1 was originally identified as a tumor suppressor, mutationally inactive in various types of epithelial cancers. Mutations in the LKB1 gene at 19p13 cause the rare autosomal dominantly inherited Peutz-Jeghers cancer syndrome (PJS), a rare autosomal dominantly inherited cancer susceptibility disorder mostly characterized by multiple hamartomatous polyps in the gastrointestinal tract and higher cancer risk (Katajisto, Vallenius et al. 2007). LKB1 was demonstrated to phosphorylate and activate AMPK in 2003 by Hawley and coworkers (Hawley, Boudeau et al. 2003) and the inhibiting effects of AMPK on mTOR are thought to play an important role in the activity of LKB1 as an tumor suppressor (Shackelford and Shaw 2009). The first identified physiological substrate for LKB1 was AMPK but LKB1 is now known to phosphorylate and activate 13 other downstream kinases (Lizcano, Goransson et al. 2004; Jaleel, McBride et al. 2005). LKB1 itself can be phosphorylated on at least eight residues and four of these residues are autophosphorylation sites (Alessi, Sakamoto et al. 2006). Although controversial, there is no specific mechanism known that causes activation/inactivation of LKB1 and the kinase has been considered constitutively active (Fogarty and Hardie 2009). LKB1 has an N-terminal nuclear localization signal and it is known that when LKB1 is not associated with its two fellow proteins, the pseudokinase STRAD (STE20-related adaptor) and the scaffolding protein MO25 (mouse protein 25), it is predominantly located in the nucleus. However, once active, the kinase is mostly located in the cytoplasm and then in complex with STRAD and MO25.

It has been suggested that binding of STRAD and MO25 to LKB1 causes LKB1 to relocalize from the nucleus to the cytoplasm, critical for activating its downstream targets (Boudeau, Scott et al. 2004). Others have demonstrated the role of SIRT1 in this process, assuming that deacetylation of LKB1 by SIRT1 in the nucleus facilitates its movement to the cytoplasm (Lan, Cacicedo et al. 2008). Still others have emphasized the role of phosphorylation of LKB1 at Ser428/Ser431 mediated by PKCz in the translocation of the kinase from the nucleus to the cytoplasm and its possibility to activate its downstream target, AMPK (Song, Xie et al. 2008; Xie, Dong et al. 2008) although the kinases PKA and p90RSK have also been implicated in phosphorylating this residue (Collins, Reoma et al. 2000; Hawley, Boudeau et al. 2003).

Kou et al. recently suggested the possibility that AMPK itself mediated this phosphorylation of LKB1 (Kou, Sartoretto et al. 2009). However, others have shown that this particular phosphorylation does neither alter the cellular location of LKB1 (Collins, Reoma et al. 2000) nor affect its activity. Interesting recent results on a short splice variant of LKB1, which does not contain this particular phosphorylation site (LKB1 short form, LKB1s), strongly suggest that phosphorylation of Ser428/431 is not involved in LKB1´s relocalization (Denison, Hiscock et al. 2009) or its ability to phosphorylate its downstream targets (Fogarty and Hardie 2009) (alternate splicing at the 3´end of the mRNA). The short variant has been shown to associate with STRAD and MO25, to be catalytically active and to activate AMPK in cells (Denison, Hiscock et al. 2009) underlining the fact that phosphorylation at this site cannot be critical for the activation of the kinase with respect to its downstream target, AMPK. A possible explanation for this discrepancy are the different cells used in the aforementioned studies and the possibility that LKB1 relies on various activation paths depending on the stimulus. However, the mechanism of the activation of LKB1 and its ability to interact with its substrates is still unclear.

1.5.5.1.1 AMPK-related kinases

The 12 known AMPK-related kinases, BRSK1, BRSK2, NUAK1, NUAK2, QIK, QSK, SIK, MARK1, MARK2, MARK3, MARK4 and MELK, are all structurally similar to AMPK. With one exception (MELK), they are all activated by LKB1 (Bright, Thornton et al. 2009). There are limited data on the upstream pathways activating these kinases. Sakamoto and coworkers demonstrated in skeletal muscle that contraction, phenformin or AICAR did not increase the activity of the AMPK-related kinases QSK, QIK, MARK2, 3 or 4, demonstrating a difference in the activation of these kinases and AMPK (Sakamoto, Goransson et al. 2004).

Also, CaMKK does not phosphorylate/activate BRSK1 or -2 (Bright, Carling et al. 2008). The role of TAK1 has not been investigated. Many of these kinases are still largly unknown and their possible role in the signaling pathways described in this thesis has not yet been identified.

1.5.5.2 CaMKK

In 2005, evidence emerged demonstrating a physiological role of the Ca^{+2}/CaM dependent kinase, CaMKK, in activating AMPK in mammalian cells. Previous work had demonstrated in 1995 that CaMKK could phosphorylate AMPK Thr172 in a cell free system (Hawley, Selbert et al. 1995). The β-isoform of CaMKK is the nearest relative in the human genome to the three protein kinases Elm1, Pak1 and Tos3 that phosphorylate the yeast ortholog of the AMPKα, Snf1, in Saccharomyces cerevisiae (Hong, Leiper et al. 2003; Sutherland, Hawley et al. 2003). It has been shown that AMPK can be markedly activated in cells lacking LKB1 (Hawley, Pan et al. 2005; Hurley, Anderson et al. 2005; Woods, Dickerson et al. 2005). Hawley and coworkers demonstrated that the β-isoform of CaMKK was the dominant isoform mediating Ca^{+2}-dependent activation of AMPK in HeLa cells (deficient in LKB1 but express both CaMKKα and –β (Ishikawa, Tokumitsu et al. 2003)) although they did not exclude the possibility that the α-isoform could activate AMPK in other cell types (Hawley, Pan et al. 2005) as suggested by Hurley and coworkers (Hurley, Anderson et al. 2005). The results of Woods and coworkers showed a role for CaMKKβ but not –α in the activation of AMPK in CCL13 cells, also lacking LKB1 (Woods, Dickerson et al. 2005). Unlike LKB1, CaMKK is not constitutively active but is activated in response to elevated Ca^{+2}.

1.5.5.3 TAK1

As previously mentioned, transforming growth factor-β-activated protein kinae-1 (TAK1), a member of the MAPK kinase kinase family, has been shown to activate AMPK in cell-free assays (Momcilovic, Hong et al. 2006). TAK1 has been shown to activate AMPK-dependent cytoprotective autophagy in human epithelial cells treated with TRAIL (tumour necrosis factor-related apoptosis-inducing ligand) (Herrero-Martin, Hoyer-Hansen et al. 2009), but its physiological role is still largely unknown.

1.5.6 Downstream targets of AMPK

As suggested by its name, AMPK is activated by increased intracellular concentrations of AMP, responding to cellular energy stress (increase in AMP/ATP levels) by shifting the balance of the cell´s metabolic processes from

anabolic to catabolic with the aim to restore cellular ATP levels. In general, activated AMPK turns on catabolic pathways to generate ATP, e.g., enhanced glucose uptake via glucose transporter type 4 (GLUT4) and GLUT1, activation of glycolysis via phosphorylation of 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase (PFKFB), β-oxidation of fatty acids via phosphorylation and inhibition of ACCβ, while switching off anabolic processes that consume ATP, e.g., glycogen synthesis by inhibition of glycogen synthase, fatty acid synthesis by inhibition of ACC1, protein synthesis by inhibition of tuberous sclerosis 2 (TSC2) and regulatory associated protein of mTOR (RAPTOR), cholesterol synthesis by inhibition of 3-hydroxy-3-methylglutaryl CoA reductase (HMG-CoA reductase), triglyceride synthesis by inhibition of glycerol phosphate acyl-transferase, rRNA synthesis by inhibition of transcription initiation factor IA (TIFIA) and mitochondrial biogenesis by inhibition of peroxisome proliferator-activated receptor-γ co-activator 1α (PCG1α) (Kahn, Alquier et al. 2005; Hardie, Ross et al. 2012). These effects of AMPK are mediated both by rapid, direct phosphorylation of target enzymes and via long-term activation/inhibition of gene and protein expression (Kahn, Alquier et al. 2005). Recently, Jeon and coworkers demonstrated a role for LKB1-AMPK-ACC in maintaining the intracellular level of NADPH, critical in the cellular response to oxidative stress (Jeon, Chandel et al. 2012).

1.6 The PI3K-Akt pathway

The serine/threonine protein kinase Akt, also known as protein kinase B, is a downstream effector of phosphoinositide 3-kinase (PI3K)-dependent signaling pathways. It is involved in embryonic vascular development and neoangiogenesis as well as in several endothelial cell functions, including activation of eNOS and promotion of endothelial cell survival. Numerous growth factors, including EGF, VEGF, insulin and HGF as well as shear stress are potent activators of Akt. G-protein activators have also been shown to activate Akt. Numerous reports have in recent years identified activated Akt as a survival signal to protect various types of cells from apoptosis induced by a variety of stresses (Datta, Brunet et al. 1999). In the vasculature, the pathophysiologic relevance is highlighted by the stimulatory effects of Akt on eNOS activity (Dimmeler, Fleming et al. 1999; Fulton, Gratton et al. 1999), critical for endothelial function and health, and the stimulatory effects of statins on Akt, eNOS activity and neovascularization (Kureishi, Luo et al. 2000).

1.7 Thrombin

Among the numerous chemical, cellular and physical affectors that the vascular endothelium is exposed to because of its strategic location at the interphase of the vascular wall and the circulating blood, thrombin is one of the key players. Recent evidence suggests that even in normal individuals thrombin is continously generated, underscoring a complex and continuous interaction between the coagulation system and the vascular wall (Borissoff, Spronk et al. 2009). The balance between synthesis and inactivation is decisive for the net amount of thrombin affecting the vascular wall, a balance that is strongly influenced by physiological and pathophysiological factors operating at each segment of the vascular tree at any given time.

Thrombin is not only the key affector of the coagulation cascade but is also the most potent of many activators of platelet aggregation. Its effects on the endothelium are both direct and indirect. The indirect effects are through intermediaries, such as platelets, fibrin and activated protein C. Platelets stimulated by thrombin release a host of vasoactive substances with effect on endothelial cells, serotonin, thromboxane A2, platelet derived growth factor and ADP, to name a few, all physiologically important in their own right (Borissoff, Spronk et al. 2009). Collectively they create an extremely complex situation. The direct effects are mediated through the G-protein coupled protease-activated receptors (PARs) (Hirano, Nomoto et al. 2007). Currently 4 members of the PAR family have been identified (PAR-1 through PAR-4). Although the signal transduction for the most part, is channeled through G-proteins activating various cascades of signaling molecules, the downstream propagation of the signal does not follow linear routes of parallel pathways but is mediated by a complex network with crosstalk between pathways and numerous feedback loops. The cellular and physiologic changes that follow downstream include changes in cell shape and permeability, changes in vascular tone, hemostasis, angiogenesis and inflammation. While having a multifaceted role that involves both disease progression and protection of vascular homeostasis, thrombin has been implicated in all stages of atherogenesis, from endothelial dysfunction to disease progression, plaque rupture and thrombosis, both through thrombotic mechanisms and through thrombin´s stimulation of transcription of proinflammatory genes.

The protease activated receptors (PARs) are members of the G-protein coupled receptor family but have a unique activation mechanism. The ligands are internal parts of the inactive receptor which is irreversibly activated when proteolytic agonists remove the amino terminus causing intramolecular activation

of the receptor by the new amino terminus. All four of the identified PARs are expressed by endothelial cells and three of them (PARs 1,3 and 4) are directly activated by thrombin (Macfarlane, Seatter et al. 2001). Knockout mice have been produced for all of the PARs and the most severe phenotype is caused by Par-1 -/- resulting in about 50% lethality at mid-gestation. Restoration of PAR-1 in the endothelial cells by the use of an endothelium specific promoter prevented the embryonic lethality (Griffin, Srinivasan et al. 2001). Thus, there is strong evidence supporting the view that PAR-1 is the major thrombin receptor expressed on endothelial cells (Hirano, Nomoto et al. 2007).

Signaling from PARs is initiated by nucleotide exchange on the α subunit of the heterotrimeric G-proteins, leading to disassociation from the βγ dimer and subsequently signaling by both the α and the βγ subunits. PAR-1 couples directly to G_q, G_i and $G_{12/13}$ (McLaughlin, Shen et al. 2005; Soh, Dores et al. 2010) and indirectly through prostacyclin generation to $G_{\alpha s}$ (Werthmann, Lohse et al. 2011). As has been found to be the case with other G protein-coupled receptors (GPCR) (Tilley 2011) some of the signals mediated by PAR-1 are propagated independently of G-proteins. Thus, both creatine kinase and heat shock protein 90 (Hsp90) have been shown to directly interact with PAR-1 and mediate cytoskeletal reorganisation (Mahajan, Pai et al. 2000; Pai, Mahajan et al. 2001) and recently it was suggested that binding of β arrestin 2 to PAR-1 is a link in the activation of NF-κB by thrombin (Delekta, Apel et al. 2010).

As PARs can signal through all the major classes of G-proteins the signals are similar to those of many other pro-inflammatory G protein-coupled receptors, such as those for histamine, platelet activating factor, angiotensin II and lysophosphatidyl choline but some of the detailed differences may stem from the unique activation mechanism of PARs. Firstly, as the activator is an enzyme, a single molecule can activate many receptors. Secondly, the ligand created by the activator is an integral part of the receptor and is not inactivated or diluted. Finally, although an activated receptor can be removed from the cell surface it cannot be re-used.

1.8 Histamine

Histamine is an important chemical in local immune response, produced by mast cell or basophils. In humans, histamine exerts its action by activating its receptors, H_1-H_4, all of which are G-protein coupled receptors (Tanimoto, Sasaguri et al. 2006). In endothelial cells histamine induces a rapid increase in vascular permeability, responsible for the edema formation in the infected

tissue. These effects of histamine are via the H_1-receptor, causing Ca^{+2}/calmodulin-dependent activation of myosin light chain kinase (MLCK), phosphorylation of myosin light chain (MLC) eventually promoting actin-myosin interaction in the cell (van Nieuw Amerongen, Draijer et al. 1998). These cytoskeletal contractile forces affect the tight junction proteins of the closely apposed endothelial cells strongly influencing cell-cell junctions as well as cell-matrix adhesion structures and thus the integrity of the endothelial barrier (Shen, Rigor et al. 2010). We did not investigate the effects of histamine on endothelial permeability in our model.

It had been demonstrated in endothelial cells that histamine generated inositol trisphosphates and raised intracellular Ca^{+2} by binding to H_1-receptors (Lo and Fan 1987; Bull, Courtney et al. 1992), confirmed by the laboratory in HUVEC (Halldorsson and Thorgeirsson 1989). It had also been shown by the laboratory that histamine caused formation of cGMP, prevented by MIBG due to inhibition of histamine binding to the H_1-receptor (Jonsson, Halldorsson et al. 1998).

2 Aims

The overall aim of the work described in this thesis was to explore the effects of the G-protein agonists histamine and thrombin on the phosphorylation and activation of eNOS in HUVEC.

Specific aims:

1. **Paper I:** To explore the signaling pathway(s) mediating eNOS phosphorylation and NO-production after treatment with histamine or thrombin. To investigate the role of PI3K-Akt in that pathway.

2. **Paper II:** To explore the role of AMPK in the phosphorylation and activation of eNOS after treatment of cells with histamine or thrombin.

3. **Papers III and IV:** To describe the conditions leading to AMPK-dependent activation of eNOS after treatment of cells with thrombin or histamine. To explore in detail the activation of AMPK caused by these agonists under different culture conditions and clarify the role of the two AMPKkinases CaMKK and LKB1 in that activation.

3 Materials and methods

3.1 Materials

EBM-2 was purchased from Clonetics. Other cell culture media, Foetal Bovine Serum and Penicillin-Streptomycin were purchased from Gibco Brl, Life Technologies. Tissue culture plates (35mm) and flasks (50mL) were from Nunc, Cryotin X (trypsin) from cod was provided by The Science Institute of Iceland. EGF, TPA, wortmannin, insulin, LPC, thrombin, histamine, A23187, CCCP, IBMX, cAMP, Rp-cAMPs, U73122, forskolin, Tempol and Trypsin-EDTA Solution were purchased from Sigma. S1P, rottlerin, Gö6976, PD98059, SB203580, H89 and GF109203X were from Biomol. AICAR, BAPTA, STO-609, Compound C, KN-93, KN-62 and Y 27632 were from Calbiochem. Hybond ECL Nitrocellulose membrane (6x8cm), ECL+PLUS Western blotting detection system, Hyperfilm ECL High performance chemiluminescence film and L-(3H) arginine (61.0 Ci/mmol) were from Amersham Pharmacia Biotech. Cyclic GMP XP Assay kit and antibodies against Akt, phospho-Akt (Ser473), phospho-PKCδ, eNOS, phospho-eNOS (Ser1177), AMPK, phospho-AMPK (Thr172), AMPKα1, AMPKα2, LKB1, phospho-LKB1, GRP78, p47phox, Pan-Actin and Anti-Rabbit IgG/HRP-linked came from Cell Signaling Technology. Antibodies against ACC and phospho-ACC (Ser79) were from Upstate. LipofectamineTM RNAiMAX Reagent and 5-(and 6)–carboxy-2´7´dichlorodihydofluorescein deacetate (carboxy-H2DCFDA) were from Invitrogen. Validated siRNA against AMPKα1 (s100), AMPKα2 (s11057) and LKB1 (s13580) came from Ambion as well as a negative control siRNA (Silencer Negative Control #1 with a sequence that does not target any gene product). Eliten kit was purchased from Promega. gp91phox antibody came from Santa Cruz. Poly-prep prefilled chromatography columns were from BioRad.

3.2 Methods

3.2.1 Cell culture

Endothelial cells were cultured from human umbilical veins by a modification of the method of Jaffe et al. (Jaffe, Nachman et al. 1973) as previously reported by our group (Gudmundsdottir, Halldorsson et al. 2001). The cells were harvested by Cryotin X trypsin digestion and seeded on 35mm culture dishes in Morgan´s medium 199 containing 20% foetal bovine serum and antibiotics (penicillin, 100 units/mL and streptomycin, 100 μg/mL). The culture

dishes were incubated at 37°C in humidified air with 5% CO2. The medium was changed 24 hours after seeding the cells and then every 2-3 days thereafter until the cell culture reached confluence (after ~7 days).

When confluent the cells were washed with the appropriate medium and placed in 1.0 mL serum free medium with or without inhibitors at the indicated concentrations. Agonist was added 10 to 20 minutes later in a concentration calculated to reach the intended concentration for each experiment and left on for additional 2 to 3 minutes. The agonists were then removed along with the medium and cellular reactions terminated by adding 250 μL SDS sample buffer. The samples were boiled for 5 minutes and centrifuged for 10 minutes at 3000 rpm. The samples were then ready to be either used or stored at -20°C.

Identification of the cells harvested and cultured by this method as being endothelial cells had previously been carried out by the demonstration of factor VIII antigen in the cells by immunofluorescence and the demonstration of Weibel-Palate bodies by electron microscopy (Thorgeirsson and Robertson 1978).

3.2.2 siRNA Transfection

Endothelial cells, grown to approximately 80% confluence in 25 cm2 tissue culture flasks, were trypsinized and diluted sixfold on to 35mm culture dishes. 24 hours later, the cells were transfected with LipofectamineTM RNAiMAX transfecting agent containing siRNA for AMPKα1, AMPKα2 or LKB1 (20nM) in an antibiotics-free EBM-2 medium containing 6% serum. Cells were cultured for 44-48 hours and protein expression analyzed by Western blotting.

3.2.3 Electrophoresis and Immunoblotting

Samples (8 μL) were resolved by SDS-PAGE (10%). The gels were blotted and the proteins thereby transferred to nitrocellulose. The membranes were hybridized with the indicated antibodies and subsequently with a secondary antibody (Anti-Rabbit IgG/HRP-linked). The immuno-complexes were detected with ECL+PLUS Western blotting detection system and developed onto a film. Equal loading was ascertained by hybridizing membranes with antibodies against unphosphorylated protein. The band intensity was quantified using Kodak 3.5 software.

3.2.4 Measurement of ATP

ATP was determined by luciferase assay. For validation HPLC was used in which case AMP was also determined (data not shown). For ATP determination using the luciferase assay the medium was removed from the

cells and the cells lyzed by adding 0.5 mL of 0.5N perchloric acid. After 30 minutes a 10 µL sample was diluted x100 in water mixed with 80 µL of a luciferase mixture from an Eliten kit by Promega.

3.2.5 Cell Morphology

Cell morphology was monitored with confocal microscopy and photographic images obtained at the indicated intervals using Leica DFC310 FX.

3.2.6 Determination of ROS Levels

For determination of ROS levels, cells were washed twice with PBS and incubated with 10 µM carboxy-H2DCFDA which is taken up by the cells and cleaved by intracellular esterases and transformed to a fluorescent dye when oxidized. After 15 minutes the cells were trypsinized and fluorescence analysed by flow cytometry.

3.2.7 cGMP Measurements

HUVEC monolayers were washed with Morgan´s medium 199 or medium1640 and placed in 1.0mL Morgan´s medium 199 or medium 1640 containing 0.25mM IBMX. The cells were incubated for 17 min. at 37°C. Histamine was then added to give a final concentration of 10µM and the cells further incubated for 3 min. The reaction was stopped by removing the medium and adding 0.5mL of 0.1M HCl. Intracellular cGMP was determined using an enzyme immunoassay kit in accordance with the manufacturer´s instructions.

3.2.8 Citrullin assay

eNOS activity in intact cells was determined by monitoring the conversion of incorporated L-(^{3}H) arginine into L-(^{3}H) citrulline as described by Schmidt and Mayer (Schmidt 1999). Briefly, the cells were washed and equilibrated for 20 min in incubation buffer (50 mM Tris buffer, pH 7.4, containing 100 mM NaCl, 5 mM KC1, 1 mM $MgCl_2$, and 2 mM $CaCl_2$) with or without BAPTA or H89. Reactions were started by the addition of L-(^{3}H) arginine (61.0 Ci/mmol, l µCi, final concentration 16 nM) and the agonist. After 3 min the cells were washed with chilled incubation buffer containing 0.1 mM EGTA instead of $CaCl_2$, followed by the addition of 1 mL of 10 mM HCl. An hour later, an aliquot was removed for determining the incorporated radioactivity. To the remaining sample a solution of 200 mM sodium acetate, 20mM NaOH containing 10 mM citrulline was added (final pH approx. 5.0) and L-(^{3}H) citrulline was separated from L-(^{3}H) arginine by cation exchange chromatography .

3.2.9 Statistical Analysis

Values are expressed as average ± S.D. Unpaired, two-tailed Student´s t-test was performed for comparisons between groups. The level of significance was set at $p<0.05$. Software used was GraphPad Prism 5.00. Results express at least three independent experiments (indicated in the figure texts in Papers I-IV).

4 Results and Discussion

4.1 Effects of thrombin and histamine on eNOS, role of Akt

4.1.1 Introduction

Previously, it had been demonstrated and published by Jonsson and co-workers in our laboratory that histamine caused cGMP production in HUVEC, an indication of eNOS activation (Jonsson, Halldorsson et al. 1998). The best characterized pathway mediating phosphorylation and activation of eNOS is the PI3K – Akt cascade (Dimmeler, Fleming et al. 1999; Fulton, Gratton et al. 1999; Fulton, Gratton et al. 2001; Fleming and Busse 2003). Paper I examines the phosphorylation of eNOS after treatment with the G-protein activators histamine and thrombin, hypothesizing that these agonists would activate the PI3K/Akt pathway and thereby cause eNOS Ser1177 phosphorylation. Also, it had previously been published by Igarashi and coworkers that the platelet-derived angiogenic lipid growth factor, sphingosine 1-phosphate (S1P), caused activation of Akt and NO-production in bovine aortic endothelial cells (Igarashi, Bernier et al. 2001). Therefore, we also tested the effects of S1P on the phosphorylation of Akt and eNOS in HUVEC.

- **Hypothesis for paper I:** In HUVEC, the G-protein activators histamine and thrombin as well as S1P cause eNOS Ser1177 phosphorylation via activation of the PI3K-Akt pathway.

4.1.2 Paper I - Results and Discussion

Histamine and thrombin inhibit the phoshorylation of Akt mediated by EGF or S1P in HUVEC by activation of PKCδ. Despite inhibiting Akt, these agonists cause phosphorylation of eNOS Ser1177.

As expected and shown in **Figure 4**, both EGF and S1P caused phosphorylation of Akt. Treatment with histamine or thrombin, on the other hand, lowered the basal phoshorylation of Akt (**Figure 4B**) and 10 minute pretreatment with histamine or thrombin prevented the phosphorylation of Akt mediated by EGF or S1P (**Figure 4C**).

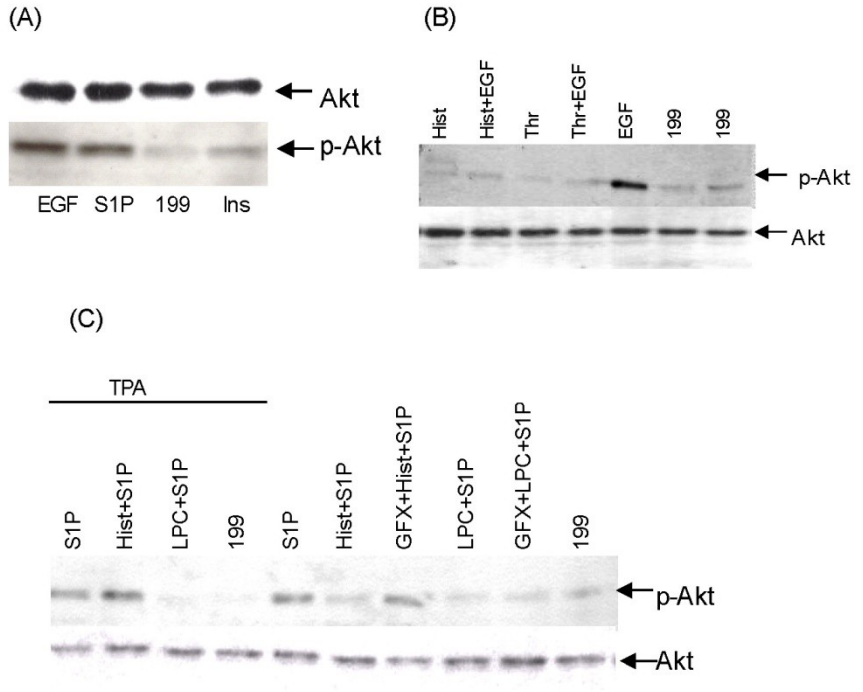

Figure 4 Phosphorylation of Akt after agonist treatment in HUVEC. (A) Effects of EGF, S1P and insulin on phospho-Akt, Ser473. (B) Effect of a 10 minute pretreatment with thrombin or histamine on EGF-mediated phosphorylation of Akt. (C) Effects of pretreatment with LPC or GF109203X on S1P mediated Akt-phosphorylation.

Thrombin and histamine activate various signaling pathways in HUVEC and we next investigated the effects of several signaling molecules generated by these pathways on EGF- or S1P-mediated Akt phosphorylation (**Figure 5**). Direct stimulation of PKC with the phorbol ester TPA inhibited EGF mediated phosphorylation of Akt as did pretreatment with lysophosphatidylcholine (LPC), generated by activation of PLA_2.

Figure 5 Inhibition of EGF-mediated Akt phosphorylation (Ser473) by PD98059 (PD), LPC, wortmannin (Wm), TPA or arachidonic acid (AA).

Inhibition of PKC with GF109203X prevented the effects of histamine and thrombin on EGF- and S1P-mediated Akt phosphorylation, supporting a possible contribution of PKC to the inhibitory effects of histamine and

thrombin (**Figure 6**). GF109203X, on the other hand, had no effect on the LPC-mediated inhibition of Akt, demonstrating different inhibitory mechanisms of these agonists.

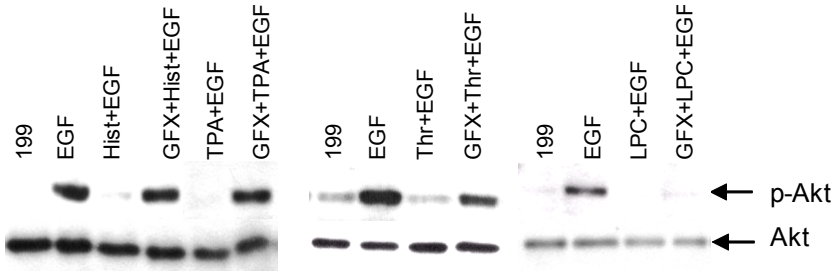

Figure 6 Involvement of PKC in the inhibition of EGF-mediated Akt phosphorylation (Ser473), effects of pretreatment with GF109203X.

Since there are at least 5 different isoforms of PKC expressed in primary cultures of HUVEC (Mattila, Majuri et al. 1994; Haller, Ziegler et al. 1996), we tested the effect of more specific PKC-inhibitors on this pathway. Gö6976 had no effect on EGF-stimulated Akt-phosphorylation, ruling out conventional isoforms of PKC (α, β, γ). Our results, using the PKCδ inhibitor rottlerin, suggested that this isoform of PKC was responsible for the inhibition seen after treatment with histamine or thrombin (**Figure 7**). The weakness of this experimental approach is the lack of absolute specificity of this pharmacological inhibitor, rottlerin (Bain, Plater et al. 2007). In support, however, is our finding that both histamine and thrombin caused phosphorylation of PKCδ Thr505 (**Figure 7**) known to greatly increase the activity of the kinase (Newton 2003; Cheng, He et al. 2007). What remains to be done is to test these results with the use of siRNA for PKCδ.

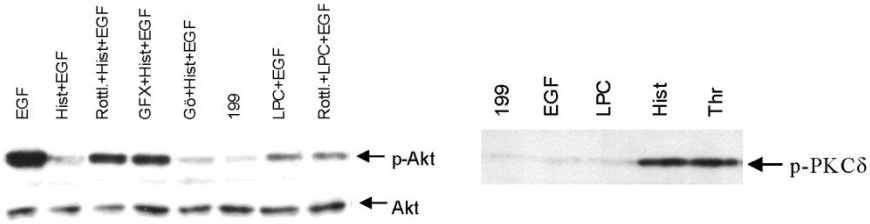

Figure 7 Effects of the isoform-specific PKC inhibitors rottlerin (Rottl.) or Gö6976 (Gö) on histamine- and LPC-mediated Akt inhibition and PKCδ phosphorylation (Thr505) after treatment with various agonists.

EGF caused eNOS Ser1177 phosphorylation as expected but in contrast to its effects on the phosphorylation of Akt, histamine caused phosphorylation

of eNOS (**Figure 8**) as did thrombin (not shown). We tested the possible role of various kinases in this pathway by the use of inhibitors (GF109203X for PKC, PD98059 for ERK, SB203580 for p38, Y27632 for ROCKII and KN-62 for CaMKII) (**Figure 8**). None of these kinases appeared to play a role in the phosphorylation of eNOS by histamine or thrombin.

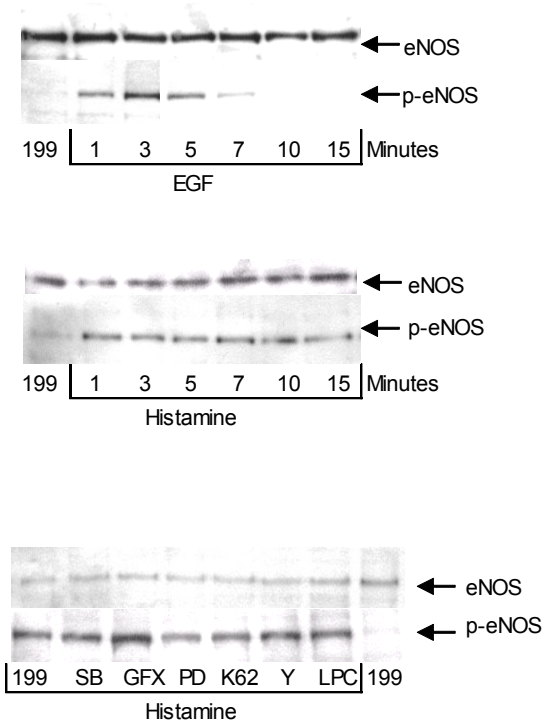

Figure 8 Phosphorylation of eNOS (Ser1177) after EGF or histamine and effects of various inhibitors on histamine-mediated eNOS phosphorylation. Pretreatment with SB203580 (SB), GF109203X (GFX), PD98059 (PD), KN-62 (KN), Y27632 (Y) or LPC.

The results presented in paper I, therefore, left us with a pathway of eNOS Ser1177 phosphorylation that was PI3K-Akt independent and had not been previously described. We hypothesized that the AMP-activated protein kinase (AMPK) might be a missing link in the signaling cascade since it was at that time already known to phosphorylate eNOS (Chen, Mitchelhill et al. 1999; Morrow, Foufelle et al. 2003) and eNOS had been reported to be in a complex with AMPK (Chen, Mitchelhill et al. 1999; Zou, Hou et al. 2002). Testing this hypothesis became the project of paper II.

4.1.3 Conclusion and Significance

This study reported on the inhibitory effects of the G-protein coupled agonists histamine and thrombin on the phosphorylation of the survival kinase Akt in HUVEC after treatment with EGF or the G-protein coupled agonist S1P. Despite the inhibitory effects on Akt phosphorylation, histamine and thrombin caused phosphorylation of eNOS Ser1177. In the vasculature, the stimulatory effects of Akt on eNOS activity are critical for normal endothelial function and health (Dimmeler, Fleming et al. 1999; Fulton, Gratton et al. 1999). S1P, the G-protein coupled agonist highly concentrated in HDL and native LDL, as opposite to oxidized LDL, has now been identified as an important activator of eNOS in the vasculature (Igarashi and Michel 2008) and its reduced levels to contribute importantly to the cytotoxic effects of ox-LDL (Kimura, Sato et al. 2001).

In atherosclerosis, oxLDL accumulates in the arterial wall where it is cytotoxic and chemotactic for monocytes fueling the inflammatory component of atherogenesis. LPC is a major phospholipid component of ox-LDL and βVLDL and is known to be increased in both atherosclerotic and inflammatory lesions (Steinberg, Parthasarathy et al. 1989; Witztum and Steinberg 1991). When we conducted this study it had been shown to selectively upregulate VCAM-1, ICAM-1 (Kume, Cybulsky et al. 1992) and growth factor gene expression (Kume and Gimbrone 1994) in EC and suppress endothelium-dependent vasorelaxation (Mangin, Kugiyama et al. 1993; Gimbrone, Cybulsky et al. 1995). LPC also mimicked the dose-dependent impairment of arterial relaxation caused by oxLDL (Kugiyama, Kerns et al. 1990; Yokoyama, Hirata et al. 1990) and was known to have inhibitory effects on endothelial cell migration by dephosphorylation of Akt (Chavakis, Dernbach et al. 2001). It was therefore a known mediator modulating endothelial function at all stages of atherogenesis. Additionally, Choi and co-workers recently demonstrated in HUVEC that while lower levels of LPC (≤20μM) inhibit eNOS Ser1177 phosphorylation, higher levels (≥100μM) induce eNOS downregulation via superoxide overload and SOD1 inhibition (Choi, Park et al. 2010). The data presented in paper I, demonstrating the inhibitory effects of LPC on the activation of Akt, by either EGF or S1P, and the downstream target eNOS, added to the list of the detrimental vascular effects of LPC. Also, our demonstration that LPC inhibited S1P-induced phophorylation of Akt in endothelial cells provided an added insight into the potential mechanisms of the cytotoxic effects of oxLDL, the key player in atherogenesis (Steinberg 1997; Ross 1999).

In paper I, we, furthermore, demonstrated a role for PKCδ in the histamine- or thrombin-mediated inhibition of Akt phosphorylation after treatment with EGF or S1P. Stimulation of PKC has repeatedly been shown to impair endothelium-dependent arterial relaxation and has been proposed as a major mechanism explaining vascular dysfunction in diabetes (Geraldes and King 2010) as well as that imposed by oxidized LDL (Kugiyama, Ohgushi et al. 1992; Chen, Liang et al. 1997). PKCδ had previously been shown to induce growth arrest or apoptosis in cultured endothelial cells, emphasizing the relevance of our results that PKCδ plays a role in the inhibitory effects of thrombin and histamine on Akt in HUVEC. Our demonstration that PKCδ mediated Akt inhibition in endothelial cells and that the G-protein agonists thrombin and histamine inhibited Akt by their stimulation of PKCδ provided another potential mechanism for consideration in relation to PKC-mediated endothelial dysfunction in general and specifically vascular stress mediated by stimulation of G-protein linked receptors. It should, however, be noticed that thrombin has been shown to cause phosphorylation and activation of eNOS via PKCδ (Motley, Eguchi et al. 2007) and to activate Akt in endothelial cells (Motley, Eguchi et al. 2007; Bair, Thippegowda et al. 2009; Minhajuddin, Bijli et al. 2009; Mi, Chen et al. 2011) quite opposite to what we demonstrated in paper I. In the paper by Motley and co-workers and Minhajuddin and co-workers, significant phosphorylation of Akt was seen after 5 minute treatment with thrombin while Bair and co-workers observed this phoshorylation after 10 minutes. We therefore tested the phosphorylation of Akt after 5-15 minutes of thrombin treatment and saw no changes in the phosphorylation status (**Figure 9**, unpublished results).

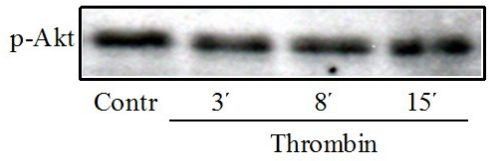

Figure 9 Effects of thrombin on Akt phosphorylation in medium 199 (unpublished results).

The discrepancy between these results and ours are yet to be explained. Fleming and coworkers had previously demonstrated histamine-stimulated activation of eNOS in PAEC mediated by CaMKII independent of PI3K-Akt (Fleming, Fisslthaler et al. 2001). We, however, later ruled out the possible involvement of CaMKII in this pathway in HUVEC (Paper II).

Bae and Rezaie recently demonstrated that in cultured endothelial cells thrombin caused phosphorylation of Akt via Gi only when endothelial protein C receptor (EPCR) was bound by protein C (Bae and Rezaie 2010). Thrombin in turn caused PAR-1-dependent protective intracellular signaling responses via Rac1, opposite to the proinflammatory pathways activated by thrombin via RhoA when EPCR was not expressed or occupied by protein C (Bae and Rezaie 2010; Rezaie 2011). Interestingly, activated protein C and even thrombin itself can activate sphingosine kinase and mediate barrier protection through transactivation of the S1P1 receptor (Tauseef, Kini et al. 2008).

Although the findings on S1P and PKC presented in paper I were very interesting considering the role of Akt in maintaining structural and functional integrity of the vascular endothelium, and raised sufficient a number of questions about the mechanisms of Akt inhibition to form a basis of a PhD project, this part of our results was left for future exploration.

4.2 Effects of thrombin and histamine on eNOS, role of AMPK

4.2.1 Introduction

The major source of NO in the endothelium is endothelial NO-synthase (eNOS) (Forstermann 2005) which is regulated by a complex battery of regulatory mechanisms. Besides subcellular localization and protein-protein interactions, coordinated phosphorylation and dephosphorylation of various sites on eNOS regulates the activity of the enzyme (Sessa 2004). Phosphorylation at Ser1177 has been most thoroughly studied and appears to enhance the sensitivity of eNOS to Ca^{+2}, thus promoting its activation. Several protein kinases have been implicated in mediating this phosphorylation, including Akt/PKB (Dimmeler, Fleming et al. 1999), PKA (Boo, Hwang et al. 2002), AMPK (Chen, Mitchelhill et al. 1999; Chen, Montagnani et al. 2003) and CaMKII (Fleming, Fisslthaler et al. 2001). The results of paper I excluded kinases known at that time to be activated by histamine or thrombin. In paper II, we tested the possible role of kinases known to phosphorylate eNOS at Ser1177. AMPK was then known to phosphorylate eNOS at Ser1177 (Chen, Mitchelhill et al. 1999; Morrow, Foufelle et al. 2003) and eNOS had been reported to complex with AMPK (Chen, Mitchelhill et al. 1999; Zou, Hou et al. 2002). Chen and co-workers had demonstrated adiponectin-mediated Akt-independent eNOS phosphorylation via AMPK in BAEC (Chen, Montagnani et al. 2003) while others had concluded that Akt had its effects downstream of AMPK in adiponectin-stimulated eNOS phosphorylation in HUVEC (Ouchi, Kobayashi

et al. 2004). In these cases, however, eNOS phosphorylation by AMPK was PI3K dependent in contrast to our conclusion in paper I. We found that the PI3K inhibitor wortmannin had no effect on eNOS phosphorylation after histamine or thrombin. Furthermore, these agonists did not cause phosphorylation of Akt. In paper II, we explore the role of various protein kinases in mediating the stimulatory effects of the G-protein activators thrombin and histamine on eNOS Ser1177 phosphorylation.

- **Hypothesis for paper II:** In HUVEC, histamine and thrombin cause eNOS Ser1177 phosphorylation via AMPK. Histamine and thrombin activate PLC, causing formation of inositol trisphosphates (IP_3) leading to a Ca^{+2} signal which activates processes that consume ATP. Activation of AMPK in response to lowered ATP would lead to eNOS activation and NO-formation.

4.2.2 Paper II – Results and Discussion

In HUVEC, the G-protein activators histamine and thrombin caused eNOS Ser1177 phosphorylation via AMPK, independent of PI3K-Akt. Inhibition of AMPK by H89 prevented the phosphorylation and activation of eNOS in HUVEC. AICAR, a known activator of AMPK, caused phosphorylation of AMPK, ACC and eNOS.

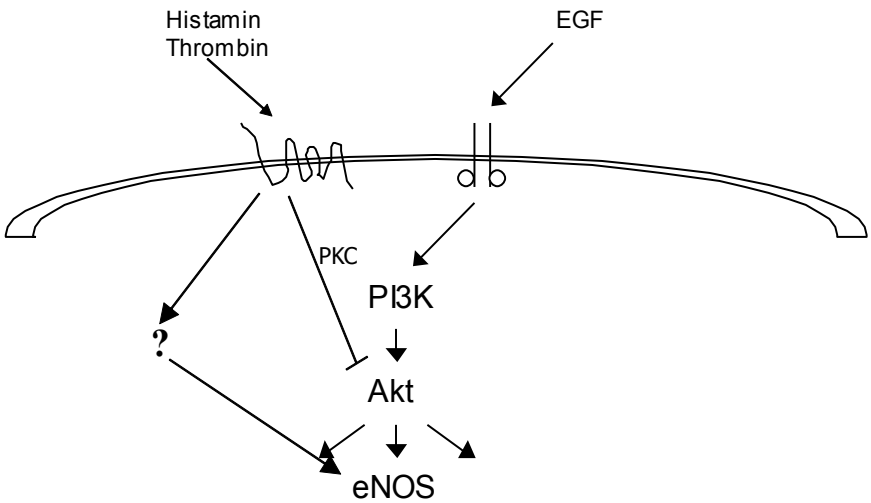

Figure 10 A proposed pathway based on the results presented in paper I. EGF causes phosphorylation of Akt via PI3K. Histamine and thrombin inhibit this phosphorylation by activation of PKCδ. However, both agonists cause Ser1177 phosphorylation of eNOS via an unknown mechanism.

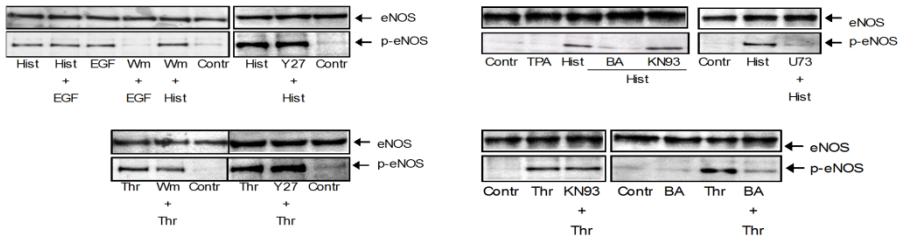

Figure 11 EGF, histamine- (Hist) or thrombin- (Thr) mediated phosphorylation of eNOS (Ser1177) and the effects of wortmannin (Wm), Y 27632 (Y27), BAPTA (BA), KN-93 and U73122 (U73).

This study examined the possible involvement of AMPK in mediating the phosphorylation of eNOS after histamine or thrombin treatment. As **Figure 11** demonstrates, the phosphorylation of eNOS mediated by histamine or or thrombin was independent of PI3K unlike that mediated by EGF which was totally blocked by the PI3K inhibitor wortmannin. The histamine-mediated phosphorylation of eNOS was inhibited by the PLCβ inhibitor U73122 as well as the calcium chelator BAPTA (also shown for thrombin) (**Figure 11**). ATP lowering after treatment with ionophore was also inhibited by BAPTA, demonstrating Ca^{+2}-dependence (44.2±5,8% lowering with A23187 vs -0.9±7,2% with A23187 and BAPTA, unpublished results).

Still this pathway was not inhibited by KN-93 and thus did not involve CaMKII (**Figure 11**) as was later suggested by Stahmann and co-workers although they did not publish any data to support that hypothesis (Stahmann, Woods et al. 2006). When ruling out various other kinases known to phosphorylate eNOS at Ser1177, we tested the effects of the PKA inhibitor H89. H89 totally inhibited eNOS phosphorylation by either histamine or thrombin (**Figure 12**). Another structurally unrelated PKA inhibitor Rp-cAMPS, however, had no effect.

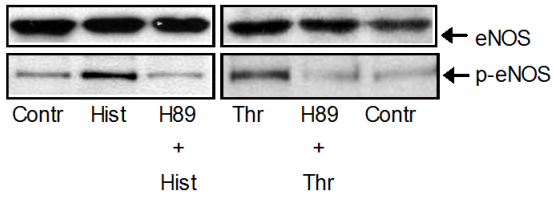

Figure 12 The possible role of PKA in eNOS phosphorylation.

Moreover, manipulations of cAMP levels (IBMX and forskolin or 8-bromo-cAMP) had no effects in our cells (figure 2B in paper II), indicating that the

involvement of PKA in this pathway was unlikely although the involvement of the kinase in eNOS phosphorylation had been demonstrated in response to shear stress (Boo, Hwang et al. 2002) and bradykinin (Bae, Kim et al. 2003). Although H89 had been marketed as “a selective and potent inhibitor of PKA”, Cohen and co-workers had shown that H89 inhibited several protein kinases to a similar degree, including AMPK (Davies, Reddy et al. 2000). As AMPK had been shown to phosphorylate eNOS in endothelial cells (Chen, Mitchelhill et al. 1999; Chen, Montagnani et al. 2003), we tested the effects of histamine and thrombin on AMPK activity. Our results presented in **Figure 13** clearly demonstrated PI3K-independent phosphorylation and activation of AMPK after treatment of cells with histamine or thrombin (AMPK and ACC phosphorylations unaffected by wortmannin). Also, manipulations known to activate AMPK (AICAR and CCCP) resulted in ACC and eNOS phosphorylation (see figure 3b,c in paper II).

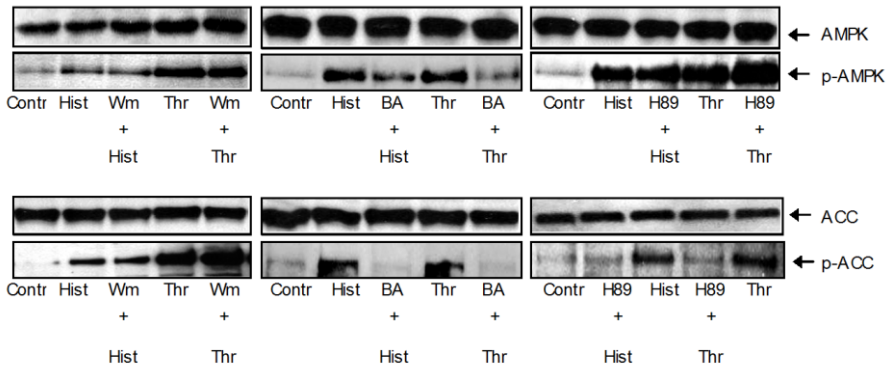

Figure 13 Histamine or thrombin mediated phosphorylation of AMPK (Thr172) or ACC (Ser79) and effects of the inhibitors wortmannin (Wm), BAPTA (BA) or H89.

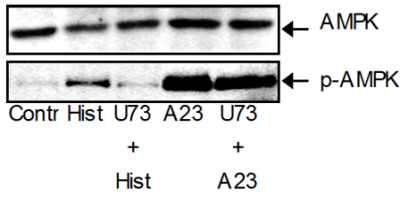

Figure 14 The involvement of PLC in AMPK phosphorylation (Thr172), effect of U73122.

The inhibitory effect of H89 on ACC phosphorylation after histamine or thrombin supported its role as an AMPK inhibitor in our system. As expected, with histamine and thrombin being known to activate PLC in HUVEC (Halldorsson, Kjeld et al. 1988), and Gq coupled receptors known to activate

AMPK (Kishi, Yuasa et al. 2000), the phosphorylation of AMPK was totally prevented by U73122 (**Figure 14**) as well as by BAPTA (**Figure 13**), consistent with the effects of these inhibitors on eNOS phosphorylation (**Figure 11**).

As has repeatedly been shown, the regulation of eNOS is a complex, multifactorial process (Fleming and Busse 2003; Mount, Kemp et al. 2007). In order to determine whether histamine or thrombin treatment led to activation of eNOS, we studied their effect on the conversion of L-(^{3}H) arginine to L-(^{3}H) citrulline. Monitoring the conversion of L-(^{3}H) arginine to L-(^{3}H) citrulline is an indirect measure of NO-formation. To confirm that the results demonstrated the activity of eNOS and to rule out the possible formation of citrullin by other means we used L-NAME that totally inhibited the formation of citrullin after histamine treatment.

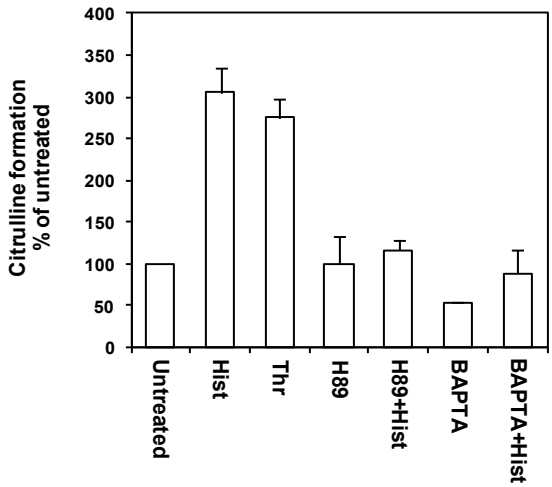

Figure 15 eNOS activation in response to histamine (Hist) or thrombin (Thr) and the effects of H89 or BAPTA (shown here for histamine).

Both agonists caused threefold increase in citrulline formation and this activation was largely prevented by pretreatment with BAPTA or H89 (**Figure 15** for histamine). This inhibition of NO-production with BAPTA or H89 not only supports the importance of Ser1177 phosphorylation in the activation of eNOS following histamine treatment but also demonstrates the importance of this pathway for eNOS activation and thus NO-production.

4.2.3 Conclusion and Significance

Figure 16 demonstrates a proposed pathway for thrombin- and histamine-mediated activation of eNOS based on results from paper II. Also, the effects

of various inhibitors are shown. Although AMPK had previously been shown to phosphorylate eNOS at Ser1177 in endothelial cells there was no agonist known to cause AMPK-mediated eNOS activation independent of PI3K-Akt.

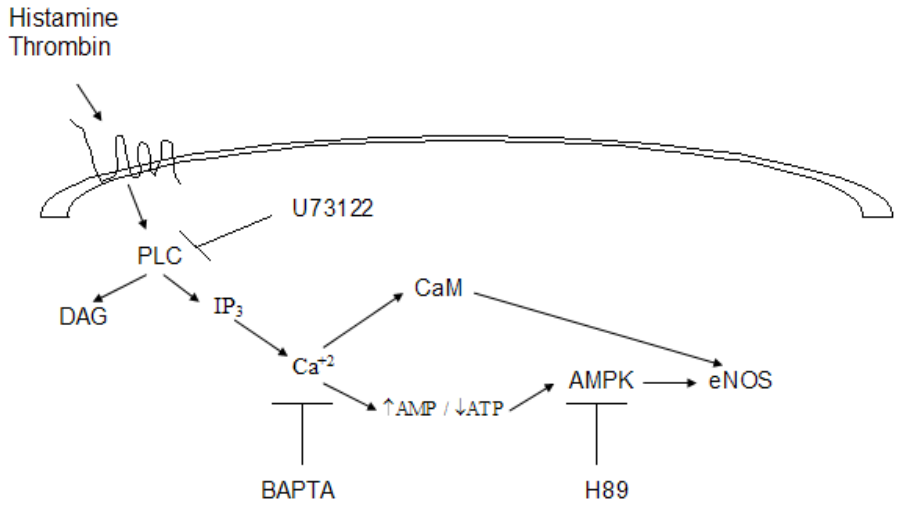

Figure 16 A proposed pathway for thrombin- and histamine-mediated eNOS activation.

AMPK was mostly known at the time for playing an important role in maintaining energy balance within cells (Carling 2004). AMPK was also emerging as an important regulator of whole body metabolism, activated by the adipocyte-hormones leptin and adiponectin in skeletal muscle increasing fatty acid oxidation (Minokoshi, Kim et al. 2002; Yamauchi, Kamon et al. 2002) or in the liver, where adiponectin causes a decrease in glucose output as well as an increase in glucose and fatty acid oxidation (Yamauchi, Kamon et al. 2002). In the hypothalamus, AMPK activity had been shown to be regulated by hormones that control feeding, involving the kinase in the regulation of appetite (Andersson, Filipsson et al. 2004; Kim, Miller et al. 2004; Kim, Park et al. 2004). However, AMPK had not been thoroughly studied in the endothelium and our results, presented in paper II, indicated an important role for AMPK in the activation of eNOS and the formation of NO, a central signaling molecule in the vasculature. In 2003, LKB1 was identified as being an important upstream AMPK kinase, phosphorylating Thr172 in response to lowered ATP/AMP ratio (Hawley, Boudeau et al. 2003; Woods, Johnstone et al. 2003). Although it was demonstrated in 1995 that CaMKK could phosphorylate AMPK Thr172 in a cell free system (Hawley, Selbert et al. 1995), it was not until 2005 that evidence emerged demonstrating a

physiological role of the Ca^{+2}/CaM dependent kinase, CaMKK, in activating AMPK in mammalian cells (Hawley, Pan et al. 2005; Hurley, Anderson et al. 2005; Woods, Dickerson et al. 2005). During our work on paper II, we therefore hypothesized that the treatment of cells with thrombin or histamine caused LKB1-mediated AMPK phosphorylation, presumably preceded by a rise in intracellular AMP. However, our results had clearly demonstrated the Ca^{+2}-dependence of the pathway, with total inhibition of AMPK, ACC and eNOS phosphorylation and activation by BAPTA as well as with the observation that all these phosphorylations were mimicked by treatment with the Ca^{+2}-ionophore A23187. We therefore hypothesized that the Ca^{+2}-elevation caused by these G-protein linked agonists initiated energy consuming events within the cell, including synthesis of prostacyclin and platelet activating factor, release of von Willenbrand factor and induction of shape change, thereby lowering intracellular ATP and in turn activating AMPK (**Figure 16**). Exploring the mechanism of thrombin- and histamine-mediated eNOS phosphorylation and the involvement of upstream kinases became the project of paper III.

4.3 Effects of thrombin and histamine on AMPK - Role of the upstream kinases CaMKK and LKB1

4.3.1 Introduction

In paper II we described a pathway in which AMPK is an important link in mediating thrombin- and histamine-induced activation of eNOS in HUVEC. As described above, AMPK was at this time (2004) mostly known as a cellular energy sensor. Therefore, although the AMPK-mediated eNOS phosphorylation shown in paper II was clearly dependent on Ca^{+2}, we hypothesized that AMPK was activated by a lowering of intracellular ATP after treatment of cells with thrombin or histamine. However, although the role of AMPK as an energy sensor had been well established in tissues such as skeletal and cardiac muscle, its precise role in endothelial cell metabolism was less well understood. There was evidence that endothelial cells existed in what was called a permanent state of “metabolic hypoxia“ (Quintero, Colombo et al. 2006) and therefore it had been suggested that in the endothelium AMPK had a more important role in maintaining endothelial function and in affecting signaling cascades than by acting as an intracellular fuel gauge (Fisslthaler and Fleming 2009). This was a new paradigm concerning the functional role of AMPK in vascular endothelium.

Although AMPK was identified more than 25 years ago (Ingebritsen, Lee et al. 1978), its activating or upstream kinases remained largely unknown until

recently. With the identification in 2003 of Elm1, Pak1 and Tos3, the upstream kinases of the AMPK homologue Snf1 in yeast (Hong, Leiper et al. 2003; Sutherland, Hawley et al. 2003), their nearest relatives in the human genome soon became known, the tumor suppressor LKB1 and the Ca^{+2}/CaM kinase kinase (CaMKK). However, as previous studies had ruled out CaMKK as an AMPKK (Hawley, Selbert et al. 1995), LKB1 was proposed by several groups to be the major upstream kinase phosphorylating and activating AMPK (Hawley, Boudeau et al. 2003; Woods, Johnstone et al. 2003; Shaw, Kosmatka et al. 2004). Therefore, when we published paper II in 2004, the role of LKB1 in phosphorylating AMPK at Thr172 had recently been demonstrated and with AMPK known as the cell´s "metabolic master switch", this fitted well into our proposed pathway of AMPK activation after histamine or thrombin.

In 2005, the role of CaMKK in phosphorylating AMPK was revisited. Results on AMPK activation in cells lacking LKB1 suggested another upstream kinase, the likeliest candidate being CaMKK. Several studies clearly demonstrated the role of CaMKK as an alternative AMPKK, in addition to LKB1 (Hawley, Pan et al. 2005; Hurley, Anderson et al. 2005; Woods, Dickerson et al. 2005). A year later, Stahmann and co-workers, in contrast to our findings, identified CaMKK as the sole upstream kinase mediating AMPK phosphorylation and activation after treatment of HUVEC with thrombin (Stahmann, Woods et al. 2006). They found no significant change in the AMP/ATP ratio upon thrombin stimulation and downregulation of LKB1 had no effect on thrombin-induced AMPK activation. In contrast to our findings in paper II, they did not find a role for AMPK in mediating thrombin-stimulated eNOS phosphorylation. These results encouraged us to explore these discrepancies on the mechanism of thrombin-mediated eNOS phosphorylation, besides further clarifying the involvement of the upstream AMPKkinases, LKB1 and CaMKK. This led to papers III and IV.

- **Hypothesis for papers III and IV:** In HUVEC, histamine and thrombin cause a lowering of intracellular ATP, thereby activating AMPK via LKB1. The elevation of intracellular Ca^{+2} caused by these agonists also activates CaMKK, another AMPKkinase, allowing phosphorylation of AMPK by both upstream kinases, LKB1 and CaMKK. Activation of eNOS by these agonists is at least in part mediated by AMPK.

4.3.2 Papers III and IV – Results and Discussion

In HUVEC, AMPK causes phosphorylation and activation of eNOS under culture conditions that allow a fall in cellular ATP after thrombin or histamine stimulation. Downregulation of LKB1 partially inhibits both AMPK

and eNOS phosphorylation and NO-synthesis under these conditions. Under culture conditions that prevent or do not allow such a fall in the energy level of the cells AMPK is still activated but exclusively via CaMKK and this AMPK-activation plays no role in the activation of eNOS.

As AMPK is a known energy sensor we tested the possibility that the activation of the kinase after treatment with histamine or thrombin involved lowering of intracellular ATP. We found that both histamine and thrombin caused a lowering of intracellular ATP levels after 3 minutes in culture medium Morgan´s 199. However, if the cells were transferred to a different culture medium, RPMI 1640, these lowering effects were not seen (**Figure 17**). The Ca^{+2}-ionophore A23187 caused similar effects on the intracellular ATP level, although with a more robust response but again with different responses in different media.

In medium 199 the CaMKK inhibitor STO-609 only partially prevented the phosphoryla- tion of AMPK, ACC and eNOS, indicating the presence and activity of another upstream AMPKkinase, most probably LKB1 (**Figure 18**). To test that possibility, we used HeLa cells which are known to be deficient in LKB1 (Tiainen, Ylikorkala et al. 1999).

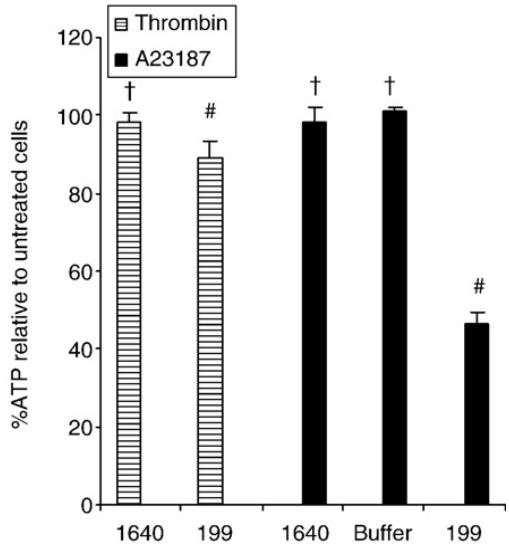

Figure 17 Effects of thrombin or A23187 on ATP levels in different media. (†ns vs. unstimulated cells; #p<0.001 vs. unstimulated cells).

Supporting this hypothesis, STO-609 totally inhibited the phosphorylation of AMPK after treatment of HeLa with histamine or A23187 (see figure 4 in

paper III). In paper IV, we further verified the role of LKB1 by using siRNA, showing a total inhibition of AMPK phosphorylation with STO-609 in cells with downregulated LKB1 (**Figure 19**).

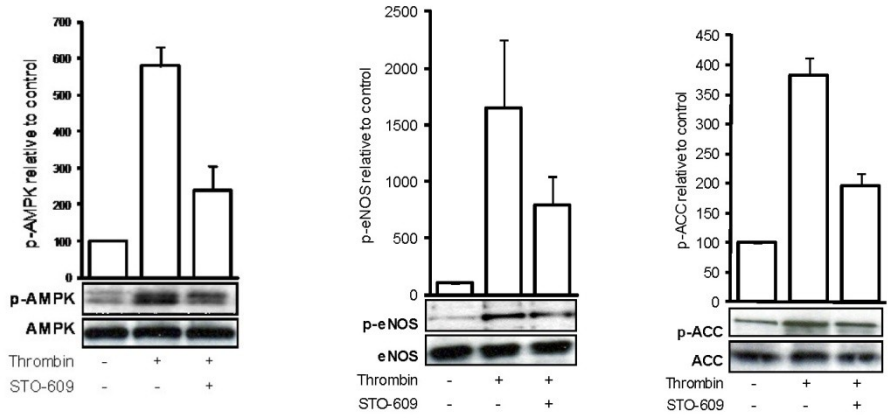

Figure 18 The effects of CaMKK inhibition on thrombin-mediated phosphorylation of AMPK, ACC and eNOS in medium 199.

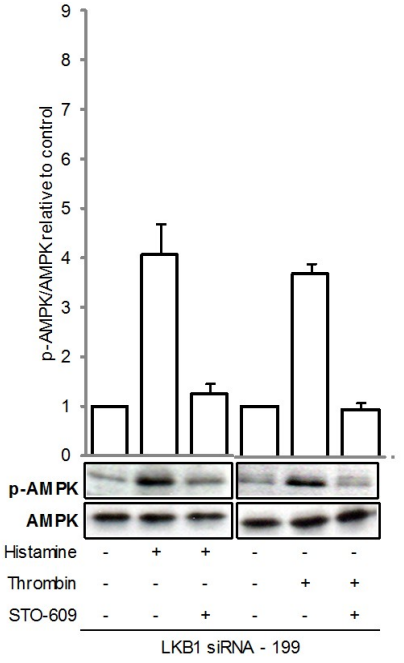

Figure 19 The role of LKB1 or CaMKK in histamine- or thrombin-mediated phosphorylation of AMPK.

We demonstrated a role for AMPK in eNOS phosphorylation in paper II using the protein kinase inhibitor H89 as well as AICAR, which stimulated the phosphorylation of AMPK, ACC and eNOS (**Figure 12**, figure 3c in paper II). In papers III and IV we used a more direct approch to verify the role of AMPK. Downregulation of both α-isoforms of AMPK simultaneously with siRNA partially inhibited both thrombin- and histamine-induced phosphorylation of eNOS as well as the formation of cGMP which we used as an indicator of NO-synthesis (shown for histamine in **Figure 20**).

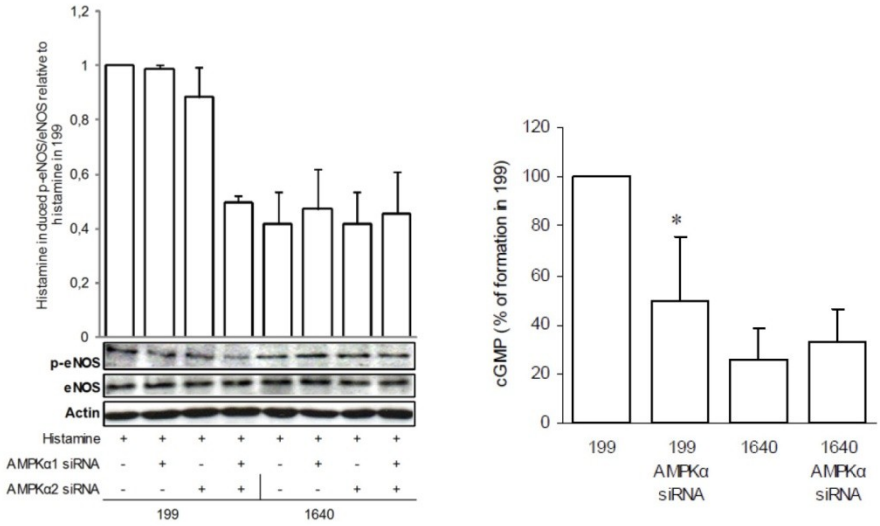

Figure 20 The role of the two AMPKα isoforms in histamine-mediated phosphorylation of eNOS (Ser1177) and cGMP formation. (*p<0.05 vs. cells treated with thrombin alone in medium 199).

An important step in reconciling our findings with those of Stahmann and coworkers (Stahmann, Woods et al. 2010) came with the observation that the composition of the culture medium played a critical role in the responses of endothelial cells to thrombin or histamine stimulation and in the signaling transduction pathways involved. If the cells were transferred from Morgan´s medium 199 that was used in the experiments described above to medium RPMI 1640 (or buffer), there was no lowering of intracellular ATP after treatment with histamine or thrombin (**Figure 17**).

In medium RPMI 1640 the phosphorylation of AMPK was totally inhibited by the CaMKK inhibitor STO-609, ruling out the involvement of LKB1 (**Figure 21**). This could be expected with unchanged AMP/ATP ratio after stimulation. eNOS phosphorylation and activation was not affected by AMPK siRNA

(shown here for histamine) (**Figure 20**) and the formation of cGMP was much lower than in Morgan´s medium 199 (**Figure 20**). If the intracellular ATP, however, was lowered by adding 2-deoxy-glucose to medium RPMI 1640, similar results were seen as previously described for Morgan´s medium 199 (figure 3D, paper III). Thus, we have demonstrated in these papers that environmental/culture conditions dictate which signal transduction pathways are activated by stimulation with thrombin or histamine. Only conditions subjecting endothelial cells to a fall in ATP after thrombin- or histamine-stimulation facilitate AMPK-dependent activation of eNOS, enhancing the magnitude of the response. This explains the differences between our results and those by Stahmann and co-workers on thrombin-mediated eNOS phosphorylation in HUVEC which was only present when we used culture medium 199 which prompted a fall in ATP after thrombin or histamine stimulation. Using the same culture conditions as reported by Stahmann and coworkers (Stahmann, Woods et al. 2010) we also found an AMPK-independent stimulation of eNOS.

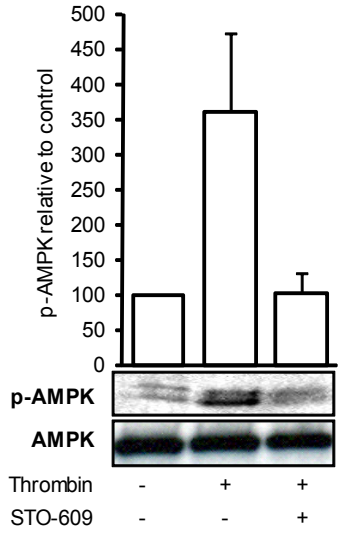

Figure 21 Effects of CaMKK inhibition on thrombin-mediated phosphorylation of AMPK in medium RPMI 1640.

eNOS was phosphorylated and activated in medium 1640 although not via AMPK, as demonstrated by Stahmann et al. and ourselves. Stahmann and co-workes suggested the responsible kinase to be CaMKII, based on the Ca^{+2}-dependency of the pathway. We have, however, ruled out CaMKII as well as several other kinases known to phosphorylate eNOS on Ser1177. The

kinase responsible for this AMPK-independent stimulation of eNOS is therefore still unknown. However, under conditions where cellular ATP was lowered LKB1 was involved in activating AMPK, as previously described, and contributed to the AMPK-dependent pathway of eNOS phosphorylation and activation after treatment with the G-protein activating agonists histamine and thrombin. In cells with downregulated LKB1, eNOS phosphorylation after histamine treatment was not affected by STO-609 or Compound C and thus CaMKK-AMPK-independent (**Figure 20**, **Figure 22**). Also, in paper IV we demonstrated that the formation of cGMP was inhibited with LKB1 siRNA to the same degree as had been shown with AMPK siRNA in paper III (**Figure 22**). These results clearly demonstrated the dependence of the AMPK-eNOS pathway on LKB1.

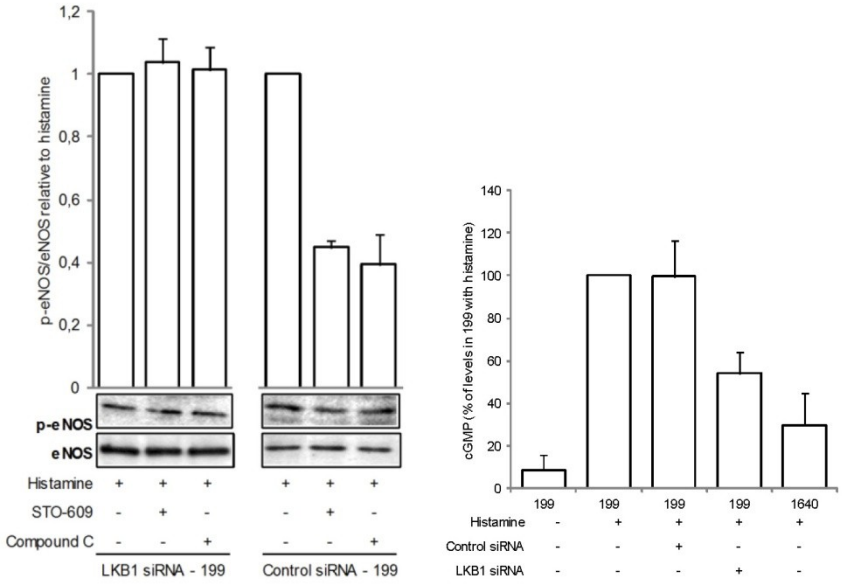

Figure 22 The role of LKB1 in histamine-mediated phosphorylation and activation of eNOS in medium 199.

Results on the effect of A23187 on ATP levels in media 199, 1640 or buffer (**Figure 17**), suggested that a constituent of medium 199 allowed or facilitated the drop in ATP levels in response to thrombin, histamine and A23187. We therefore examined several possibilities that could explain the different responses to stimulation in the different media (the composition of the two media is listed in Appendix I). These included the presence of ATP, UTP, adenosine, xanthine and hypoxanthine, cholesterol and vitamins in medium 199 and their absence in medium 1640. However, reduction of cellular ATP levels after A23187 treatment in medium 199 manufactured

without purines, ribose and deoxyribose was identical to that in normal medium 199 (data not shown). Adding the lipid soluable vitamins or cholesterol to medium 1640 in various combinations had no effect either (not shown). However, as shown in **Figure 23**, treatment of medium 199 with 1 U apyrase, used to hydrolyze nucleoside triphosphates and diphosphates, markedly reduced the ionophore mediated lowering of cellular ATP, the apyrase also prevented the ATP fall in cells in medium 199 manufactured without purines, ribose and deoxyribose (data not shown). The explanation for these effects has not yet been found.

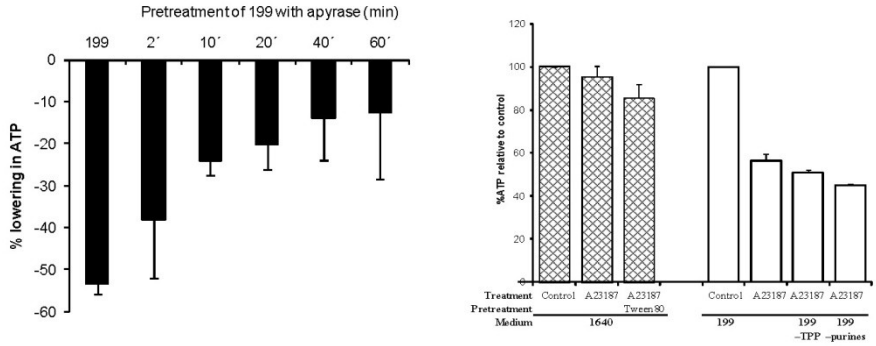

Figure 23 Effects of A23187 on ATP levels in different media (TPP = tocopheryl phosphate).

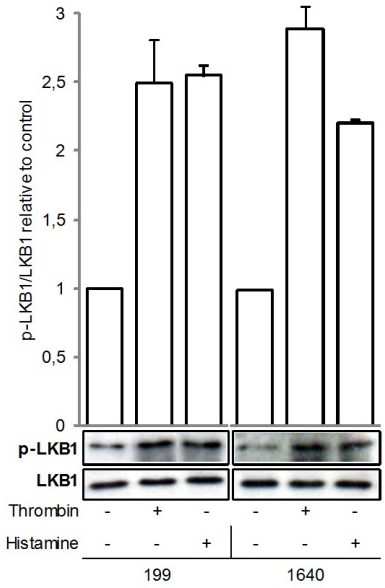

Figure 24 Thrombin- or histamine-mediated phosphorylation of LKB1.

Conversely, after adding tween 80 to medium 1640 in the miniscule amounts (20 µg/mL) that are present in medium 199, subsequent treatment with A23187 lowered intracellular ATP significantly (**Figure 23**) and similar activation of downstream pathways was demonstrated after thrombin treatment (AMPK and eNOS phosphorylation) as in cells stimulated in medium 199 (figure 7, paper III). Addition of triton X-100, another non-ionic detergent, to medium 1640, had similar effects as the addition of tween 80 (data not shown), suggesting that the detergent properties contribute to the ATP fall after stimulation of HUVEC with ionophore, histamine or thrombin although not affecting ATP in unstimulated cells.

It has been suggested that phosphorylation of LKB1 at Ser428 facilitates activation of AMPK (Song, Xie et al. 2008; Xie, Dong et al. 2008). In our study, we found that the treatment of HUVEC with histamine or thrombin caused similar phosphorylation of LKB1 at Ser428 in medium 199 and medium 1640 (**Figure 24**). Thus, differences in LKB1 phosphorylation after stimulation cannot be invoked as an explanation for the differences in eNOS phosphorylation in the two media.

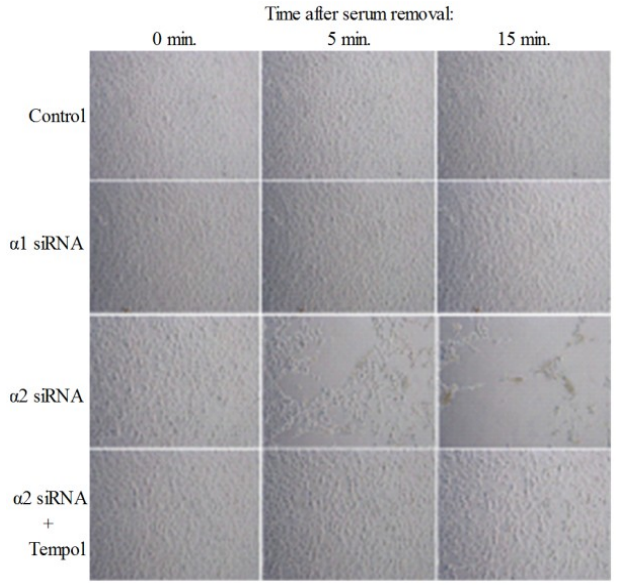

Figure 25 The role of the two AMPKα isoforms in maintaining endothelial monolayer integrity.

In recent years, it has been demonstrated that AMPK has an important function in maintaining intracellular homeostasis independent of energy status by responding to various stress challenges and cellular damage

(Alexander and Walker 2011). Activation of AMPK as a stress response has been linked to cellular adaptation of cardiovascular tissues to pathologic conditions such as the endothelial dysfunction seen in many cardiovascular diseases (Li and Keaney 2010). As reviewed by Wang and coworkers AMPK has been shown to regulate ROS/redox balance, autophagy, cell proliferation, cell apoptosis, cellular polarity mitochondrial function and genotoxic response by numerous downstream pathways (Wang, Song et al. 2012). In paper IV, we demonstrated a role for AMPKα2 in maintaining endothelial monolayer integrity (**Figure 25**).

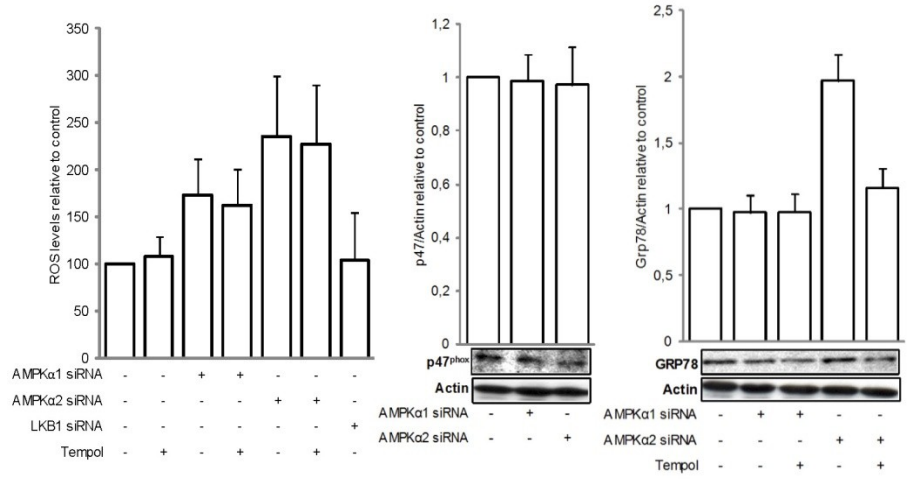

Figure 26 The effects of LKB1, AMPKα1 and/or -α2 siRNA on ROS levels, expression of p47phox or GRP78 in HUVEC.

Downregulation of this isoform for 67 hours caused deterioration of monolayer integrity, with shrinkage of cells and loss of contact both between cells and with the culture substrate when the cells were transferred to serum free media for up to 30 minutes. Cells lacking the α1-isoform showed no morphological changes nor did control cells or cells with downregulated LKB1 (not shown) demonstrating activation of AMPKα2 independent of LKB1. The SOD-mimetic Tempol markedly inhibited these morphological changes, suggesting a role for AMPKα2 in the regulation of antioxidant status as previously demonstrated by Kukidome and coworkers (Kukidome, Nishikawa et al. 2006). However, the level of reactive oxygen species (ROS) as measured by DCF fluorescence was increased after downregulation of either isoform and not affected by treatment with Tempol (**Figure 26**). AMPKα1 or –α2 downregulation had no effect on the expression of the $p47^{phox}$ subunit of NADPH-oxidase (**Figure 26**) nor the expression of the $gp91^{phox}$ subunit (data not shown)

demonstrating an increase in ROS level independent of increased expression of NADPH oxidase in AMPKα2 downregulated cells. The expression of the chaperone GRP78, an indicator of ER stress (Ron and Walter 2007), was greatly enhanced after downregulation of AMPKα2 whereas downregulation of AMPKα1 had no such effect (**Figure 26**). Treatment with Tempol prevented the increase of GRP78 expression in α2-downregulated cells.

4.3.3 Conclusion and Significance

In **Figure 27** an attempt is made to summarize the findings of our studies published in papers III and IV. A pathway is proposed for thrombin- and histamine-mediated activation of eNOS in cultured endothelial cells. In these papers, we demonstrated an LKB1-AMPK-eNOS signaling pathway, activated under conditions where intracellular ATP was lowered after stimulation. These findings reconcile the conflicting results on the role of AMPK in eNOS activation and clarify the role of the two upstream AMPKkinases, LKB1 and CaMKK.

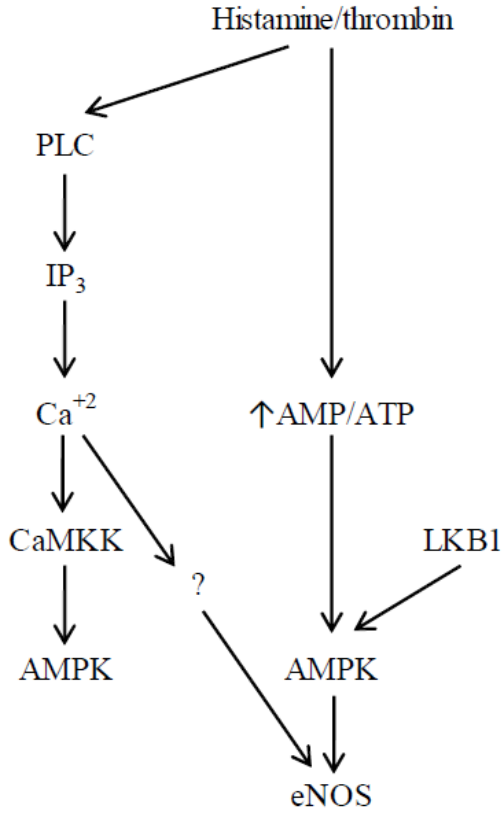

Figure 27 A proposed pathway for thrombin- and histamine-mediated eNOS activation.

There are recent reports on AMPK-mediated eNOS phosphorylation in endothelial cells. Reihill et al. (Reihill, Ewart et al. 2007) concluded, based on the effects of dominant negative AMPK and wortmannin, that in human aortic endothelial cells both AMPK and Akt contributed to eNOS phosphorylation and activation after VEGF treatment. Conversely, Stahmann and coworkers quite convincingly demonstrated an AMPK-independent eNOS phosphorylation after VEGF in HUVEC (Stahmann, Woods et al. 2010). In contrast to our results with histamine or thrombin, the AMPK contribution of eNOS phosphorylation in the VEGF treated cells reported by Reihill et al., was mediated only by CaMKK and not by LKB1 as it was totally prevented by CaMKK siRNA. Martinelli et al. recently reported that ICAM-1, which does not activate PI3K-Akt, also causes AMPK-dependent eNOS phosphorylation via CaMKK in microvascular endothelial cells (Martinelli, Gegg et al. 2009).

The role of LKB1 in our model is clearly demonstrated in cells treated with LKB1 siRNA. Results from Stahmann and co-workers (Stahmann, Woods et al. 2006) revealed that a functional LKB1/AMPK pathway existed in endothelial cells although not contributing to thrombin-stimulated AMPK activation. AICAR, mimicking the effect of AMP, activated AMPK via LKB1 and these effects were totally inhibited in cells with downregulated LKB1. Under these circumstances, CaMKK played no role in the phosphorylation of AMPK since intracellular Ca^{+2} levels were not elevated after stimulation. In our model, however, both Ca^{+2} and AMP levels rise after treatment, causing activation of CaMKK- and LKB1-mediated effects, respectively. LKB1 is considered to be constitutively active (Lizcano, Goransson et al. 2004) and to have increased phosphorylating and activating effects on AMPK when the intracellular level of AMP rises and in turn inhibits Thr172 dephosphorylation on AMPK by phosphatases (Sanders, Grondin et al. 2007). In culture medium 199, where the ATP/AMP level is lowered after treatment with histamine or thrombin, we clearly demonstrated a role for LKB1 in AMPK phosphorylation, both basal and after stimulation. Also, the phosphorylation of eNOS and NO-formation are LKB1-dependent only when cellular ATP is lowered, demonstrating that only AMPK activated by LKB1 mediates eNOS phosphorylation and activation. This was clearly demonstrated in LKB1-deficient cells (after siRNA) where further inhibition of AMPK by STO-609 or Compound C had no effect on eNOS phosphorylation (**Figure 22**). However, under circumstances where cellular ATP was lowered after stimulation, STO-609 still partially inhibited eNOS phosphorylation (**Figure 18**), contrary to our hypothesis of the non-involvement of the CaMKK-AMPK pathway in activating eNOS. This can possibly be explained by the inhibiting effects of

STO-609 on AMPK itself (Bain, Plater et al. 2007), thus partially inhibiting the phosphorylation of eNOS in culture medium 199, where AMPK is involved in mediating eNOS phosphorylation, whereas having no effect in cells with downregulated LKB1, where AMPK has no role in activating eNOS. It would be possible to address this directly and use siRNA for CaMKK to escape the unspecific effects of the inhibitor STO-609. We would expect to see no effect of CaMKK siRNA on eNOS phosphorylation or activation in neither media but this experiment has not been carried out yet.

We found a clear difference in ATP levels within cells after stimulation depending on the presence of the non-ionic detergent tween 80 in the medium. Adding tween 80 (or triton X-100) to medium 1640 resulted in a significant fall in cellular ATP after ionophore treatment as well as in a similar activation of downstream pathways after thrombin treatment as in cells stimulated in medium 199. Tween 80 is added to the culture media in miniscule amounts to facilitate solubilisation of cholesterol. Its addition obviously represents an artificial condition without a definite physiological or pathophysiological significance. However, in this study the experimental conditions including tween 80 allowed energy deprivation within endothelial cells after thrombin or histamine stimulation thus uncovering the two pathways for AMPK activation, one of which mediates the downstream activation of eNOS. Also, the conditions for the activation of these pathways, i.e. energy deprivation possibly secondary to Ca^{+2}-release, were identified. Energy deprivation is of obvious interest and potential importance in pathophysiology.

We tested the phosphorylation of LKB1 in our system using a phospho-specific LKB1 antibody. Our hypothesis was that since AMPK is dependent on LKB1 only in medium 199 (consistent with the canges in AMP/ATP level), LKB1 might be phosphorylated by thrombin under these circumstances only. However, thrombin caused phosphorylation of LKB1 at Ser428 in both medium 199 and 1640. We tested the effect of various inhibitors on this phosphorylation, especially the so-called AMPK-inhibitor Compound C. We had previously seen effects of this inhibitor on the phosphorylation of AMPK itself (although by its identity as an AMPK inhibitor it should only affect the phosphorylation of its downstream targets) and examined whether the phosphorylation of LKB1 might be downstream of AMPK as previously shown by Kou et al. (Kou, Sartoretto et al. 2009). Thus we tested the effects of thrombin on LKB1 Ser428 in cells treated with siRNA for AMPKα1 and/or -α2. None of these treatments had any effect on the phosphorylation of LKB1 (not shown) convincingly confirming that this is an AMPK-independent pathway.

The effects of LKB1 on the phosphatase PTEN in the endothelium (Song, Wu et al. 2007) is of interest in the context of the signaling pathways that we have been studying. Song et al. demonstrated the role of LKB1 in suppressing Akt signaling via PTEN when HUVEC were exposed to either peroxynitrite or high glucose. Since we had already demonstrated that thrombin inhibited Akt in HUVEC and had later seen that thrombin caused LKB1-phosphorylation and LKB1-dependent cellular events, we tested the possibility that the inhibition of Akt was mediated by LKB1-dependent upregulation of PTEN. However, we saw no changes in the phosphorylation of PTEN after treatment with thrombin, histamine or A23187 (**Figure 28**, unpublished results) nor did we detect any changes in thrombin-mediated inhibition of Akt-phosphorylation after treatment with EGF in LKB1 siRNA cells. Therefore, we concluded that LKB1 was not mediating the inhibitory effects on Akt in our model.

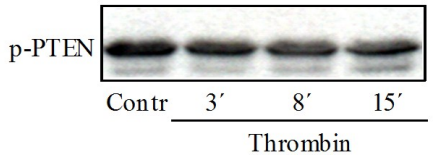

Figure 28 Phosphorylation of PTEN (Ser380/Thr382/383) after thrombin treatment.

Although the α1-isoform of AMPK is expressed to a greater extent in endothelial cells than the α2-isoform (Morrow, Foufelle et al. 2003; Dong, Zhang et al. 2010) and it has even been questioned whether the latter is expressed at all (Mount, Hill et al. 2005; Colombo and Moncada 2009), we found in paper III that both isoforms are present in primary cultures of HUVEC and that both contribute to the phosphorylation of eNOS. It has been shown that the downregulation of AMPKα2 caused ER stress in HUVEC and although the α1-isoform was expressed at much higher levels than the α2-isoform, downregulation of AMPKα2 caused greater ER stress (Dong, Zhang et al. 2010). Although we find that both isoforms of AMPKα have to be downregulated to prevent eNOS phosphorylation after either histamine or thrombin stimulation, we observed that the stress of serum removal produced almost instant morphologic changes in confluent monolayers of endothelial cells with downregulated AMPKα2 but not in cells with downregulated AMPKα1 or LKB1. These dramatic changes were markedly delayed by the SOD-mimetic Tempol. Downregulation of AMPKα2, but not α1, also caused ER stress as measured by an increase in GRP78 expression, also inhibited by Tempol. However, in contrast to the morphological changes and ER stress, the effect of AMPKα downregulation on ROS levels was observed in AMPKα1 and –α2 downregulated cells and was not

prevented by Tempol. Colombo and Moncada have recently shown that α1 AMPK is a regulator of the antioxidant status of endothelial cells (Kukidome, Nishikawa et al. 2006; Colombo and Moncada 2009). They observed that silencing of AMPKα1 in HUVEC caused a decrease in the expression of genes involved in antioxidant defences, including MnSOD, catalase and thioredoxin and an accumulation of ROS.

Regulation of antioxidant status by AMPK is also suggested by increased levels of antioxidant enzymes by agents such as metformin and AICAR which cause an increase in AMPK activity (Kukidome, Nishikawa et al. 2006). An increase in ROS levels could also result from an increase in production and Wang et al. found increased expression of various components of NADPH oxidase in AMPKα2 downregulated cells (Wang, Zhang et al. 2010). However, we did not observe any increase in the expression of gp91phox or p47phox in our experiments. More work is needed to clarify the role of AMPKα2 in maintaining endothelial monolayer integrity.

Since a fall in the cellular energy level is a well known consequence of events such as ischemia, hypoxia and infection (Kahn, Alquier et al. 2005), LKB1-dependent vasodilation via AMPK suggests an important role for LKB1-AMPK in causing a robust NO-production by activation of eNOS under pathological conditions. However, as discussed in the chapter on the limitations of this work, results from in vitro studies as those described in this thesis need ultimately be validated in vivo. A recent report on the activation of eNOS by AMPK in the mouse aorta is a strong testimony to the importance of AMPK as an important activator of eNOS in the vascular endothelium (Cacicedo, Gauthier et al. 2011). In their paper, Cacicedo and coworkers demonstrated that acute exercise increased the phosphorylation of eNOS at Ser1177 in the aorta in correlation with increased AMPK-phosphorylation whereas the phosphorylation of Akt was lowered. The results showed activation of both upstream kinases, LKB1 and CaMKKβ. In their discussion, Cacicedo and coworkers propose that the AMPK-eNOS pathway may be a critical component of the signaling events required to increase blood flow in a physically active tissue. Also, they suggest that this activation of eNOS and the NO release from the endothelium may counterbalance the hypercoagulant state caused by aerobic-type exercise associated with increases in platelet count, plasma catecholamines, thromboxane A2 and endothelin-1 release. Although more complex and involving more factors, this is a state somewhat reminiscent of the thrombin-endothelial interaction in our system.

4.4 Limitations

The introduction of a method to study isolated endothelial cells *in vitro* marked the beginning of an era during which our knowledge of endothelial biology has been revolutionized. However, as Aird pointed out, as one can "never predict the behavior of an ant colony by studying an individual ant in isolation, one cannot rely solely on isolated endothelial cells to fully understand the endothelium in health and disease" (Aird 2005). He compared the endothelium to a chameleon, "marching to the tune of the local microenvironment", emphasizing the capacity of this tissue to adapt to its extracellular milieu. The consequent phenotypic drift of the endothelial cells taking place when transferred from its native environment must be kept in mind when interpreting results from *in vitro* studies and should ultimately be validated *in vivo.* Also, the extracellular environment of various types of endothelial cells are different between different organs and even within the same organ and are very different from the artificial circumstances found in most *in vitro* models. This is unquestionably the main limitation of our work that aims at enhancing our understanding of the physiological role of the vascular endothelium. In particular, in our work and that of others using stationary cultures of isolated endothelial cells, important hemodynamic effects on the endothelium are not taken into account nor the interaction with other cell types.

When our work was initiated, the main method of studying the role of a specific protein in a given pathway was to use pharmacological inhibitors to prevent the activity of that protein. However, as mentioned before, these inhibitors are almost never fully specific and affect other intracellular proteins giving results that are hard to interpret without support from other results (Davies, Reddy et al. 2000). The question therefore had to be addressed from more than one side e.g. as was done in paper I when exploring the role of PKCδ in the inhibition of Akt, using the inhibitor rottlerin and monitoring the phosphorylation of the kinase indicative of its activity. Therefore, the use of siRNA was a great step forward in inhibiting a specific protein although it must be kept in mind that inhibiting the activity of a protein is different from preventing the synthesis of the protein since it can still remain as a scaffold for other protein(s) inside the cell although inactive when inhibited by a pharmacological inhibitor, unlike when downregulated with siRNA.

The measurement of eNOS activity using the conversion of arginine to citrullin as an indicator is not as reliable as measuring the synthesis of NO directly. The available amount of NO in the cell is not clearly indicated by this measurement since produced NO can react with free O_2^- within the cell to form $ONOO^-$ under certain circumstances (Forstermann 2005).

5 General discussion

The vascular endothelium, the monolayer that forms the inner lining of blood vessels is, because of its unique location, constantly exposed to a variety of stimulatory interactions, physical, humoral and cellular. The signaling mechanisms involved in linking exposure to endothelial response have in recent years attracted considerable attention. The transduction of these signals is critical for an appropriate cellular response while derailment of the signaling can be a key feature of a pathologic response or disease. Endothelial cells play an important role in controlling vascular tone, inflammation and cell proliferation within the vascular wall. By secretion or expression of surface molecules the endothelium ensures appropriate regulation of blood flow, counteracting intravascular activation of platelets and coagulation. Under pathological conditions a critical change in phenotype can occur. A vascular surface that normally is thromboresistant, anti-inflammatory, vasodilatory and anti-proliferative can turn into a surface that is thrombogenic, pro-inflammatory, vasoconstrictive and stimulatory of smooth muscle cell proliferation. Often this change is transient and reactive and may contribute to successful restoration of vascular homeostasis. However, in certain disease states such as atherosclerosis, hypertension and diabetes such changes may involve a chronically perturbed vascular behaviour critical for disease progression (Yang and Ming 2006). One of the crucial responses of the vascular endothelium to a variety of signals is the production of nitric oxide (NO) from arginine through the activation of endothelial NO-synthase (eNOS) (Li and Forstermann 2000).

The work presented in this thesis aimed at exploring in depth the effects of the G-protein agonists thrombin and histamine on vascular endothelial cells (HUVEC) with emphasis on the regulation of eNOS. A critical requirement for eNOS activation is phosphorylation at the most thoroughly studied site, Ser1177 (Sessa 2004). This site is phosphorylated by most if not all factors activating the enzyme (Mount, Kemp et al. 2007). At the beginning of our work, the best characterized pathway mediating this phosphorylation of eNOS after activation was the PI3K-Akt pathway (Fulton, Gratton et al. 2001; Fleming and Busse 2003). With our studies on the effects of thrombin and histamine, we were able to describe another pathway that had not been prevously described in which AMPK is an important link in mediating

phosphorylation and activation of eNOS after treatment with these agonists (paper II, paper III). At this time, AMPK was well known as a „metabolic masterswitch“ in tissues such as the skeletal and cardiac muscle while its precise role in endothelial cell metabolisms was less well understood (Fisslthaler and Fleming 2009). The overall goal of this thesis was thus accomplished, although with a number of new questions arising from new findings, clearly not finalised. Furthermore, the specific aims for each step on the way were radically modified with the new and unexpected findings as is discussed more thoroughly below. The most influential of these findings for the continuing progress of the work were that thrombin/histamine mediated activation of eNOS was independent of PI3K-Akt but dependent on AMPK under conditions that allowed or facilitated a fall in cellular ATP upon stimulation. Also, the differential role of the AMPKkinases LKB1 and CaMKK, dependent on culture conditions.

The aim of paper I was to describe the possible role of Akt in eNOS phosphorylation and activation after treatment of cells with thrombin or histamine. It had previously been demonstrated in our laboratory that histamine caused production of cGMP, an indicator of eNOS activation (Jonsson, Halldorsson et al. 1998). We showed that while activating eNOS, the basal phosphorylation of Akt as well as EGF- and S1P-mediated phosphorylation of Akt was inhibited by these agonists. This phosphorylation was also prevented by pretreatment with LPC. We demonstrated a role for the δ-isoform of PKC in the inhibition caused by thrombin and histamine whereas PKCδ played no role in the inhibition by LPC. Our unexpected results presented in paper I on the inhibition of Akt after treatment with thrombin or histamine and the wortmannin-insensitive phosphorylation of eNOS caused by these agonists led to the search of the kinase mediating this pathway. This became the aim of paper II.

At this time, AMPK was known to cause phosphorylation of eNOS at Ser1177 in endothelial cells (Chen, Mitchelhill et al. 1999; Morrow, Foufelle et al. 2003) but there was no agonist known to cause AMPK-mediated eNOS activation independent of PI3K-Akt. We demonstrated in HUVEC that histamine and thrombin caused phosphorylation of AMPK. We suggested a possible role for AMPK in mediating eNOS phosphorylation after treatment with these agonists by demonstrating inhibitory effects of H89, an inhibitor of PKA and AMPK although with a limited specificity. We supported these results by showing phosphorylation of both ACC (known to be downstream of AMPK) and eNOS after treatment with AICAR, a known activator of AMPK. When we published these results in 2004 (paper II), the only kinase known to activate AMPK in mammals was LKB1. We

therefore hypothesized in paper II that thrombin and histamine caused LKB1-dependent activation of AMPK, presumably preceded by a rise in AMP, caused by Ca^{+2}-mediated energy consuming events previously suggested to be activated by these agonists. In this paper, we demonstrated the Ca^{+2}-dependency of these events with the inhibition caused the Ca^{+2}-chelator BAPTA on the thrombin- and histamine-mediated phosphorylation of AMPK, ACC and eNOS as well as eNOS activation measured by the formation of citrullin. In 2005, another upstream kinase was shown to activate AMPK in mammals, the Ca^{+2}/calmoduline kinase kinase (CaMKK) (Hawley, Pan et al. 2005; Hurley, Anderson et al. 2005; Woods, Dickerson et al. 2005). A year later, Stahmann and coworkers published their results on thrombin-mediated phosphorylation and activation of AMPK in HUVEC where they found CaMKK to be the only kinase upstream of AMPK (Stahmann, Woods et al. 2006). They found no significant change in the AMP/ATP ratio upon thrombin stimulation and downregulation of LKB1 had no effect on thrombin-induced AMPK activation. Also, in contrast to our results in paper II, they demonstrated AMPK-independent phosphorylation of eNOS after thrombin. We therefore explored the role of LKB1 in our system as well as the possible changes in the AMP/ATP ratio after treatment of HUVEC with thrombin. This became the aim of papers III and IV.

In paper III we showed a role for CaMKK in the phosphorylation of AMPK after treatment with thrombin with partial prevention of this phosphorylation with the CaMKK-inhibitor STO-609. We also confirmed, using siRNA, AMPK-dependent phosphorylation and activation of eNOS after thrombin. In paper IV, using siRNA, we also demonstrated a role for LKB1 in the phosphorylation of AMPK and the involvement of LKB1-AMPK in the phosphorylation and activation of eNOS, quite different from the results of Stahmann and coworkers (Stahmann, Woods et al. 2006). The most notable aspect of this work was the discovery that this particular pathway, LKB1-AMPK-eNOS, was only activated under culture conditions that allowed a sharp but brief fall in cellular ATP after thrombin stimulation (medium 199). Under culture conditions that prevented or did not allow such a fall in the energy level of the endothelial cells (medium 1640) AMPK was still activated but exclusively via a different upstream pathway (CaMKK-mediated activation of AMPK) and downstream this AMPK-activation played no role in the activation of eNOS, identical to that seen by Stahmann and coworkers (Stahmann, Woods et al. 2006). The kinase responsible for that phosphorylation is still unknown although we have excluded several kinases.

The results showing that environmental/culture conditions dictate which signal transduction pathways are activated by thrombin or histamine stimulation suggested that a constituent of medium 199 allowed or facilitated

the drop in ATP levels in response to thrombin or histamine. We therefore examined several possibilities that could explain the different responses to stimulation in the different media (paper III). Treatment of medium 199 with 1 U apyrase markedly reduced the ionophore-mediated lowering of cellular ATP (the ionophore, A23187, mimicked the effects of thrombin on ATP levels in different media). Conversely, after adding tween 80 to medium 1640 in the amounts that are present in medium 199, subsequent treatment with ionophore lowered intracellular ATP significantly and similar activation of downstream pathways was demonstrated after thrombin treatment (AMPK and eNOS phosphorylation) as in cells stimulated in medium 199. The explanation for these effects has not yet been found but our recent experiments have suggested involvement of caveolae as treatment with the cholesterol depleating agent methyl-β-cyclodextrin causes ATP-lowering after A23187 treatment in medium 1640 and increases the response seen in medium 199 (unpublished results). It remains to be seen if this treatment increases the eNOS response and if this contributes to the increased NO-production caused by statin treatment as has been demonstrated (Martinez-Gonzalez and Badimon 2007).

Another important finding of paper IV was the effect of AMPK downregulation on the integrity of the endothelial monolayer. It has been demonstrated that AMPK has an important function in maintaining intracellular homeostasis independent of energy status by responding to various stress challenges and cellular damage (Alexander and Walker 2011). AMPK has been shown to regulate ROS/redox balance, autophagy, cell proliferation, cell apoptosis, cellular polarity, mitochondrial function and genotoxic response by numerous downstream pathways (Wang, Song et al. 2012). In paper IV, we demostrated that downregulation of this isoform for 67 hours caused deterioration of monolayer integrity, with shrinkage of cells and loss of contact both between cells and with the culture substrate when the cells were transferred to serum free media for up to 30 minutes. Cells lacking the α1-isoform showed no morphological changes nor did control cells or cells with downregulated LKB1. The SOD-mimetic Tempol markedly inhibited these morphological changes, suggesting a role for AMPKα2 in the regulation of antioxidant status as previously demonstrated by Kukidome and coworkers (Kukidome, Nishikawa et al. 2006). The expression of the chaperone GRP78, an indicator of ER stress (Ron and Walter 2007), was also greatly enhanced after downregulation of AMPKα2 whereas downregulation of AMPKα1 had no effect. In contrast to the morphological changes and ER stress, the level of reactive oxygen species (ROS) was elevated in both AMPKα1 and AMPKα2

downregulated cells and this increase was not affected by Tempol. We found no increase in the expression of $gp91^{phox}$ or $p47^{phox}$ subunits of NADPH oxidase leaving the reason for the increase in ROS levels unexplained. More work is needed to clarify the role of AMPKα2 in maintaining endothelial monolayer integrity.

The work described in this thesis clarifies the effects of the G-protein agonists histamine and thrombin on the phosphorylation and activation of eNOS in HUVEC. We confirm a pathway dependent on CaMKK causing activation of eNOS under culture conditions where intracellular ATP is unchanged without the involvement of (LKB1-)AMPK, as described by others (Stahmann, Woods et al. 2006). However, we also describe a previously unknown pathway, activated by a rise in intracellular AMP/ATP ratio, causing LKB1-AMPK-dependent phosphorylation and activation of eNOS by these agonists. Our fundamental discovery that environmental condions dictate which pathway is activated is interesting but even more so the key role played by the ATP-level, a fall in the cellular energy level being a well known consequence of pathologic conditions such as ischemia, hypoxia and infection. Although thrombin can activate eNOS through an AMPK-independent pathway the activation of the LKB1-AMPK pathway greatly enhances the NO-production and may therefore be of major importance for endothelial function and endothelial health.

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Appendix I

RPMI Medium 1640, with L-Glutamine / Formulation

COMPONENTS	Conc. (mg/L)
INORGANIC SALTS	
Calcium nitrate (Ca(NO3)2 4H2O)	100.00
Potassium chloride (KCl)	400.00
Magnesium sulfate (MgSO4)	48.84
Sodium chloride (NaCl)	6000.00
Sodium bicarbonate (NaHCO3)	2000.00
Sodium Phosphate (Na2HPO4)	800.85
OTHER COMPONENTS	
Glucose	2000.00
Glutathione Reduced	1.00
Phenol red	5.00
AMINO ACIDS	
L-Arginine	200.00
L-Asparagine-H20	50.00
L-Aspartic Acid	20.00
L-Cystine	50.00
L-Glutamic Acid	20.00
L-Glutamine	300.00
Glycine	10.00
L-Histidine	15.00
L-Hydroxyproline	20.00
L-Isoleucine	50.00
L-Leucine	50.00
L-Lysine hydrochloride	40.00
L-Methionine	15.00
L-Phenylalanine	15.00
L-Proline	20.00
L-Serine	30.00
L-Threonine	20.00
L-Tryptophan	5.00
L-Tyrosine	20.00
L-Valine	20.00
VITAMINS	
Biotin	0.20
D-Ca Pantothenate	0.25
Choline Chloride	3.00
Folic Acid	1.00
i-Inositol	35.00
Niacinamide	1.00
p-Aminobenzoic Acid (PABA)	1.00
Pyridoxine HCl	1.00
Riboflavin	0.20
Thiamine HCl	1.00
Vitamin B12	0.005

Medium M-199 Earle`s Salts Base / Formulation

COMPONENTS	Concentration (mg/L)
INORGANIC SALTS	
Calcium chloride (CaCl2)(anhyd.)	200.00
Ferric nitrate (Fe(NO3)3-9H2O)	0.72
Potassium chloride (KCl)	400.00
Magnesium sulfate (MgSO4)	97.67
Sodium chloride (NaCl)	6800.00
Sodium bicarbonate (NaHCO3)	2200.00
Sodium phosphate, mono. (NaH2PO4-H2O)	140.00
OTHER COMPOUNDS	
Adenine sulfate	10.00
Adenosine 5'-triphosphate,disodium salt	1.00
Adenylic Acid	0.20
Cholesterol	0.20
Deoxyribose	0.50
D-Glucose	1000.00
Glutathione (reduced)	0.05
Guanine hydrochloride	0.30
Hypoxanthine-Na	0.40
Phenol red	20.00
Ribose	0.50
Sodium acetate	50.00
Thymine (5-Methyluracil)	0.30
Tween 80	20.00
Uracil	0.30
Xanthine-Na	0.34
AMINO ACIDS	
L-Alanine	25.00
L-Arginine hydrochloride	70.00
L-Aspartic acid	30.00
L-Cysteine HCl-H2O	0.11
L-Cystine	20.00
L-Glutamic Acid	75.00
L-Glutamine	100.00
Glycine	50.00
L-Histidine HCl H2O	21.88
L-Hydroxyproline	10.00
L-Isoleucine	20.00
L-Leucine	60.00
L-Lysine hydrochloride	70.00
L-Methionine	15.00
L-Phenylalanine	25.00
L-Proline	40.00
L-Serine	25.00
L-Threonine	30.00
L-Tryptophan	10.00
L-Tyrosine	40.00
L-Valine	25.00
VITAMINS	
Ascorbic acid	0.050
alpha-Tocopherol Phosphate	0.010
d-Biotin	0.010
Calciferol (Vitamin D2)	0.100
D-Calcium pantothenate	0.010

Choline chloride	0.500
Folic acid	0.010
i-Inositol	0.050
Menadione (Vitamin K3)	0.010
Niacin	0.025
Niacinamide	0.025
Para-aminobenzoic acid	0.050
Pyridoxal hydrochloride	0.025
Pyridoxine hydrochloride	0.025
Riboflavin	0.010
Thiamine hydrochloride	0.010
Vitamin A (acetate)	0.100

Original publications

Paper I

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Inhibition of Akt phosphorylation by thrombin, histamine and lysophosphatidylcholine in endothelial cells Differential role of protein kinase C

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Abstract

The protein kinase Akt is involved in embryonic vascular development and neoangiogenesis as well as in several endothelial cell functions, including activation of endothelial NO-synthase (eNOS) and promotion of endothelial cell survival. We have examined the effects of G-protein activators thrombin and histamine as well as lysophosphatidylcholine (LPC) on Akt phosphorylation in cultured human umbilical vein endothelial cells (HUVEC). Akt phosphorylation was analyzed with the phosphospecific Akt (Ser473) antibody by Western blotting. While epidermal growth factor (EGF) was a potent stimulator of Akt phosphorylation histamine, thrombin and LPC blocked its activation when used in cotreatment with EGF. Following inhibition or downregulation of protein kinase C (PKC), the inhibitory effect of both histamine and thrombin on the endothelial response to EGF was prevented. Furthermore, stimulation of PKC, using short-term 12-*O*-tetradecanoylphorbol-13-acetate (TPA) treatment, markedly inhibited the stimulatory effects of EGF on Akt phosphorylation. Rottlerin, an inhibitor of the PKCδ, but not Gö6976, which is an inhibitor of α, β, γ and isoforms, reversed the inhibitory effects of histamine. Conversely, inhibition or downregulation of PKC did not prevent the inhibitory effect of LPC. Akt phosphorylation was also increased by sphingosine 1-phosphate (S1P) treatment and this activity was influenced by the various cotreatments in the same way as the activation by EGF. Overall, this study demonstrated that the G-protein activators thrombin and histamine inhibited both EGF- and S1P-mediated Akt phosphorylation in HUVEC by activation of PKCδ, while the inhibitory effects of LPC were independent of PKCδ.

Keywords: Akt; Protein kinase C; Lysophosphatidylcholine; Sphingosine 1-phosphate; Endothelial cells

1. Introduction

The serine/threonine protein kinase Akt, also known as protein kinase B, is a downstream effector of phosphoinositide 3-kinase (PI3K)-dependent signaling pathways [1,2]. It is involved in embryonic vascular development and neoangiogenesis [3] as well as in several endothelial cell functions, including activation of endothelial NO-synthase (eNOS) [4,5] and promotion of endothelial cell survival [6]. Numerous growth factors, including epidermal growth factor (EGF), vascular endothelial growth factor (VEGF), insulin and hepatocyte growth factor as well as shear stress are potent activators of Akt [1]. In some tissues, G-protein activators also have been shown to activate Akt [7–9].

Recently, the bioactive lipid sphingosine 1-phosphate (S1P) which is a ligand for several of the endothelial differentiation gene (EDG) family of G-protein-coupled

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receptors and promotes endothelial cell survival, proliferation and migration [10], was found to activate Akt and NO-production in endothelial cells [11,12]. S1P is concentrated in both HDL and LDL in human plasma [13]. Kimura and co-workers have shown that oxidation of LDL results in marked reduction in S1P level in LDL, while lysophosphatidylcholine (LPC) concurrently increases. Furthermore, S1P, HDL and native LDL had cytoprotective effects on both serum-deprived endothelial cells in culture and endothelial cells exposed to oxidized LDL (ox-LDL). The protective effect of HDL was markedly reduced after removal of S1P by charcoal treatment. These results prompted the suggestion that S1P is accountable for the cytoprotective effects of HDL and native LDL and that a decrease in S1P along with accumulation of LPC contribute importantly to the cytotoxic effects of ox-LDL [14]. The inhibitory effects of ox-LDL on endothelial cell migration have been shown to be mediated by dephosphorylation of Akt [15].

Inhibition of Akt phosphorylation by thromboxane A2 was recently demonstrated in endothelial cells in association with induction of apoptosis and reversal of angiogenesis [16]. Furthermore, TNFα counteracts insulin-induced phosphorylation of Akt in endothelial cells [17]. 12-*O*-tetradecanoylphorbol-13-acetate (TPA) treatment of insulin-stimulated 3T3-LI cells [18], IGF-stimulated PC12 cells [19] as well as ceramide-induced apoptosis in several cell types have been related to Akt inhibition [20,21].

Here, we report on the inhibitory effects of thrombin, histamine and LPC on both EGF and S1P-mediated stimulation of Akt in endothelial cells and explore the role of protein kinase C (PKC) in mediating the inhibitory effects.

2. Materials and methods

2.1. Materials

Morgan's medium 199, Foetal Bovine Serum and Penicillin–Streptomycin were purchased from Gibco Brl, Life Technologies. Tissue culture plates (35 mm) were from Nunc, Cryotin X (collagenase) was provided by The Science Institute of Iceland. EGF, TPA, histamine, thrombin, wortmannin (Wm), insulin and LPC were purchased from Sigma. S1P, rottlerin, Gö6976, PD98059, SB203580 and GF109203X were from Biomol. KN-62 and Y27632 were from Calbiochem. Myo-[^{3}H]inositol, Hybond ECL Nitrocellulose membrane (6 cm × 8 cm), ECL+PLUS Western blotting detection system and Hyperfilm ECL High performance chemiluminescence film were from Amersham Pharmacia Bioteck. Antibodies against Akt, Phospho-Akt (Ser473), phospho-extracellular-signal-regulated kinase (ERK1/2), phospho-eNOS, phospho-PKCδ, phospho-AMPK and Anti-Rabbit IgG/HRP-linked came from Cell Signaling Technology. eNOS antibody came from Transduction Laboratories. Anion-exchange resin (AG 1-x8) was from Biorad.

2.2. Endothelial cell culture

Endothelial cells were cultured from human umbilical veins by a modification of the method of Jaffe et al. as previously reported [22]. The cells were harvested by 0.1% collagenase digestion and seeded on 35 mm culture dishes in medium 199 containing 20% foetal bovine serum and antibiotics (penicillin, 100 Units/ml and streptomycin, 100 μg/ml). The culture dishes were incubated at 37 °C in humidified air with 5% CO_2. The medium was changed 24 h after seeding the cells and every 2–3 days thereafter until the cell culture reached confluence (after ∼7 days).

When confluent the cells were washed with Morgan's medium 199 and then placed in 0.9 ml Morgan's medium 199 with or without inhibitors at the indicated concentrations. Ten minutes later 0.1 ml of agonist was added to reach the intended concentration and left on for additional 1–30 min. The agonists were then removed along with the medium and cellular reactions terminated by adding 250 μl SDS sample buffer. The samples were boiled for 5 min and centrifuged for 10 min at 3000 rpm. After that the samples were ready to be used or could be stored at −20 °C.

2.3. Electrophoresis and immunoblotting

Samples (8 μl) were resolved by SDS-PAGE (10%). The gels were blotted and the proteins thereby transferred to nitrocellulose. The membranes were hybridized with the indicated antibodies and later with a secondary antibody (Anti-Rabbit IgG/HRP-linked). The immunocomplexes were detected with ECL+PLUS Western blotting detection system and developed onto a film.

2.4. Formation of inositol phosphates

Confluent cell cultures were incubated for 24 h in 1 ml of Morgan's medium 199 containing 20% fetal calf serum, antibiotics, and 3 μCi of myo-[^{3}H]inositol per milliliter. Before the experiments were carried out, the cells were washed with medium containing 20 mmol/l LiCl. The experiments were performed in 1 ml of this solution with agonists and/or inhibitors at the indicated concentrations. After 20 min, the medium was removed and 1 ml of ice-cold TCA was added to terminate reactions. IPs were separated on columns of anion-exchange resin and quantified by liquid scintillation counting [23].

3. Results

3.1. Effects of EGF and S1P on Akt (Ser473) phosphorylation

We first characterized the times and doses needed for optimal activation of Akt in primary cultures of human umbilical vein endothelial cells (HUVEC). The results for EGF-mediated phosphorylation of Akt are shown in Fig. 1A. A dose of 1.0 ng/ml caused a clear activation and at 10 ng/ml there was a robust response which peaked at 5 min and was down to control level at 15 min (Fig. 1B).

Fig. 1C shows that S1P (1 μM) caused a similar response as EGF (2 ng/ml), while insulin at 1 μM caused a much less activation. We also characterized the time and dose–response to S1P (results not shown). Maximal response was seen after 5 min of 1 μM S1P.

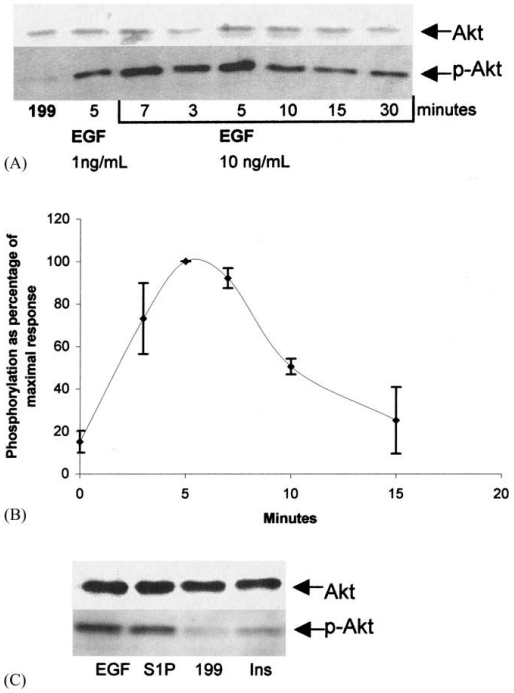

Fig. 1. Phosphorylation of Akt after agonist treatment of HUVEC. (A) Time- and dose-dependent effect of EGF on Akt phosphorylation. (B) Optical density measurements of the Western blots. The data are expressed as percentage of maximal phosphorylation and are the means ± S.D. of three separate experiments. (C) Effects of EGF (2 ng/ml, 5 min), S1P (1 μM, 5 min) and insulin (1 μM, 5 min). Confluent endothelial cells were stimulated as indicated, lysates were electrophorized and blotted as described in Section 2 and detected using antibody against Akt and the Ser473 phosphorylated Akt. Each Western blot is representative of three independent experiments.

3.2. Effects of thrombin, histamine and LPC on EGF-stimulated Akt phosphorylation

Since the effects of S1P are mediated through G-protein-coupled receptors, and thrombin and histamine are known to act on G-protein-coupled receptors in HUVEC, activating phospholipase C (PLC), PLA_2 and PKC, we tested the effects of these agonists on the phosphorylation of Akt. Neither thrombin nor histamine caused any phosphorylation at several doses and times up to 30 min (data not shown) and even caused a decrease in Akt phosphorylation compared with untreated cells. We, therefore, tested the effect of thrombin and histamine pretreatment on EGF-induced activation of Akt. As shown in Fig. 2A, both thrombin and histamine totally blocked EGF-induced activation while the mass of Akt in the cells was constant.

As thrombin and histamine activate various signaling pathways in HUVEC, we next investigated the effect of several signaling molecules generated by these pathways on EGF-mediated Akt phosphorylation. As shown in

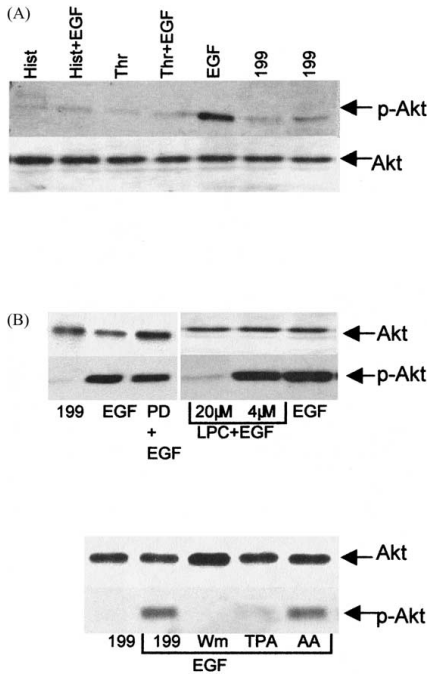

Fig. 2. Inhibition of EGF-mediated (2 ng/ml) Akt phosphorylation. (A) Effect of a 10 min pretreatment with thrombin (1 U/ml) or histamine (11 μM) on Akt phosphorylation. (B) Effect of a 10 min pretreatment with PD98059 (20 μM), LPC (4 and 20 μM), Wm (50 nM), TPA (100 ng/ml) or AA (20 μM). Samples were separated by SDS-PAGE and analyzed by Western blotting. Each Western blot is representative of three independent experiments.

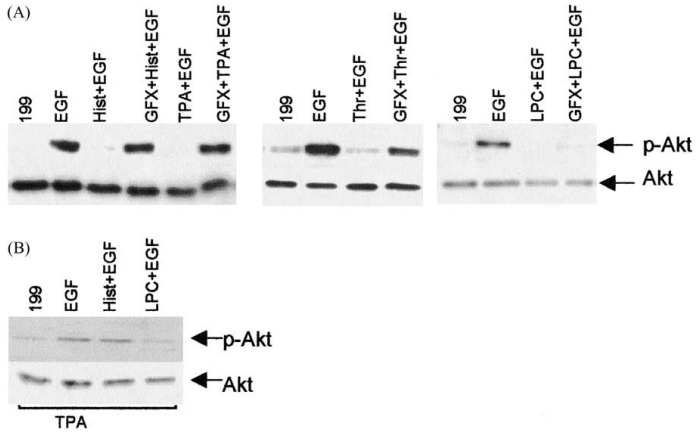

Fig. 3. Involvement of PKC in the inhibition of EGF-mediated Akt phosphorylation. (A) PKC inhibition using GF109203X. Cells were treated with GF109203X (1 μM) for 10 min before treatment with histamine (11 μM), thrombin (1 U/ml), TPA (100 ng/ml) or LPC (20 μM) as indicated. EGF (2 ng/ml) was added 10 min later and Akt phosphorylation determined after 5 min. (B) PKC downregulation using long-term TPA treatment. Cells were treated for 21 h with TPA (200 ng/ml) before treatment with histamine, LPC and EGF as in A. Samples were separated by SDS-PAGE and analyzed by Western blotting. Each Western blot is representative of three independent experiments.

Fig. 2B arachidonic acid (AA) at 20 μM had no effect whereas LPC, the other product of PLA_2 activation, caused some inhibition at 4 μM and total inhibition at 20 μM. Direct stimulation of PKC with TPA (100 ng/ml) also caused a complete inhibition without affecting total Akt (Fig. 2B). PD98059 (20 μM), which totally inhibits ERK1/2 activation in HUVEC [22] had no effect either on EGF-mediated Akt activation (Fig. 2B) or its inhibition by thrombin or histamine (data not shown). As expected, treatment with PI3K inhibitor, Wm, totally inhibited phosphorylation of Akt.

3.3. Role of PKC in mediating the inhibitory effects of thrombin, histamine and LPC on EGF- or S1P-induced Akt phosphorylation

As shown in Fig. 3A, PKC inhibitor GF109203X prevented the inhibition of EGF-mediated Akt phosphorylation caused by histamine or thrombin, while there was no effect on the inhibition caused by LPC, suggesting different inhibitory mechanisms. GF109203X also prevented TPA-mediated inhibition of EGF response, demonstrating its PKC inhibition at the dose used. Differences in inhibitory mechanisms were further demonstrated by downregulation of PKC with long-term TPA treatment which totally abolished the histamine-mediated inhibition of Akt phosphorylation, whereas LPC-mediated inhibition remained unaltered (Fig. 3B).

We next investigated, whether histamine or LPC affected S1P-mediated Akt phosphorylation. As shown in Fig. 4, both compounds caused total inhibition. Therefore, we also tested the effects of PKC inhibition or downregulation. Again the histamine-mediated inhibition was abolished by both treatments, whereas the inhibition caused by LPC was unaffected (Fig. 4).

3.4. Effects of LPC on inositol phosphate formation and ERK activation

Both thrombin and histamine are potent activators of PLC in HUVEC [24] and this activation is inhibited by TPA. To further compare the endothelial effects of LPC to those of thrombin or histamine, we studied the effects of LPC on PLC activity. As shown in Table 1, LPC only caused a very slight activation of PLC but, like TPA, caused inhibition of histamine-mediated activation. However, unlike TPA effect, LPC-mediated inhibition was not PKC-dependent and was prevented neither by GF109203X pretreatment nor by downregulation of PKC.

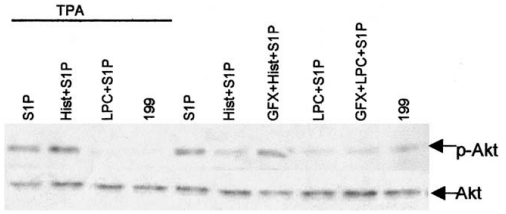

Fig. 4. Involvement of PKC in the inhibition of S1P-mediated Akt phosphorylation. Pretreatment times and dosages as in Fig. 3. S1P (1 μM) treatment time was 5 min. Samples were separated by SDS-PAGE and analyzed by Western blotting. Each Western blot is representative of three independent experiments.

Table 1
Inhibition of histamine-induced IP generation by LPC is independent of PKC

Treatment	Pretreatment		
	199	GF109203X	Long-term TPA
Histamine	100	100	100
LPC	7±2		
TPA+histamine	43±1	93±10	79±22
LPC+histamine	33±15	38±24	23±16

Prelabeled cells were washed and incubated for 10 min with or without GF109203X (2 μM). TPA (200 ng/ml) or LPC (20 μM) was then added as indicated. After additional 10-min histamine (11 μM) was added. After 20 min, the medium was removed and TCA added and the inositol phosphates quantitated. Long-term TPA treatment was used for PKC downregulation. Cells were treated for 24 h with TPA (200 ng/ml) before the experiment. The results are expressed as percentage of the response to histamine without TPA or LPC and are the means±S.D. of three experiments. The average count in inositol phosphates of untreated cells was 220 cpm and the histamine response was 803±71%.

We have previously shown that PKC causes ERK activation in HUVEC [22]. As LPC has been shown to activate PKC in several cell types [25–27], we tested whether LPC mimicked TPA-induced activation of ERK. As shown in Fig. 5, LPC, contrary to TPA, did not cause any ERK activation, indicating that LPC is not activating PKC.

3.5. PKCδ mediates the inhibition of Akt phosphorylation

In order to identify the isoform of PKC responsible for the inhibition of Akt phosphorylation, we used isoform-specific PKC inhibitors. As shown in Fig. 6, PKCδ inhibitor rottlerin reversed the inhibitory effect of histamine on EGF-stimulated Akt phosphorylation. Conversely, Gö6976 which is an inhibitor of α, β and γ isoforms had no effect. As expected rottlerin had no effect on LPC-mediated inhibition.

To further examine the role of PKCδ in the inhibition of Akt phosphorylation, an antibody against phosphorylated PKCδ (Thr505) was used. As shown in Fig. 6B, both histamine and thrombin caused PKCδ phosphorylation, whereas EGF or LPC had no effect.

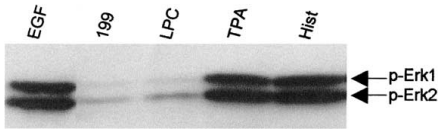

Fig. 5. LPC does not activate ERK. Cells were treated for 5 min with EGF (2 ng/ml), LPC (20 μM), TPA (200 ng/ml) or histamine (11 μM) and phosphorylation of ERK1/2 determined by electrophoresis and Western blotting using an ERK1/2 (Ser202/Tyr204) phosphorylation-specific antibody as described in Section 2. The results shown are representative of three independent experiments.

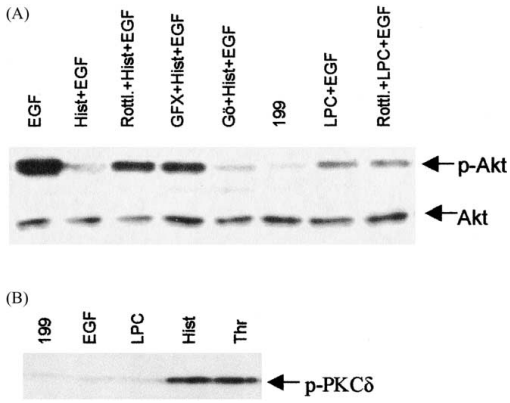

Fig. 6. Effects of isoform-specific PKC inhibitors on histamine and LPC-mediated Akt inhibition. (A) Cells were treated with the general PKC inhibitor GF109203X (2 μM), PKCδ-specific inhibitor rottlerin (10 μM) or the conventional isoform-specific inhibitor Gö6976 (10 μM) for 10 min before treatment with histamine (11 μM) or LPC (20 μM) as indicated. EGF (2 ng/ml) was added 10 min later and Akt phosphorylation determined after 5 min. (B) Effects of EGF (2 ng/ml), LPC (20 μM), histamine (11 μM) or thrombin (1 U/ml) on PKCδ phosphorylation. Samples were separated by SDS-PAGE and analyzed by Western blotting using total Akt, Akt Ser473 phosphorylation or PKCδ Thr505 phosphorylation-specific antibody as described in Section 2 and are representative of three independent experiments.

3.6. Effects on eNOS phosphorylation

After investigating the effects of various agonists on Akt phosphorylation, their downstream effect on eNOS was examined. EGF treatment caused phosphorylation of eNOS on Ser1179 as expected (Fig. 7A), as did S1P (data not shown). The maximum effects of EGF were seen after 3 min and decreased after that.

In contrast to its inhibition of Akt phosphorylation, histamine caused phosphorylation of Ser1179 on eNOS (Fig. 7B), as did thrombin (data not shown). The maximum effects were seen after only 1 min. These effects of histamine and thrombin were not affected by Wm (not shown), GF109203X, PD98059, LPC, the p38 inhibitor SB203580, the Rho-dependent protein kinase inhibitor Y27632 or the calmodulin kinase II inhibitor KN-62 (Fig. 7C). Conversely, Wm as well as LPC totally blocked the stimulatory effects of EGF on eNOS (Fig. 7D).

4. Discussion

In this article, we demonstrate that the G-protein activators thrombin and histamine as well as LPC

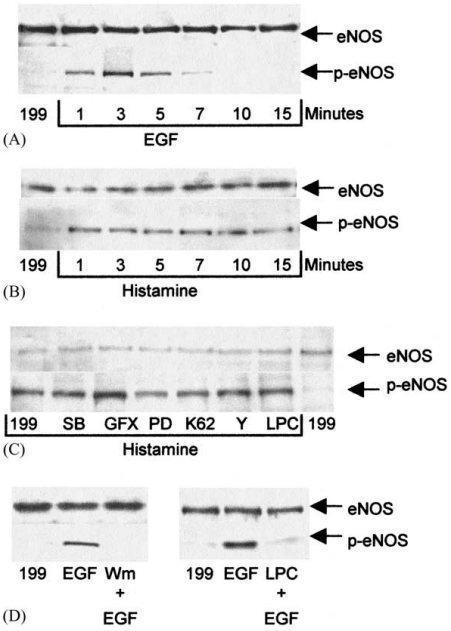

Fig. 7. Phosphorylation of eNOS. (A) Time course for EGF-mediated (10 ng/ml) phosphorylation of eNOS at Ser1179. (B) Time course for histamine-induced (11 μM) eNOS phosphorylation at Ser1179. (C) Effect of a 10 min pretreatment with SB203580 (10 μM), GF109203X (2 μM), PD98059 (20 μM), KN-62 (10 μM), Y27632 (10 μM) or LPC (20 μM) on histamine-mediated eNOS phosphorylation. (D) Effect of a 10 min pretreatment with Wm (50 nM) or LPC (20 μM) on EGF-mediated eNOS phosphorylation. Samples were separated by SDS-PAGE and analyzed by Western blotting using total eNOS and eNOS Ser1179 phosphorylation-specific antibody as described in Section 2 and are representative of three independent experiments.

inhibit both EGF- and S1P-mediated Akt phosphorylation in HUVEC. Furthermore, our results demonstrate the differential mechanisms of Akt inhibition. Thrombin and histamine inhibited Akt phosphorylation by activation of PKCδ, while the inhibitory effects of LPC were PKC-independent. We also show that direct stimulation of PKC by short-term TPA treatment inhibits EGF-mediated Akt phosphorylation.

Previously, agonist-induced inhibition of Akt in endothelial cells was reported by Gao et al. [16]. They found that the thromboxane A_2 mimetic IBOP-induced apoptosis of cultured HUVEC and reversed in vitro tube formation, at least partly through inhibition of Akt activation. Such inhibition has also been reported after treatment of endothelial cells with TNFα [17] and ox-LDL [15]. Increased intracellular ceramide generation has been demonstrated after both these treatments and numerous investigators have found that the ceramide decreases Akt phosphorylation. Several potential mechanisms have been discovered, including activation of protein phosphatase A2-like activity [28], activation of PKCζ [29] as well as direct effects of ceramide, disrupting interaction between lipids and the PH-domains [30].

A flurry of reports has in recent years identified activated Akt as a survival signal to protect various types of cells from apoptosis induced by a variety of stresses [1]. In the vasculature, the pathophysiologic relevance is highlighted by the stimulatory effects of Akt on eNOS activity [4,5], critical for endothelial function and health, and the stimulatory effects of statins on Akt, eNOS activity and neovascularization [31].

Recently, it was demonstrated that S1P is an activator of Akt [11,12] and possibly accountable for the cyto-protective effects of HDL and native LDL but only present in markedly reduced amounts in ox-LDL [14]. S1P is a polar sphingolipid metabolite, which has been proposed to act both as an extracellular mediator and as an intracellular second messenger [10]. It is stored and released from platelets and many other cell types, and affects such diverse biological processes as cell growth, differentiation, migration and apoptosis [10]. The effects of S1P on Akt and eNOS phosphorylation are mediated by transactivation of VEGF receptor [32].

Our demonstration that LPC inhibits S1P-induced phosphorylation of Akt in endothelial cells provides an added insight into the potential mechanisms of the cytotoxic effects of ox-LDL which for years has been recognized as a key player in atherogenesis. While oxidation of LDL produces numerous compounds in addition to LPC that may play a role in vascular toxicity and atherosclerosis [33,34], the dose-dependent impairment of arterial relaxation is mimicked by LPC [35,36]. In atherosclerosis, ox-LDL accumulates in the arterial wall where it is cytotoxic and chemotactic for monocytes fueling the inflammatory component of atherogenesis [37,38]. Stimulation of PKC has repeatedly been shown to impair endothelium-dependent arterial relaxation and has been proposed as a major mechanism explaining vascular dysfunction in diabetes [39] as well as that imposed by ox-LDL [40,41].

There are numerous potential mechanisms by which PKC could affect endothelial function and integrity. Our demonstration that PKC mediates Akt inhibition in endothelial cells and that the G-protein agonists thrombin and histamine inhibit Akt by their stimulation of PKC provides another potential mechanism for consideration in relation to PKC-mediated endothelial dysfunction in general and specifically vascular stress mediated by stimulation of G-protein linked receptors.

Bommakanti et al. [42] have shown in HEK-293 and COS-7 cells transiently transfected by G-protein subunits that G-proteins can regulate Akt through two opposing mechanisms: activation by Gβγ subunits and inhibition by Gαq. Our findings with different G-protein

activators are in good agreement with their model. Thrombin and histamine both cause pertussis toxin-resistant PLC activation which is Gαq mediated [43]. Conversely, S1P induces PI3K activation through EDG receptor via Gβγ subunits [10,12].

PKC is a family of at least 11 isoforms divided into three classes according to sequence homology and mode of activation [44]. Primary cultures of HUVEC express five PKC isoforms: α, δ, ε, θ and ξ [45,46]. All these isoforms apart from ξ are activated by DAG and downregulated by long-term TPA treatment. Haller et al. [45] studied the effect of TPA and thrombin on PKC isoforms in HUVEC and found that both agents increased the immunoreactivity of isoforms α and δ. Despite the lack of absolute specificity [47] our results, showing no effect of the conventional PKC inhibitor Gö6976 but a reversal of histamine-mediated inhibition of Akt phosphorylation by PKCδ-specific inhibitor, rottlerin, strongly suggest that δ isoform of PKC is responsible for the inhibition. Furthermore, we show that histamine and thrombin stimulate phosphorylation of PKCδ, whereas LPC or EGF has no such effect. Although phosphorylation of PKCδ is not required for activity, it greatly increases signal strength [48]. PKCδ has previously been shown to induce growth arrest [49,50] or apoptosis [51,52] in cultured cells and recently it was shown to mediate VEGF-resistant apoptosis in sinusoidal endothelial cells [53].

LPC is a major phospholipid component of ox-LDL and βVLDL [54,55]. It is increased in both atherosclerotic and inflammatory lesions and it has been shown to selectively upregulate VCAM-1, ICAM-1 [56] and growth factor gene expression [57] in cultured endothelial cells and suppresses endothelium-dependent vasorelaxation [58]. It has thus emerged as an important mediator modulating endothelial function at all stages of atherogenesis. The data presented here establish that LPC also inhibits the activation of Akt, whether mediated by EGF or S1P, possibly adding importantly to its list of detrimental vascular effects.

Although LPC has been shown to activate PKC [25,26], this is not its mechanism of action in our experiments. Firstly, inhibition or downregulation of PKC does not prevent LPC effect despite totally blocking the inhibition of thrombin, histamine or TPA. Secondly, in contrast to PKC activator TPA, it did not cause ERK activation and, unlike thrombin or histamine, it caused very little IP formation. Thirdly, LPC did not cause phosphorylation of PKCδ, unlike TPA, thrombin or histamine.

Finally, we have shown that LPC inhibits EGF-mediated eNOS phosphorylation, indicating a downstream consequence of LPC-mediated Akt inhibition. Similarly, Wm inhibited EGF-mediated eNOS phosphorylation. Conversely, both thrombin and histamine were found to stimulate eNOS phosphorylation in spite of their demonstrated inhibitory effect on Akt and this stimulation was unaffected by Wm. The histamine-induced phosphorylation of eNOS was also unaffected by a variety of inhibitors of several signaling pathways (Fig. 7B). These findings are in line with numerous observations emphasizing complex enzymatic control mechanisms of eNOS activity [59].

We are currently studying the involvement of AMP-Kinase, which has been shown to phosphorylate eNOS [60]. Preliminary results indicate that AMPKinase is activated after thrombin or histamine treatment.

In conclusion, we demonstrate that thrombin, histamine and LPC inhibit both EGF- and S1P-mediated Akt phosphorylation in cultured HUVEC. While PKCδ mediated the inhibitory effects of thrombin and histamine the inhibitory effects of LPC were independent of PKC. The implications of our findings relate to the role of Akt in maintaining structural and functional integrity of vascular endothelium and may provide added insight into potential mechanisms for the cytotoxic effects of ox-LDL and chronically activated PKC from any cause.

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Paper II

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Thrombin and histamine stimulate endothelial nitric-oxide synthase phosphorylation at Ser1177 via an AMPK mediated pathway independent of PI3K-Akt

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Abstract Histamine and thrombin cause phosphorylation and activation of endothelial NO-synthase (eNOS) on Ser1177. We tested the role of various protein kinases in mediating this effect in human umbilical vein endothelial cells. Inhibition of the Ca^{2+}/calmodulin-dependent protein kinase II or phosphoinositide 3-kinase (PI3K) had no effect. H89, an inhibitor of both protein kinase A (PKA) and 5′-AMP-activated protein kinase (AMPK), strongly inhibited phosphorylation and activity of eNOS. Conversely, the PKA inhibitor Rp-adenosine 3′5′-cyclic monophosphate (cAMPS) had no effect and eNOS was not phosphorylated by treatments that affect cAMP levels. Thrombin and histamine caused phosphorylation of AMPK on Thr172 as well as on its downstream target acetyl-CoA carboxylase. Activation of AMPK using AICAR or CCCP also resulted in eNOS phosphorylation. We conclude that histamine and thrombin cause eNOS phosphorylation in an AMPK mediated manner, independent of P13K-Akt.

Keywords: eNOS; AMPK; Histamine; Thrombin; Endothelial cell

1. Introduction

Given the central position of the vascular endothelium in early atherogenesis and the vast number of blood borne chemical, cellular and rheological stimuli that continuously affect endothelial cells, the signal transduction mechanisms involved in linking exposure to endothelial response have in recent years attracted considerable attention. Among the numerous biologically important molecules produced by the vascular endothelium, nitric oxide (NO) seems to play a particularly important role in regulating cardiovascular homeostasis. It affects blood vessel dilation and hence vascular resistance and blood pressure, angiogenesis, apoptosis, adhesion of platelets and monocytes to the endothelium, endothelin-1 generation and vascular smooth muscle cell proliferation [1].

The major source of NO is endothelial NO-synthase (eNOS) which is regulated by a complex battery of regulatory mechanisms, including subcellular localization in caveoli where interaction of eNOS with caveolin and heat shock protein 90 exemplifies a regulatory protein-protein interaction [2]. Regulation with phosphorylation has also been reported and recently specific sites for phosphorylation of eNOS and specific protein kinases mediating the phosphorylation have been identified. These include protein kinase B (PKB)/Akt [3], protein kinase A (PKA) [4], protein kinase C (PKC) [5], 5′-AMP-activated protein kinase (AMPK) [6,7] and Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII) [8], Michell and coworkers have published data suggesting that regulation of eNOS activity involves coordinated phosphorylation and dephosphorylation of Ser1179 and Thr497 residues on bovine eNOS by multiple protein kinases and phosphatases with stimulation, involving phosphorylation at Ser1179 and dephosphorylation at Thr497 [5]. Bradykinin has been shown to activate eNOS phosphorylation at Ser1179 either in an Akt [9] or CaMKII [8] dependent manner while stimulating dephosphorylation of Thr497 through a calcineurin mediated, Akt independent mechanism [9]. Adiponectin was also recently shown to cause eNOS phosphorylation at Ser1179. This phosphorylation was inhibited by wortmannin but was still Akt independent and mediated partially by AMPK [7]. Others have implicated Akt in eNOS phosphorylation caused by adiponectin, demonstrating a cross-talk between AMPK and Akt in endothelial cells [10].

AMPK is the central component of a protein kinase cascade that plays an important role in the regulation of energy metabolism, often referred to as the cell's metabolic master switch [11]. In response to a decrease in the energy state of a cell, AMPK is phosphorylated and activated by a still not fully characterized upstream pathway. Once activated, AMPK phosphorylates multiple targets, all aimed at restoring adenosine 5′-triphosphate (ATP) levels. These include acetyl CoA carboxylase (ACC), hydroxymethylglutaryl-CoA

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Abbreviations: ACC, acetyl-CoA carboxylase; AICAR, 5-aminoimidazole-4-carbozamide-1-β-4 ribofuranoside; AMP, adenosine 5′-monophosphate; AMPK, 5′-AMP-activated protein kinase; ATP, adenosine 5′-triphosphate; CaMKII, Ca^{2+}/calmodulin-dependent protein kinase II; cAMP, adenosine 3′5′-cyclic monophosphate; EGF, epidermal growth factor; eNOS, endothelial NO-synthase; HUVEC, human umbilical vein endothelial cells; NO, nitric oxide; PI3K, phosphoinositide 3-kinase; PKA, protein kinase A; PKC, protein kinase C; PLC, phospholipase C; ROCK, Rho-dependent protein kinase

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(HMG-CoA) reductase, glycogen synthase and eNOS [11]. The phosphorylation of eNOS at Ser1177 by AMPK is Ca^{2+}/CaM dependent [6].

We have previously shown in primary cultures of human umbilical vein endothelial cells (HUVEC) that thrombin and histamine, although inhibiting Akt phosphorylation when applied in cotreatment with epidermal growth factor, stimulate eNOS phosphorylation at Ser1177 [12].

In this paper, we explore the role of various protein kinases in mediating the stimulatory effects of the G-protein activators thrombin and histamine on eNOS Ser1177 phosphorylation. Our results demonstrate that AMPK mediates the stimulatory effects of both agonists through a phosphoinositide 3-kinase (PI3K)-Akt independent pathway.

2. Materials and methods

2.1. Materials

Morgan's medium 199, fetal bovine serum and Penicillin–Streptomycin were purchased from Gibco-BRL, Life Technologies. Tissue culture plates (35 mm) were from Nunc, Cryotin X (collagenase) from cod was provided by The Science Institute of Iceland. Epidermal growth factor (EGF), 12-*O*-tetradecanoylphorbol-13-acetate (TPA), histamine, thrombin, carbonyl cyanide *m*-chlorophenylhydrazone (CCCP), 1-isobutyl-3-methyl xanthine (IBMX), 8-bromoadenosine 3′5′-cyclic monophosphate (cAMP), Rp-cAMPS, A23187, U73122, forskolin and wortmannin were purchased from Sigma. H89 was from Biomol. 5-aminoimidazole-4-carbozamide-l-β-4 ribofuranoside (AICAR), BAPTA/AM, KN-93 and Y 27632 were from Calbiochem. Myo[^{3}H]inositol, Hybond ECL Nitrocellulose membrane (6×8 cm), ECL + PLUS Western blotting detection system, Hyperfilm ECL High performance chemiluminescence film and L-(^{3}H) arginine (61.0 Ci/mmol) were from Amersham Pharmacia Bioteck. Antibodies against phospho-eNOS (Ser1177), AMPK, phospho-AMPK (Thrl72) and Anti-Rabbit IgG/HRP-linked came from Cell Signaling Technology. eNOS antibody came from Transduction Laboratories and pACC (Ser79) and ACC antibodies from Upstate, Poly-prep prefilled chromatography columns were from BioRad.

2.2. Endothelial cell culture

Endothelial cells were cultured from human umbilical veins by a modification of the method of Jaffe et al. as previously reported [13]. The cells were harvested by Cryotin X collagenase digestion and seeded on 35 mm culture dishes in medium 199 containing 20% fetal bovine serum and antibiotics (penicillin, 100 units/mL and streptomycin, 100 μg/mL). The culture dishes were incubated at 37 °C in humidified air with 5% CO_2. The medium was changed 24 h after seeding the cells and every 2–3 days thereafter until the cell culture reached confluence (after ~7 days).

When confluent, the cells were washed with Morgan's medium 199 and then placed in 1.0 mL Morgan's medium 199 with or without inhibitors at the indicated concentrations. 10–20 min later, agonist was added in a concentration calculated to reach the intended concentration for each experiment and left on for additional 2–20 min. The agonists were then removed along with the medium and cellular reactions terminated by adding 250 μL SDS sample buffer. The samples were boiled for 5 min and centrifuged for 10 min at 3000 rpm. After that, the samples were ready to be used or could be stored at −20 °C.

2.3. Electrophoresis and immunoblotting

Samples (8 μL) were resolved by SDS–PAGE (10%). The gels were blotted and the proteins thereby transferred to nitrocellulose. The membranes were hybridized with the indicated antibodies and later with a secondary antibody (Anti-Rabbit IgG/HRP-linked). The immuno-complexes were detected with ECL+PLUS Western blotting detection system and developed onto a film. Equal loading was ascertained by hybridizing membranes with antibodies against unphosphorylated protein.

2.4. Determination of eNOS activity

eNOS activity in intact cells was determined by monitoring the conversion of incorporated L-(^{3}H) arginine into L-(^{3}H) citrulline as described by Schmidt and Mayer [14]. Briefly, the cells were washed and equilibrated for 20 min in incubation buffer (50 mM Tris buffer, pH 7.4, containing 100 mM NaCl, 5 mM KC1, 1 mM $MgCl_2$, and 2 mM $CaCl_2$) with or without BAPTA or H89. Reactions were started by the addition of L-(^{3}H) arginine (61.0 Ci/mmol, 1 μCi, final concentration 16 nM) and the agonist. After 3 min the cells were washed with chilled incubation buffer containing 0.1 mM EGTA instead of $CaCl_2$, followed by the addition of 1 mL of 10 mM HCl. An hour later, an aliquot was removed for determining the incorporated radioactivity. To the remaining sample a solution of 200 mM sodium acetate, 20 mM NaOH containing 10 mM citrulline was added (final pH approx. 5.0) and L-(^{3}H) citrulline was separated from L-(^{3}H) arginine by cation exchange chromatography [14].

3. Results

3.1. Effects of EGF, histamine and thrombin on eNOS phosphorylation

Fig. 1 demonstrates the effects of EGF, histamine and thrombin on Serl177 phosphorylation of eNOS. As seen, the PI3K inhibitor wortmannin totally blocked the effect of EGF whereas phosphorylation by histamine and thrombin was unaffected, demonstrating different pathways mediating phosphorylation by these agonists.

3.2. Histamine and thrombin mediated eNOS phosphorylation is dependent on Ca^{2+} but independent of CaMKII

As shown in Fig. 2A, U73122, an inhibitor of phospholipase Cβ (PLCβ), inhibited the histamine mediated phosphorylation of eNOS. Conversely, the effects of the Ca^{+2} ionophore A23187 were not affected. The calcium chelator BAPTA blocked the phosphorylation by thrombin and histamine as well as that caused by A23187 (Fig. 2A).

One of the downstream effectors of Ca^{2+}/CaM is CaMKII, which has been implicated in eNOS phosphorylation after bradykinin treatment [8]. As seen in Fig. 2A, the CaMKII inhibitor KN-93 had no effect on eNOS phosphorylation caused by histamine or thrombin. The results suggest that

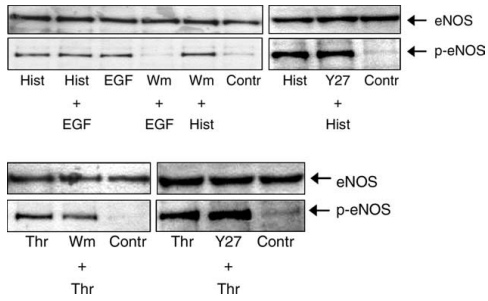

Fig. 1. The effect of EGF, histamine or thrombin on eNOS phosphorylation (Ser1177). Cells were treated with wortmannin (100 nM) for 10 min before treatment with EGF (10 ng/mL, 5 min), histamine (10 μM, 10 min) or thrombin (1 U, 5 min). The effect of Y 27632 (10 μM, 20 min) on histamine or thrombin mediated eNOS phosphorylation. Confluent endothelial cells were stimulated as indicated, lysates were electrophorized and blotted as described in methods and detected using antibody against eNOS and the Serl177 phosphorylated eNOS. Each Western blot is representative of three independent experiments.

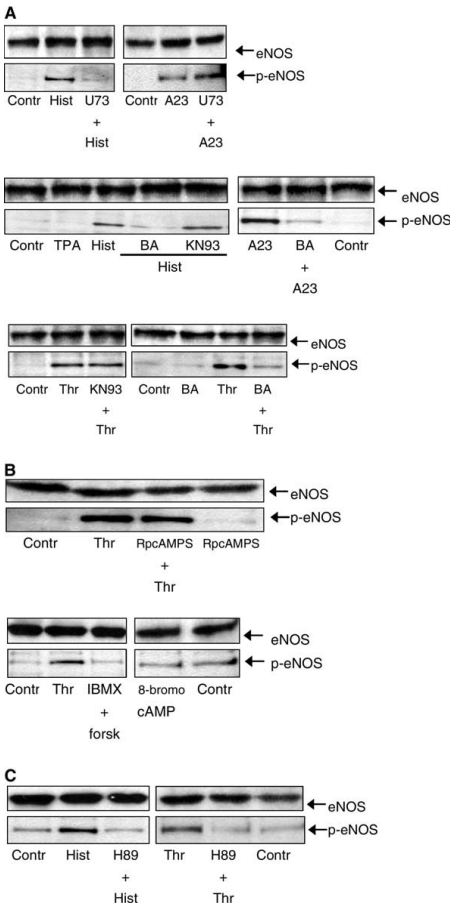

Fig. 2. The role of various protein kinases in eNOS phosphorylation (Serl177). (A) The cells were treated with U73122 (10 μM) for 20 min before treatment with histamine (10 μM, 3 min) or A23187 (200 nM, 3 min). The effect of a 20 min pre-treatment with BAPTA (30 μM) or KN-93 (30 μM) on histamine (10 μM, 5 min), thrombin (1 U, 5 min) or A23198 (200 nM, 3 min) mediated eNOS phosphorylation. The effect of TPA (100 ng/mL, 7 min) on eNOS phosphorylation. (B) The effect of Rp-cAMPS (10 μM, 20 min) on eNOS phosphorylation after thrombin (1 U, 2 min). The effect of BMX + forskolin (100 μM/10 μM, 20 min), thrombin (1 U, 10 min) and 8-bromo-cAMP (100 μM, 10 min) on eNOS phosphorylation. (C) Cells were treated with H89 (20 μM) for 10 min before treatment with histamine (10 μM, 3 min) or thrombin (1 U, 10 min). Samples were separated by SDS–PAGE and analyzed by Western blotting. Each Western blot is representative of three independent experiments.

histamine and thrombin mediated phosphorylation of eNOS is dependent on a Ca^{2+} signal without involving CaMKII.

3.3. PKC and PKA are not involved in histamine or thrombin-induced eNOS phosphorylation at Ser 1177

In addition to Ca^{2+} elevation, activation of PI specific PLC in HUVEC leads to activation of PKC. Therefore, we tested the effect of the PKC activator TPA on eNOS phosphorylation. As seen in Fig. 2A, TPA had no effect.

To test the possible effect of PKA on eNOS phosphorylation, the cells were treated either with substances increasing intracellular cAMP (IBMX and forskolin or 8-bromo-cAMP) or with the PKA inhibitors Rp-cAMPS or H89. None of the substances increasing cAMP levels had any effect on eNOS phosphorylation nor did Rp-cAMPS prevent the phosphorylation of eNOS after thrombin treatment (Fig. 2B). Conversely, H89 totally inhibited both histamine and thrombin stimulated eNOS phosphorylation (Fig. 2C). The inhibition was 96±4% based on scanning three independent gels.

3.4. The role of AMPK in eNOS phosphorylation caused by histamine or thrombin

Although marketed as "a selective and potent inhibitor of PKA", Cohen and coworkers [15] have shown that H89 inhibits several protein kinases to a similar degree, including AMPK. As AMPK has been shown to phosphorylate eNOS [6,7], we tested the effects of histamine and thrombin on AMPK activity.

Both histamine and thrombin caused phosphorylation of AMPK (Thrl72) and the AMPK substrate ACC (Ser79) (Fig. 3A). The phosphorylation of AMPK and ACC was unaffected by wortmannin, but inhibited by the Ca^{2+} chelator BAPTA (Fig. 3A). Furthermore, H89 inhibited ACC phosphorylation caused by histamine or thrombin (97 ± 3% inhibition, quantified by scanning three independent gels), whereas AMPK phosphorylation was not affected (Fig. 3A). AMPK, eNOS and ACC phosphorylation was also caused by CCCP, which activates AMPK by lowering the ATP/AMP ratio [16] (Fig. 3B). AICAR, which mimicks the activating effect of AMP on AMPK without affecting the AMP/ATP ratio [17] also caused phosphorylation of AMPK, eNOS and ACC (Fig. 3C). All the effects of thrombin, histamine and CCCP on eNOS phosphorylation were independent of PI3K (Figs. 1 and 3B), whereas H89 inhibited phosphorylation of eNOS after all these treatments (Figs. 2C and 3B). Finally, the Ca^{2+} ionophore A23187 mimicked the effects of thrombin and histamine on the phosphorylation of AMPK, eNOS and ACC (Fig. 4). These effects of A23187 on eNOS phosphorylation were inhibited by H89 and BAPTA but unaffected by wortmannin (Figs. 2A and 4A). BAPTA also prevented the phosphorylation of AMPK by A23187 (Fig. 4B). As with eNOS, the PLC inhibitor U73122 prevented the histamine mediated AMPK phosphorylation but had no effect on the phosphorylation mediated by A23187 (Fig. 4B).

As H89 has been shown to inhibit Rho-dependent protein kinase II (ROCK-II) [15] as well as AMPK, and ROCK-II can regulate eNOS expression and phosphorylation [18], we tested the effects of the ROCK-II inhibitor Y 27632. As seen in Fig. 1, inhibiting ROCK-II had no effect on eNOS phosphorylation.

3.5. eNOS activation in response to histamine or thrombin

In order to determine whether histamine or thrombin treatment led to the activation of eNOS, we studied their effect on the conversion of L-(^{3}H) arginine to L-(^{3}H) citrulline. Both agonists caused threefold increase in citrulline formation (Fig. 5) and this activation was largely prevented by pretreatment with H89 or BAPTA. BAPTA also caused a decrease in the basal rate of conversion to L-(^{3}H) citrulline. As

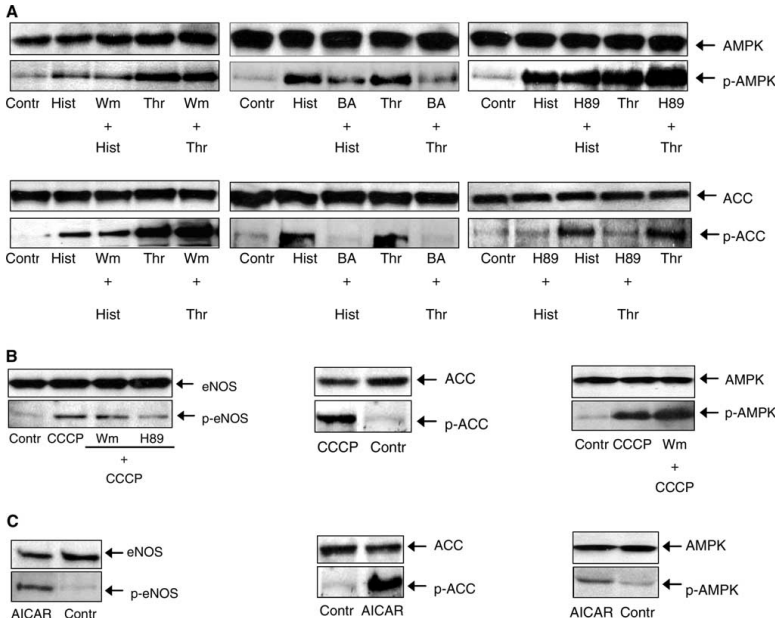

Fig. 3. The role of AMPK in eNOS phosphorylation (Ser1177). (A) The effect of wortmannin (100 nM, 10 min), BAPTA (30 μM, 10 min) or H89 (20 μM, 20 min) on histamine (10 μM, 5 min) or thrombin (1 U, 5 min) mediated phosphorylation ofAMPK (Thrl72) or ACC (Ser79). (B) The effect of a 20 min pre-treatment with wortmannin (100 nM) or H89 (20 μM) on eNOS or AMPK phosphorylation caused by CCCP (10 μM, 3 min). The effect of CCCP (10 μM, 10 min) on ACC phosphorylation. (C) Cells were treated with AICAR (2 mM) for 4 h (eNOS, ACC) or 6 h (AMPK). The AICAR treatments were performed in medium containing 20% serum. Samples were separated by SDS–PAGE and analyzed by Western blotting. Each Western blot is representative of three independent experiments.

described by Wagner et al. [19], using bradykinin or ATP as agonists, the activation was shortlived, being almost over in 3 min (not shown).

4. Discussion

In this paper, we show that both histamine and thrombin cause eNOS phosphorylation at Ser1177 in HUVEC in an AMPK mediated manner, independent of PI3K-Akt. Several protein kinases have been implicated in eNOS phosphorylation at Ser1177. We demonstrate that the phosphorylation caused by histamine and thrombin is Ca^{2+} dependent, without the involvement of PKC or CaMKII. Also, results from various manipulations affecting cAMP levels weighed against the possibility that PKA mediated the eNOS phosphorylation caused by these G-protein agonists. It has been shown that AMPK can phosphorylate eNOS on Ser1177 [6,20] and eNOS has been reported to be in a complex with AMPK [6,21]. We show that histamine and thrombin cause phosphorylation of AMPK on Thrl72 and that wortmannin had no effect on histamine or thrombin-mediated phosphorylation of either eNOS or AMPK. Other substances, such as the mitochondrial uncoupler CCCP and AICAR, which cause AMPK phosphorylation [16,17], also caused eNOS phosphorylation in our cells further supporting our conclusion that histamine and thrombin stimulate phosphorylation of eNOS in an AMPK-dependent manner. Using an L-(^{3}H) citrulline conversion assay, we demonstrate that treatment with histamine or thrombin actually leads to eNOS activation. The inhibition of AMPK phosphorylation as well as eNOS phosphorylation by U73122 suggests that histamine and thrombin mediate their activation by G_q. Histamine and thrombin are known to activate PLC in HUVEC [22] and G_q coupled receptors cause activation of AMPK [23].

Previously, it has been reported that the G-protein activator bradykinin stimulates phosphorylation of eNOS on Ser1177 via CaMKII [8]. However, while we found that histamine and thrombin mediated phosphorylation of eNOS was Ca^{2+} dependent, inhibited by the Ca^{2+} chelator BAPTA and mimicked by the Ca^{2+} ionophore A23187, it was unaffected by the CaMKII inhibitor KN-93. Treatment with TPA had no effects on eNOS Ser1177 phosphorylation, thereby ruling out PKC mediated effects of histamine or thrombin. The inhibition by H89, however, suggested a possible involvement of PKA as had been demonstrated for eNOS phosphorylation caused by shear stress and bradykinin [4,24]. However, H89 is not a specific PKA inhibitor and has been reported to inhibit several other protein kinases including AMPK and ROCK-II [15]. Also, Rp-cAMPS, a structurally unrelated PKA inhibitor, was without effect on eNOS phosphorylation. Finally, manipulation of cAMP levels had no effect on eNOS phosphorylation in our cells, indicating that the involvement of PKA in this pathway is unlikely. Therefore, we conclude that the H89 in-

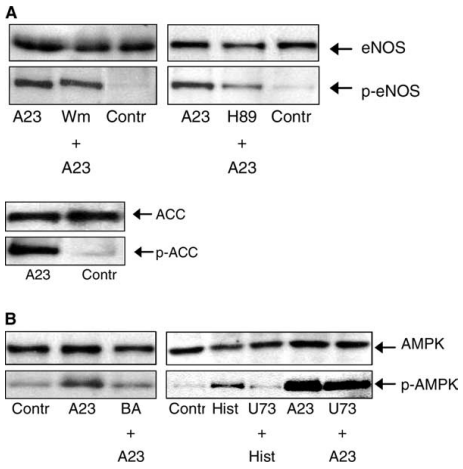

Fig. 4. The involvement of Ca^{2+} in eNOS (Ser1177) or AMPK (Thrl72) phosphorylation. (A) The effect of A23187 (200 nM, 3 min). The cells were treated with wortmannin (100 nM) or H89 (20 μM) for 20 min before treatment with A23187. (B) The cells were treated with BAPTA (30 μM) for 20 min before treatment with A23187 (200 nM, 3 min). The effect of U73122 (10 μM, 20 min) on histamine (10 μM, 3 min) or A23187 (200 nM, 3 min) mediated AMPK phosphorylation. Samples were separated by SDS–PAGE and analyzed by Western blotting. Each Western blot is representative of three independent experiments.

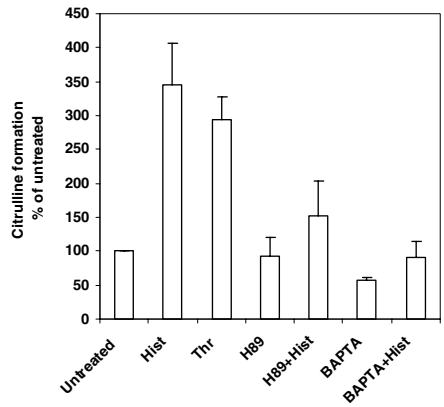

Fig. 5. eNOS activation in response to histamine or thrombin. Effect of histamine (10 μM, 3 min) or thrombin (1 U, 3 min) on eNOS activation and the effect of 20 min pre-treatment with H89 (20 μM) or BAPTA (30 μM) on eNOS activation caused by histamine. The results show the average ± S.D. of three experiments each done in duplicate.

hibition of eNOS phosphorylation is due to AMPK inhibition. This is further supported by our finding that H89 inhibits the phosphorylation of the AMPK substrate ACC but not the phosphorylation of AMPK itself.

It has repeatedly been shown that regulation of eNOS is a complex, multifactorial process [2]. Harris et al. [9] concluded that bradykinin stimulates eNOS phosphorylation via PI3K/Akt. After bradykinin treatment they demonstrated Akt phosphorylation and eNOS phosphorylation which were inhibited by wortmannin [9]. However, others have demonstrated wortmannin sensitive phosphorylation of eNOS which is Akt independent. Thus, shear stress mediated eNOS phosphorylation is mediated by PKA [4] and adiponectin mediated eNOS phosphorylation is mediated by AMPK [7]. Further complications were recently reported by Ouchi et al. [10] who demonstrated that Akt functions downstream of AMPK in adiponectin stimulated endothelial cells, suggesting the following signaling pathway: adiponectin-AMPK-PI3K-Akt-eNOS. However, in all those cases, eNOS phosphorylation mediated by AMPK was P13K dependent. In contrast, in the experiments reported in this paper, wortmannin did not inhibit eNOS phosphorylation caused by histamine or thrombin, clearly demonstrating a P13K-independent pathway distinct from those activated by the other G-protein agonists, adiponectin and bradykinin. Our finding that BAPTA inhibits the phosphorylation of AMPK, eNOS and ACC together with the observation that all these phosphorylations are mimicked by treatment with the Ca^{2+} ionophore A23187, suggests that a Ca^{2+} signal is upstream of AMPK phosphorylation in thrombin or histamine stimulated endothelial cells.

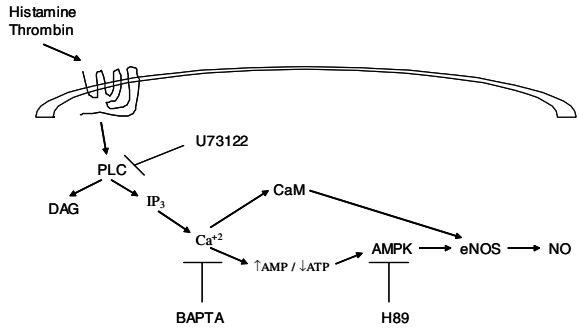

Fig. 6. A proposed pathway for thrombin and histamine-mediated eNOS activation. Thrombin and histamine activate PLC, causing formation of IPs and release of Ca^{2+} initiating energy requiring processes and activation of AMPK. AMPK phosphorylates eNOS and this together with increased Ca^{2+} levels activates eNOS to produce NO.

Finally, the inhibition by H89 and BAPTA in the citrulline assay suggests the importance of Ser1177 phosphorylation in the activation of eNOS following histamine treatment.

Thrombin, histamine and A23187 have previously been shown to cause a transient phosphorylation of elongation factor 2 (eEF2) [25] which is downstream of AMPK [26], and thus to temporarily inhibit protein synthesis. This effect is mimicked by treatments that lower ATP levels (oligomycin and CCCP) [16]. We speculate (Fig. 6) that the activation of AMPK that we describe in HUVEC is mediated by lowered ATP levels. Both thrombin and histamine cause activation of phospholipase C leading to a Ca^{2+} signal resulting in synthesis of prostacyclin and platelet activating factor, release of von Willenbrands factor and induction of shape change, all processes which require ATP. Inhibition of PLC by U73122 prevented the phosphorylation of both AMPK and eNOS after histamine stimulation but not after treatment with the Ca^{2+} ionophore A23187 (PLC independent).

In conclusion, in HUVEC we have demonstrated a role for AMPK in a pathway mediating stimulatory signals from G-protein coupled receptors causing eNOS phosphorylation in a PI3K-Akt independent manner.

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Paper III

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Mechanism of thrombin mediated eNOS phosphorylation in endothelial cells is dependent on ATP levels after stimulation

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ABSTRACT

Conflicting results have been reported concerning the role of AMP-activated protein kinase (AMPK) in mediating thrombin stimulation of endothelial NO-synthase (eNOS). We examined the involvement of two upstream kinases in AMPK activation in cultured human umbilical endothelial cells, LKB1 stimulated by a rise in intracellular AMP/ATP ratio, and Ca^{+2}/CaM kinase kinase (CaMKK) responding to elevation of intracellular Ca^{+2}. We also studied the effects of AMPK activation on the downstream target eNOS. In culture medium 1640 the level of intracellular ATP was unchanged after thrombin stimulation and the CaMKK inhibitor STO-609 totally inhibited phosphorylation of AMPK and acetyl coenzyme A carboxylase (ACC) but not eNOS. In Morgan's medium 199 thrombin caused a significant lowering of intracellular ATP and STO-609 only partially inhibited the phosphorylation of AMPK, ACC and eNOS. Inhibition of AMPK by Compound C or AMPK downregulation using siRNA partially inhibited the phosphorylation of eNOS in medium 199 but not in 1640, underscoring a clear difference in the pathways mediating thrombin-stimulated eNOS phosphorylation in different culture media. Thus, conditions subjecting endothelial cells to a fall in ATP after thrombin stimulation facilitate activation of pathways partly dependent on AMPK causing downstream phosphorylation of eNOS. In contrast, under culture conditions that do not facilitate a fall in ATP after stimulation, AMPK activation is exclusively mediated by CaMKK and does not contribute to the phosphorylation of eNOS.

1. Introduction

The vascular endothelium regulates vascular tone by releasing numerous vasodilators and vasoconstrictors. The most important vasodilator is nitric oxide (NO) produced from arginine by endothelial nitric oxide synthase (eNOS). Besides causing vasodilation, NO inhibits platelet aggregation, leukocyte adherence and smooth muscle cell proliferation. These effects exert a profound influence on blood flow, vascular remodeling and angiogenesis. Loss of endothelial function due to either decreased production or increased degradation of NO is associated with several cardiovascular disorders, including atherosclerosis [1].

eNOS activity is regulated by numerous stimuli, including shear stress, growth factors, vasoactive compounds and hypoxia. The activity is influenced by the subcellular location of the enzyme and the formation of complexes with regulatory proteins, by Ca^{+2} levels and phosphorylation at several sites by a variety of protein kinases [2]. Phosphorylation at Ser1177 has been most thoroughly studied and appears to enhance the sensitivity of eNOS to Ca^{+2} thus promoting its activation. Recently, phosphorylation at this site was shown to modulate vascular reactivity and outcome of cerebral ischemia in vivo [3]. Several protein kinases have been implicated in the phosphorylation at Ser1177 [4].

AMP-activated protein kinase (AMPK) is an energy sensor often referred to as a cellular metabolic master switch. It is activated by any stress causing an increase in the AMP/ATP ratio [5,6]. Once activated, AMPK maintains intracellular energy balance by turning down energy consuming pathways and switching on ATP generating pathways. AMPK also plays a role in whole body energy balance and food intake [6]. Activation of AMPK requires phosphorylation at threonine 172 of the catalytic α subunit caused by an upstream AMPK kinase. A known tumor suppressor, LKB1 phosphorylates this site in response to lowered ATP/AMP ratio. By this phosphorylation mediated by LKB1,

Abbreviations: ACC, acetyl coenzyme A carboxylase; AICAR, 5-aminoimidazole-4-carboxamide-1-β-4 ribofuranoside; AMPK, AMP-activated protein kinase; CaMKK, Ca^{+2}/CaM kinase kinase; eNOS, endothelial NO-synthase; HUVEC, human umbilical vein endothelial cells; L-NA, *N*(G)-nitro-L-arginine; NO, nitric oxide

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AMPK is activated in response to any stress lowering the energy state within the cell, thereby inhibiting ATP consumption and increasing ATP production.

Recently, it has been shown that activation of AMPK is not exclusively associated with perturbations in the ATP/AMP ratio. The Ca^{+2}/calmodulin dependent kinase kinase β (CaMKKβ) has been shown to phoshorylate and activate AMPK in response to elevation of intracellular Ca^{+2} [7–11]. Among the mammalian kinases CaMKK is the closest relative to the AMPKK orthologues in *Saccharomyces cerevisiae* [10]. da Silva et al. [12] identified CaMKK as the kinase involved in extracellular nucleotide mediated AMPK activation in endothelial cells. Furthermore, they demonstrated that the nucleotides activated AMPK in HeLa cells known to be deficient in LKB1.

We have previously reported that the G-protein activators thrombin and histamine stimulate NO-production and phosphorylation of eNOS at Ser1177 in human umbilical vein endothelial cells (HUVEC) in a Ca^{+2}-dependent but PI3 K/Akt independent manner and provided evidence that AMPK is involved in mediating the stimulation [13]. In contrast to our findings, Stahmann et al. [14] did not find a role for AMPK in mediating thrombin-stimulated eNOS phosphorylation. They identified CaMKKβ as the sole upstream kinase responsible for AMPK phosphorylation since downregulation of LKB1 did not affect thrombin-induced AMPK activation and there was no significant change in the AMP/ATP ratio upon thrombin stimulation. Inhibition of either CaMKKβ or AMPK by pharmacological inhibition or protein downregulation using RNA interference, showed that thrombin-induced eNOS phosphorylation was not mediated by the CaMKKβ/AMPK pathway. Motley et al. obtained similar results and further demonstrated by the use of adenovirus encoding dominant-negative mutants of protein kinase C δ (PKCδ) and PKC inhibitors the involvement of PKCδ in eNOS phosphorylation after thrombin treatment [15]. There are important methodological differences between these two and our previous studies. The aim of the present study therefore was to explore these discrepancies on the mechanism of thrombin mediated eNOS phosphorylation, besides further clarifying the involvement of upstream kinases. The results presented here demonstrate that two pathways are involved in activating AMPK after treatment with thrombin, phosphorylation stimulated by lowering of the ATP/AMP ratio and CaMKK dependent activation. Only when stimulated by a fall in ATP/AMP ratio does AMPK mediate stimulation of eNOS. When the experimental conditions do not allow or facilitate a fall in ATP after thrombin stimulation the upstream activation of AMPK is exclusively through CaMKK and while stimulating the phosphorylation of acetyl-CoA carboxylase (ACC) it does not affect eNOS phosphorylation or activation.

2. Materials and methods

2.1. Materials

EBM-2 was purchased from Clonetics. Other cell culture media, Foetal Bovine Serum and Penicillin-Streptomycin were purchased from Gibco Brl, Life Technologies. Tissue culture plates (35 mm) and flasks (50 mL) were from Nunc, Cryotin×(trypsin) from cod was provided by The Science Institute of Iceland. Thrombin, A23187, L-NA, Trypsin-EDTA Solution, Direct cGMP enzyme immunoassay kit, isobutylmethylxanthine (IBMX), apyrase and acid phosphatase were purchased from Sigma. STO-609 and Compound C were from Calbiochem. Hybond ECL Nitrocellulose membrane (6×8 cm), ECL+PLUS Western blotting detection system and Hyperfilm ECL High performance chemiluminescence film were from Amersham Pharmacia Bioteck. Antibodies against eNOS, phospho-eNOS (Ser1177), AMPK, phospho-AMPK (Thr172), AMPKα1, AMPKα2, Pan-Actin and Anti-Rabbit IgG/HRP-linked came from Cell Signaling Technology. Antibodies against ACC and phospho-ACC (Ser79) were from Upstate. Lipofectamine™ RNAiMAX Reagent was from Invitrogen. AMPKα1 and AMPKα2 siRNA came from Ambion.

2.2. Cell culture

Endothelial cells were cultured from human umbilical veins by a modification of the method of Jaffe et al. as previously reported [16]. The cells were harvested by Cryotin X trypsin digestion and seeded on 35 mm culture dishes in Morgan's medium 199 containing 20% foetal bovine serum and antibiotics (penicillin, 100 U/mL and streptomycin, 100 μg/mL). The culture dishes were incubated at 37 °C in humidified air with 5% CO_2. The medium was changed 24 h after seeding the cells and then every 2–3 days thereafter until the cell culture reached confluence (after ~7 days).

When confluent the cells were washed with the appropriate medium and placed in 1.0 mL medium with or without inhibitors at the indicated concentrations. Agonist was added 10 to 20 min later in a concentration calculated to reach the intended concentration for each experiment and left on for additional 2 to 20 min. The agonists were then removed along with the medium and cellular reactions terminated by adding 250 μL SDS sample buffer. The samples were boiled for 5 min and centrifuged for 10 min at 3000 rpm. The samples were then ready to be either used or stored at −20 °C.

HeLa cells were cultured in medium 199 containing 10% fetal bovine serum and antibiotics (penicillin, 100 U/mL and streptomycin, 100 μg/mL).

2.3. siRNA transfection

Endothelial cells grown to ca 80% confluence in 50 mL tissue culture flasks were trypsinized and deluted sixfold on to 35 mm culture dishes. 24 h later, the cells were transfected with Lipofectamine™ RNAiMAX transfecting agent containing siRNA for AMPKα1 and/or AMPKα2 (20 nM) in antibiotics-free EBM-2 medium containing 6% serum. Cells were cultured for 44 h and protein expression analyzed by Western blotting.

2.4. Electrophoresis and immunoblotting

Samples (8 μL) were resolved by SDS-PAGE (10%). The gels were blotted and the proteins thereby transferred to nitrocellulose. The membranes were hybridized with the indicated antibodies and subsequently with a secondary antibody (Anti-Rabbit IgG/HRP-linked). The immuno-complexes were detected with ECL+PLUS Western blotting detection system and developed onto a film. Equal loading was ascertained by hybridizing membranes with antibodies against unphosphorylated protein. The band intensity was quantified using Kodak 3.5 software.

2.5. Measurement of ATP

ATP was determined by luciferase assay and for validation by HPLC in which case AMP was also determined. For ATP determination using the luciferase assay the medium was removed from the cells and the cells lyzed by adding 0.5 mL of 0.5 N perchloric acid. After 30 min a 10 μL sample was diluted×100 in water mixed with 80 μL of a luciferase mixture from an Eliten kit by Promega. For HPLC analysis, the cells were killed by adding 0.25 mL of 0.5 M perchloric acid. The cells were scraped off the culture dishes, put into Eppendorf tubes, neutralized with 220 μL of 5 N KOH and centrifuged at 10,000 *g* for 10 min. 225 μL of the supernatant was transferred to another tube containing 25 μL of 1 M KH_2PO_4 pH 6.0. The nucleotides were determined by reversed phase HPLC in an Agilent 1100 apparatus using a 15 cm Zorbax C8 column and a mobile phase which was a variable mixture of 100 mM KH_2PO_4 pH 6.0 and 8 mM TMA counterion and the same solution containing 10% methanol. According to this method the level of cellular AMP was 3% of the ATP levels and when cells were stimulated with the Ca^{+2} ionophore A23187 the AMP levels doubled for every 22% lowering of ATP (not shown).

2.6. cGMP measurements

HUVEC monolayers were washed with Morgan's medium 199 or medium1640 and placed in 1.0 mL Morgan's medium 199 or medium 1640 containing 0.25 mM IBMX. The cells were incubated for 17 min. at 37 °C. Then thrombin was added to give a final concentration of 1 U/mL and the cells further incubated for 3 min. The reaction was stopped by removing the medium and adding 0.5 mL of 0.1 M HCL. Intracellular cGMP was determined using an enzyme immunoassay kit (Sigma) in accordance with the manufacturer's instructions.

2.7. Statistical analysis

Values are expressed as average ± S.D. Unpaired, two-tailed Student's *t*-test was performed for comparisons between groups. The level of significance was set at $p<0.05$. Software used was GraphPad Prism 5.00.

3. Results

3.1. Effects of thrombin or A23187 on cellular ATP content in different media

To investigate the upstream mechanisms of AMPK activation in endothelial cells the levels of cellular ATP were measured following treatment with thrombin or the Ca^{+2} ionophore A23187 in different media. As the Ca^{+2} ionophore was more potent in lowering the ATP level it was used in further studies of intracellular ATP. In medium 199, treatment with 1 U/mL of thrombin for 3 min lowered intracellular ATP by 10.7 ± 4.1%, whereas in medium 1640 the effect of thrombin was negligible (lowering of 2.0 ± 2.6%) (Fig. 1A). Treatment with A23187 at 0.6 μM revealed a marked difference between responses in the two media, a 53.3 ± 2.5% lowering of ATP in medium 199 compared to no decrease in medium 1640 (2.0 ± 4.0%) (Fig. 1A). Fig. 1A also shows that treatment with A23187 in buffer had

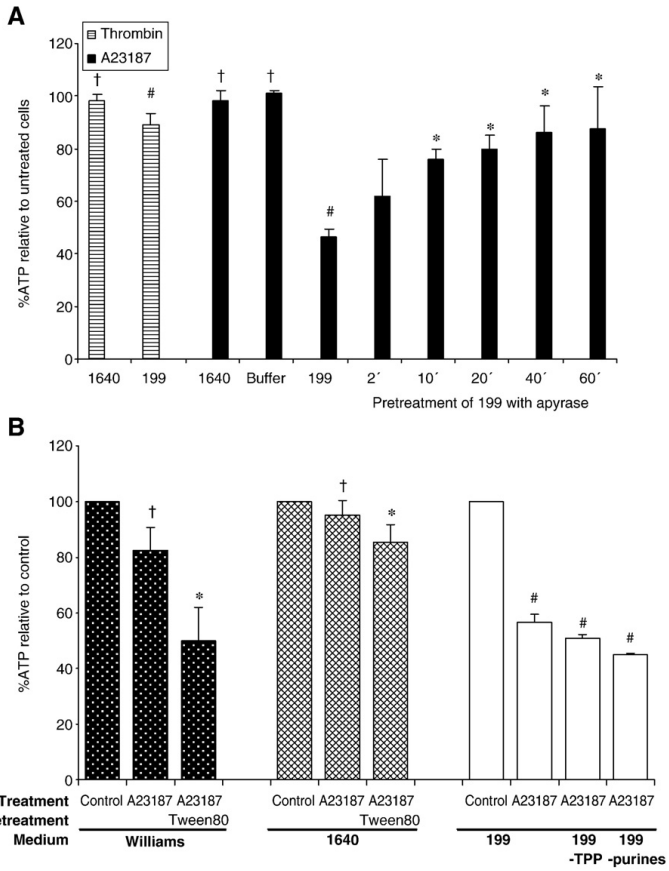

Fig. 1. Effects of thrombin or A23187 on ATP levels in different media. (A) Comparison between the effects of 3 min treatment with thrombin (1 U/mL) and A23187 (0.6 μM) on ATP levels of cells in media 199, 1640 or buffer. Effect of 2–60 min pre-treatment of medium 199 with apyrase (1 U/mL) on the decrease in cellular ATP levels caused by 3 min treatment with A23187. (†ns vs. unstimulated cells; #$p<0.001$ vs. unstimulated cells, *$p<0.001$ vs. cells stimulated with A23187 in medium 199 not treated with apyrase). (B) Effects of culture media on ATP levels of HUVEC after treatment with the Ca^{+2} ionophore A23187. The cells were cultured in normal medium 199 and subsequently transferred to the various indicated media. After 20 min the cells were treated with 0.6 μM A23187 for 3 min and ATP levels determined. (†ns vs control; *$p<0.05$ vs. cells treated with A23187 alone; #$p<0.01$ vs. control). The cells were cultured in medium 199. 20 min before treatment, the medium was replaced either with fresh media 1640 or 199 or medium 199 treated with apyrase (1 U/mL) at 37 °C for the indicated times, boiled for 2 min and cooled to 37 °C. Results are expressed as percent ATP level relative to control (unstimulated cells) and show the average ± S.D. of at least three independent experiments each done in duplicate. TPP, tocopheryl phosphate.

no effect on ATP levels, suggesting that a constituent of medium 199 allowed or facilitated the drop in ATP levels in response to both thrombin and Ca^{+2} ionophore A23187.

A potentially important difference in the composition of the two media is the presence of ATP and other purines in medium 199 and their absence in 1640. As shown in Fig. 1A treatment of medium 199 with 1 U apyrase for 1 h followed by boiling and cooling to 37 °C before adding the medium to cells markedly reduced the ionophore mediated lowering of cellular ATP. However, adding ATP to medium 1640 or to medium 199 treated with apyrase did not restore the lowering effect of A23187 on cellular ATP (data not shown). Furthermore, the reduction of cellular ATP after A23187 treatment was identical in medium 199 manufactured without purines, ribose and deoxyribose to that in normal medium 199 (Fig. 1B) and the effect was also reduced by apyrase pre-treatment.

As the commercially available apyrase contains acid phosphatase we tested the effects of pure acid phosphatase on the cellular responses to stimulation in medium 199. After 1 min treatment of 199 with phosphatase no detectable ATP was present in the medium and pre-treating the cultured endothelial cells with phosphatase in fresh medium 199 decreased the ionophore stimulated fall in cellular ATP from 53±3% to 9±1% of control values. However, the phosphatase also prevented the ATP fall in cells in medium 199 manufactured without purines, ribose and deoxyribose. Taken together, these results excluded the possibility that ATP in the medium or the products of apyrase-mediated ATP hydrolyzation in medium 199 could be the explanation for different cell responses in different media.

Another difference between the two media is the presence of vitamins A, D, C and K, tocopheryl phosphate, cholesterol and the non-ionic detergent tween 80 in medium 199 and their absence in 1640. In view of our results with phosphatase treatment the presence of tocopheryl phosphate in medium 199 was potentially interesting. However, the response in medium 199 manufactured without tocopheryl phosphate was no different from that in ordinary medium 199 (Fig. 1B) and adding tocopheryl phosphate to medium 1640 had no effect on ATP levels after A23187 treatment. Adding all the lipid soluble vitamins or cholesterol to medium 1640 in various combinations had no effect either (data not shown). However, as shown in Fig. 1B, after adding tween 80 to medium 1640 subsequent treatment with A23187 lowered intracellular ATP to 85.4±6.4% of control cells ($p<0.05$). Similar effects of tween 80 were seen in Williams medium which contains the same vitamins as medium 199 but no cholesterol and no tween 80. In this medium the ionophore caused ATP reduction to 82.5±8.4% of control and pre-treatment with tween 80 increased this effect to an ATP lowering of 50.0±12.0% of control (Fig. 1B).

3.2. Thrombin mediated phosphorylation of AMPK, ACC and eNOS in different culture media

The effects of the CaMKK inhibitor STO-609 on AMPK, ACC and eNOS phosphorylation after treatment with thrombin are shown in Fig. 2. When the experiments were carried out in culture medium 199 there was a sixfold increase in the phosphorylation of AMPK (Fig. 2A). STO-609 partially inhibited this phosphorylation (72±11% reduction) and also the thrombin mediated phosphorylation of ACC (66±4% reduction) (Fig. 2B) and eNOS (54±14% reduction) (Fig. 2C).

When the cells were transferred to medium 1640 30 min before thrombin treatment there was a fourfold activation of AMPK phosphorylation (compared to sixfold increase in medium 199) which was totally inhibited by STO-609 (94.8±6.4% reduction) (Fig. 3A), suggesting activation through CaMKK only and no dependence on another pathway. The phosphorylation of ACC was also completely inhibited by STO-609 (104.5±12.8% reduction) (Fig. 3B) in contrast to the eNOS phosphorylation which was unaffected (1.2±13.9% reduction) (Fig. 3C). However, when cellular ATP was lowered by pre-

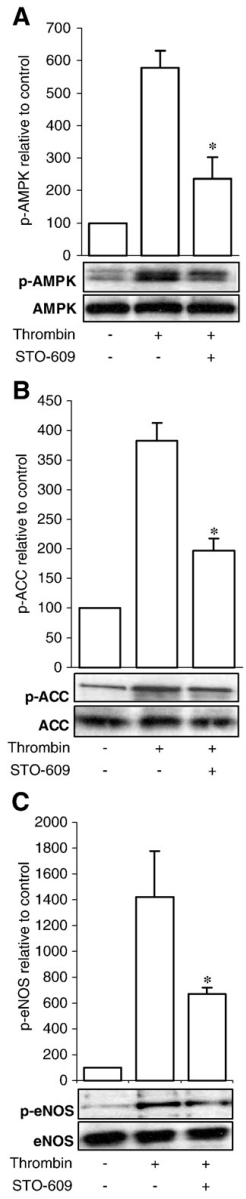

Fig. 2. The effects of CaMKK inhibition on thrombin mediated phosphorylation of AMPK, ACC and eNOS in medium 199. The effects of 20 min pre-treatment with STO-609 (10 μM) on (A) AMPK (Thr172), (B) ACC (Ser79) and (C) eNOS (Ser1177) phosphorylation caused by thrombin (1 U/mL, 2 min) in medium 199. Confluent endothelial cells were stimulated as indicated, lysates were electrophorized and blotted as described in Materials and methods and detected using antibodies against AMPK, Thr172 phosphorylated AMPK, eNOS, Ser1177 phosphorylated eNOS, ACC and Ser79 phosphorylated ACC. The results are expressed as percent phosphorylation relative to unstimulated cells and show the average±S.D. of three independent experiments. (*$p<0.05$ cells treated with STO-609 and thrombin vs. cells stimulated with thrombin alone).

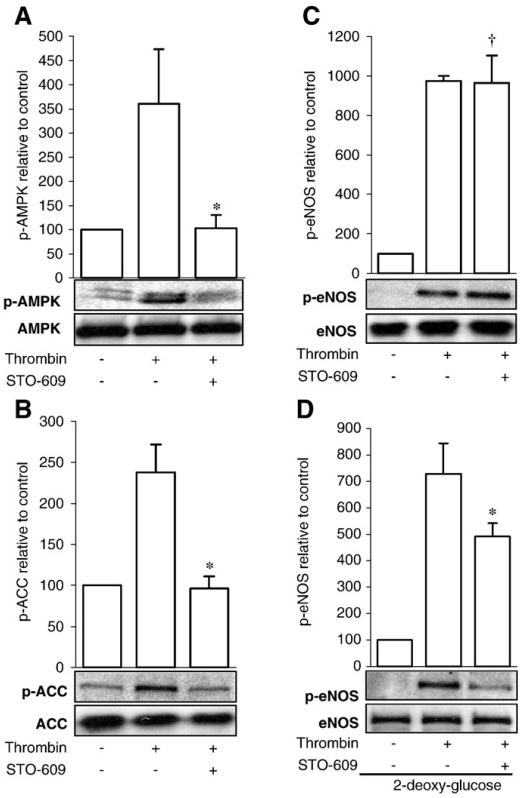

Fig. 3. The effects of CaMKK inhibition on thrombin mediated phosphorylation of AMPK, ACC and eNOS in medium 1640. The effects of 20 min pre-treatment with STO-609 (10 μM) on (A) AMPK (Thr172), (B) ACC (Ser79) and (C) eNOS (Ser1177) phosphorylation caused by thrombin (1 U/mL, 2 min) in medium 1640. (D) Modification of the effects of STO-609 on thrombin mediated eNOS phosphorylation by 2-deoxy-glucose (20 mM, 4 min) in medium 1640. Cells were treated and results expressed as described in Fig. 2 and show the average±S.D. of three independent experiments. (†ns/*$p<0.05$ cells treated with STO-609 and thrombin vs. cells treated with thrombin alone).

treatment with 2-deoxy-glucose, STO-609 inhibited eNOS phosphorylation by 33.7±3.3% in medium 1640 (Fig. 3D).

To test the possibility that LKB1 is responsible for the STO-609 resistant activation of AMPK we used HeLa cells which are known to be deficient in LKB1 [17]. The HeLa cells were cultured and tested in medium 199. STO-609 totally inhibited the phosphorylation of AMPK caused by A23187 (98.0±4.9% inhibition) (Fig. 4A). Since HeLa cells do not have a receptor for thrombin [18], we tested the effects of STO-609 on histamine mediated phosphorylation of AMPK and as seen with the Ca^{+2} ionophore, there was a total inhibition (100.9±2.5%) (Fig. 4B).

To test the possibility of eNOS being upstream of AMPK in the activation cascade, we treated endothelial cells with thrombin after 20 min pre-treatment with the nitric oxide inhibitor *N*(G)-nitro-L-arginine (L-NA). Although L-NA totally inhibited cGMP formation in the cells, it had no effect on thrombin mediated phosphorylation of AMPK, ACC or eNOS (data not shown).

3.3. The role of AMPK in eNOS phosphorylation and NO-production

To further investigate the possible involvement of AMPK in eNOS phosphorylation in different media, we tested the effects of the AMPK inhibitor, compound C, on eNOS phosphorylation after treatment with thrombin. As seen in Fig. 5A, compound C partially inhibited eNOS phosphorylation in medium 199 while having no effect in medium 1640. In contrast, compound C inhibited the phosphorylation of ACC in both media (Fig. 5B).

When acid phosphatase was added to medium 199, inhibition of either CaMKK (STO-609) or AMPK (Compound C) had no effect on eNOS phoshorylation, identical to the response in medium 1640 (Fig. 5C). Conversely, AMPK phosphorylation was totally inhibited by STO-609 when phosphatase was added to medium 199, also identical to that seen in medium 1640 (Fig. 5D).

Since the inhibitors used could possibly have off-target effects, we used siRNA to suppress the expression of AMPKα. We transfected validated siRNA constructs complementary to AMPKα1 and/or -α2

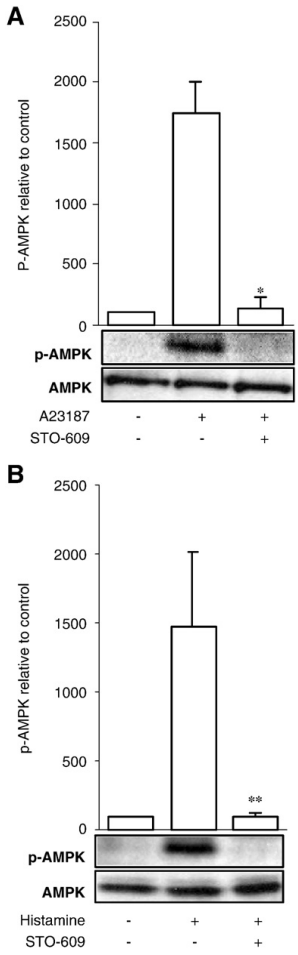

Fig. 4. Effects of STO-609 on AMPK phosphorylation in HeLa cells in medium 199. The effects of 20 min pre-treatment with STO-609 (10 μM) on AMPK phosphorylation (Thr172) in HeLa cells after treatment with A23187 (0.2 μM, 3 min) or histamine (10 μM, 3 min). Cells were treated and results expressed as described in Fig. 2 and show the average±S.D. of four independent experiments. (*$p<0.0001$ cells treated with STO-609 and A23187 vs. cells treated with A23187 alone, **$p<0.01$ cells treated with STO-609 and histamine vs. cells treated with histamine alone) .

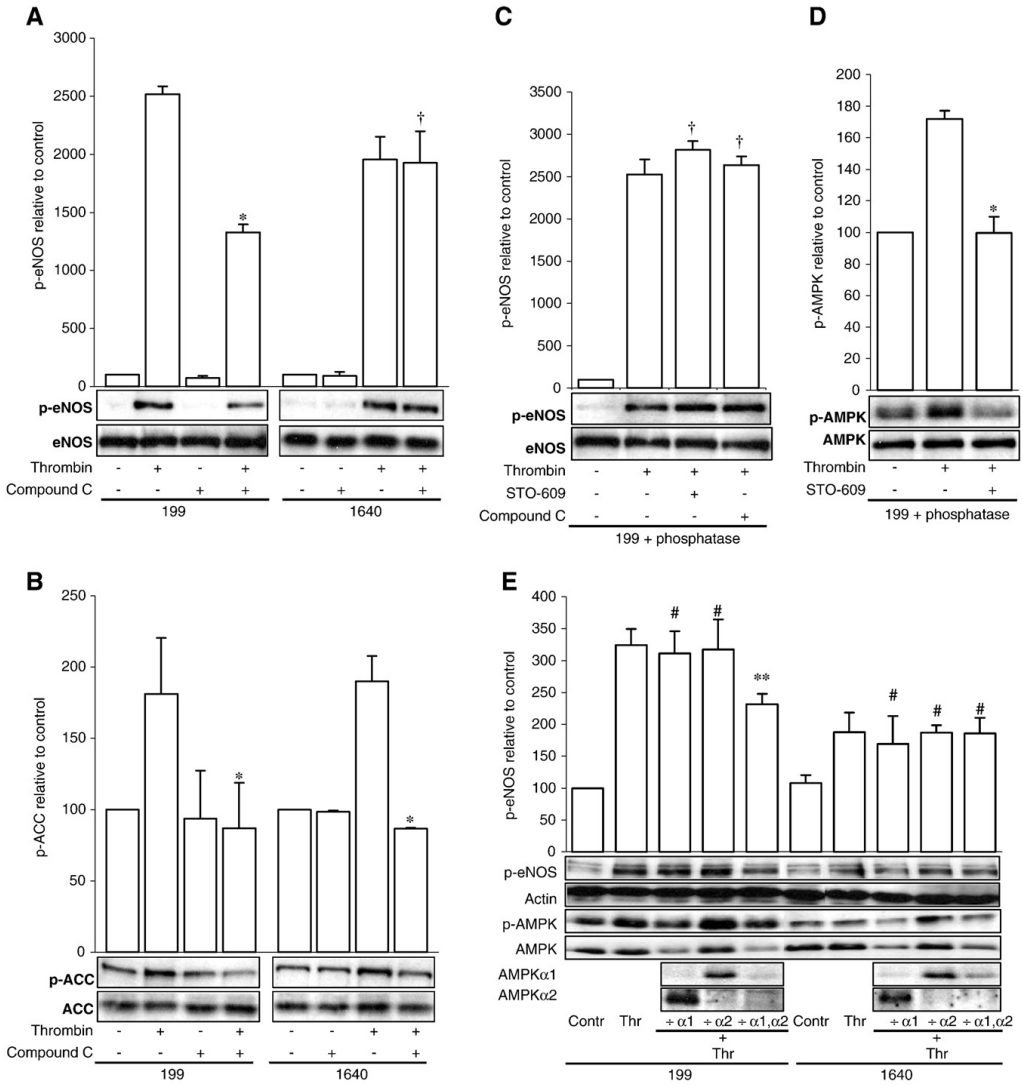

Fig. 5. AMPK inhibition in different media and the effects on thrombin mediated phosphorylation of ACC and eNOS. The effects of 20 min pre-treatment with compound C (10 mM) on the phosphorylation of (A) eNOS (Ser1177) and (B) ACC (Ser79) mediated by thrombin (1 U/mL, 2 min). Modification by phosphatase (0.2 mg/mL, 20 min) of the effects of Compound C or STO-609 (10 μM) on thrombin-induced phosphorylation of (C) eNOS and (D) AMPK (Thr172) in medium 199. (E) Total AMPK, AMPKα1 and AMPKα2 expression and thrombin-stimulated phosphorylation of AMPK and eNOS after treatment of cells with siRNA for AMPKα1 (-α1) or AMPKα2 (-α2) or both simultaneously (-α1,α2) in medium 199 or 1640. Cells were treated and results expressed as described in Fig. 2 and show the average±S.D. of three independent experiments. (†ns/*$p<0.05$ cells treated with inhibitor and thrombin vs. cells treated with thrombin alone; #ns/**$p<0.05$ cells treated with thrombin and AMPK siRNA vs. cells treated with thrombin alone).

and measured AMPKα1 and AMPKα2 expression by Western blot analysis using isoform specific antibodies. The results are shown in Fig. 5E. siRNA for AMPKα1 and -α2 suppressed the expression of AMPKα1 and AMPKα2, respectively. siRNA downregulation of either isoform had no effect on eNOS phosphorylation in media 199 or 1640. However, simultaneous downregulation of both AMPKα1 and AMPKα2 partially inhibited the phosphorylation of eNOS in medium 199 but had no effect in medium 1640.

The effect of AMPKα siRNA on NO-production was tested by monitoring the accumulation of cGMP in cells after thrombin stimulation in different media. The results are shown in Fig. 6. In agreement with the phosphorylation results, there was greater cGMP-production in medium 199 than in medium 1640. Furthermore, AMPKα siRNA reduced the cGMP-production after thrombin stimulation only in medium 199.

In view of our results with tween 80 and ATP levels in medium 1640, we tested the effects of tween 80 on AMPK and eNOS phosphorylation. As seen in Fig. 7A, adding tween 80 to medium 1640 changed the effects of STO-609 on thrombin mediated AMPK phosphorylation towards the response seen in medium 199 (Fig. 2A).

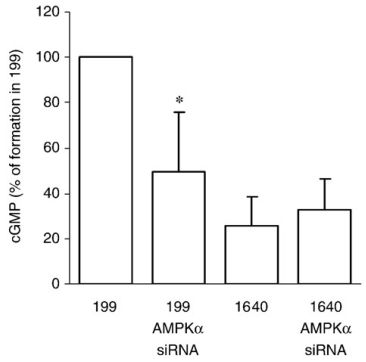

Fig. 6. Thrombin-induced cGMP formation. Effect of simultaneous pre-treatment with AMPKα1 and AMPKα2 siRNA in medium 199 and 1640. Endothelial cells were stimulated by thrombin (1 U/mL, 2 min) with or without pre-treatment with AMPKα1 and -α2 siRNA. Intracellular cGMP was determined using an enzyme immunoassay kit as described in Materials and methods. The results are expressed as percent of the response in medium 199 without siRNA pre-treatment and show the average±S.D. of four independent experiments each done in duplicate. (*$p<0.05$ vs. cells treated with thrombin alone in medium 199).

Also, under these conditions thrombin mediated eNOS phosphorylation was partially inhibited by STO-609 and compound C (Fig. 7B) as seen in medium 199 (Figs. 2C and 5A). The effects of these inhibitors on eNOS phosphorylation also changed in Williams medium after the addition of tween 80 (Fig. 7C). Similarly, when tween 80 was added to Williams medium, AMPK phosphorylation was partially inhibited by STO-609 in contrast to a total inhibition in Williams medium alone (Fig. 7D). Table 1 summarises our findings allowing comparison of the effects of STO-609 on thrombin-induced phosphorylation of AMPK and eNOS as well as the effect of A23187 on ATP levels in different media.

4. Discussion

Data presented in this paper show that in cultured endothelial cells, two upstream pathways are involved in mediating the effects of thrombin stimulation on AMPK phosphorylation and activation, one pathway involving stimulation of CaMKK and another dependent on a rise in AMP/ATP ratio presumably involving LKB1. Furthermore, we demonstrate that environmental/culture conditions dictate which signal transduction pathways are activated by thrombin stimulation. Under culture conditions that do not allow or facilitate a fall in ATP after stimulation with thrombin AMPK activation is exclusively mediated by CaMKK and does not contribute to the phosphorylation of eNOS. In contrast, conditions subjecting endothelial cells to a fall in ATP when stimulated with thrombin facilitate activation of AMPK-dependent signal transduction pathways which stimulate downstream phosphorylation of eNOS.

Thus, our results suggest that only AMPK activated in response to a fall in ATP is involved in the phosphorylation of eNOS. A possible explanation may be provided by the allosteric effects that AMP has on AMPK [19–21], independent of the upstream AMPK kinase. Sanders et al. [19] have proposed a revised model for activation of AMPK that accounts for activation of AMPK by two distinct signals: a Ca^{+2}-dependent pathway, mediated by CaMKKβ and an AMP-dependent pathway, mediated by LKB1. In circumstances, where AMP and Ca^{+2} rise in concert, both pathways could operate. In such conditions both CaMKKβ and LKB1 would contribute to AMPK activation as we have demonstrated in medium 199 and in 1640 when the cells were pre-treated with 2-deoxy-glucose. Thus, the activation of AMPK based on elevation of AMP may be critical for AMPK-dependent eNOS phosphorylation mediated by thrombin in endothelial cells but this proposed mechanism needs further study.

In view of marked differences between the composition of medium 199 and medium 1640 we examined several possibilities that could explain the different responses to stimulation in the different media. These included the presence of purines (ATP, UTP, adenosine, xanthine and hypoxanthine), cholesterol and vitamins, including tocopheryl phosphate, in medium 199. However, after these were excluded we found a clear difference in ATP levels within cells after stimulation depending on the presence of the non-ionic detergent tween 80 in the medium. Adding tween 80 (0.2 mg/L) to medium 1640 resulted in a 15% fall in cellular ATP after ionophore treatment. Also, the addition of tween 80 to medium 1640 resulted in a similar activation of downstream pathways after thrombin treatment (AMPK, ACC and eNOS phosphorylation) as in cells stimulated in medium 199. However, tween 80 is not responsible for the entire ATP lowering observed after ionophore treatment in medium 199. The non-involvement of nucleotides was particularly unexpected as the ATP lowering was prevented by apyrase or phosphatase pre-treatment.

Tween 80 is added to culture media in miniscule amounts to facilitate solubilisation of cholesterol. Its addition obviously represents an artificial condition without a definite physiological or pathophysiological significance. However, in this study the experimental conditions including tween 80 allowed energy deprivation within endothelial cells after thrombin stimulation thus uncovering the two pathways for AMPK activation, one of which mediates the downstream activation of eNOS. Also, the conditions for the activation of these pathways, i.e. energy deprivation or Ca^{+2}-release, were identified. Finally, energy deprivation is of obvious interest and potential importance in pathophysiology.

Effects of histamine and thrombin on intracellular energy status have previously been reported. It has been shown that thrombin increases the extracellular acidification rate of endothelial cells, suggesting its stimulation of rapid metabolic responses [22]. Similar results have been demonstrated after treatment with histamine [23]. These reports are consistent with our findings that thrombin lowers cellular ATP levels in endothelial cells which we suggest plays a role in an AMPK-dependent pathway mediating stimulatory signals from G-protein coupled receptors to eNOS phosphorylation in medium 199.

While the basis for the different downstream effects of AMPK depending on its mode of activation is unclear, a possible explanation might involve isoform specific phosphorylation of eNOS. In skeletal muscle, heart and liver, the two isoforms of AMPK are differently activated [24–30]. The α_2 isoform has in several reports been shown to be activated by LKB1 [24,26] or the adenosine analogue 5-aminoimidazole-4-carboxamide-1-β-4 ribofuranoside (AICAR) [27,28], while the α_1 isoform is stimulated by CaMKK [29] independently of AMP concentration [26]. It has also been observed in vitro that complexes containing the α_2 rather than the α_1 isoform have a greater dependence on AMP both in direct allosteric activation and in reactivation by an upstream kinase [30]. Tzatsos and Tsichlis recently demonstrated, using several agonists, that in HEK293 cells, activation of the α_1 subunit was mediated by CaMKKβ whereas the activation of α_2 was mediated by LKB1 [31]. Finally, two reports have just been published, demonstrating isoform specific phosphorylation of AMPK targets. McGee et al. demonstrated in skeletal muscle that the α1 isoform, independently of LKB1, mediated the phosphorylation of the mammalian target of rapamycin complex 1 (mTORC1) leading to inhibition of muscle growth [32]. Qin and De Vries demonstrated that although phosphorylation of the α1 isoform is dominant in RPE cells after H_2O_2 treatment, the phosphorylation of the AMPK substrate ACC as well as the effects on phagocytosis are mediated by the α2 subunit [33].

Endothelial cells also dominantly express the α1 isoform of AMPK [34] and it has even been doubted whether they express the α2 isoform at all [35]. While in the present study probing with

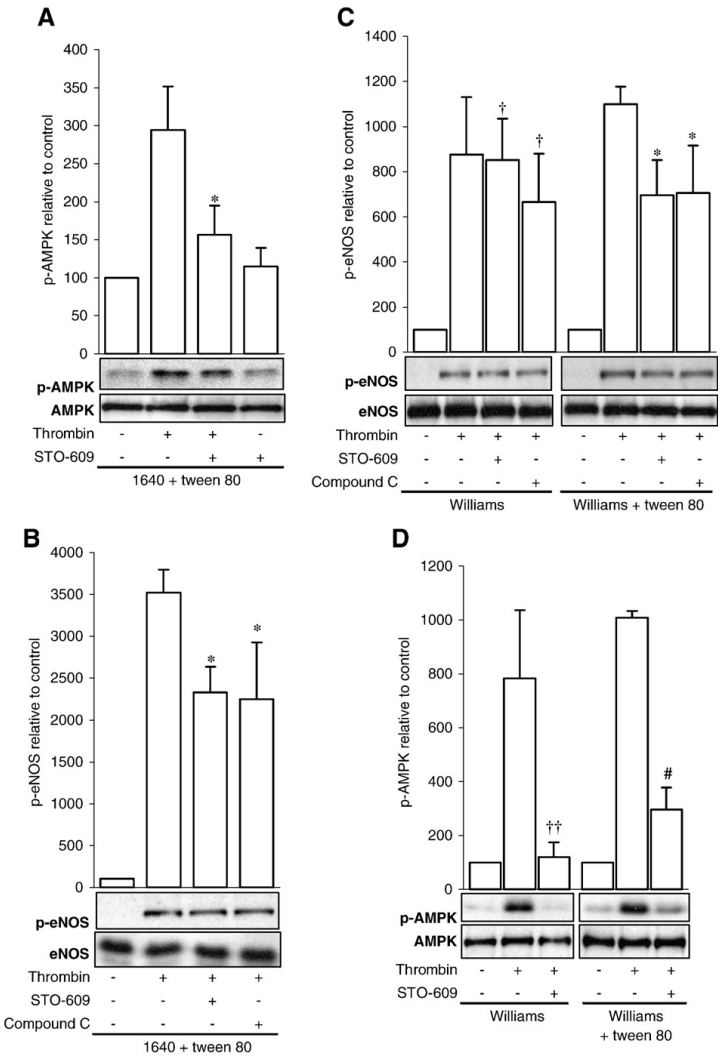

Fig. 7. The effects of tween 80 on the phosphorylation of eNOS and AMPK in different media. (A) The effects of 20 min pre-treatment with STO-609 (10 μM) on AMPK phosphorylation (Thr172) after thrombin (1 U/mL, 2 min) in 1640 with tween 80 (20 mg/L). (B) The effects of 20 min pre-treatment with STO-609 or compound C (10 μM) on thrombin mediated eNOS phosphorylation (Ser1177) in medium 1640 with tween 80. (C) eNOS phosphorylation after thrombin treatment and the effects of 20 min pre-treatment with STO-609 or compound C in Williams medium with or without tween 80. (D) AMPK phosphorylation after thrombin and the effects of 20 min pre-treatment with STO-609 in Williams medium with or without tween 80. Cells were treated and results expressed as described in Fig. 2 and show the average ± S.D. of three independent experiments. (†ns/*$p<0.05$ cells treated with inhibitor and thrombin vs. cells treated with thrombin alone; ††ns/#$p<0.05$ vs. unstimulated cells).

phosphospecific antibody showed most of the phosphate to be on the α1 subunit the expression of both isoforms was clearly demonstrated by the use of isoform specific antibodies. Furthermore, each isoform could be downregulated separately by isoform specific siRNA. Although such separate interference with either isoform did not affect eNOS phosphorylation simultaneous treatment with both α1 and α2 siRNA caused 42% inhibition of eNOS phosphorylation and a significant reduction in cGMP-production in cells maintained in culture medium 199. This treatment had no effect on eNOS phosphorylation or activity in medium 1640. These results strengthen the conclusions from the work with the AMPK inhibitor Compound C but do not support isoform specific phosphorylation of eNOS. Other isoform specific effects are, however, not excluded and we observed that treatment with α2 siRNA for more than 48 h caused morphological changes of the cells not caused by α1 siRNA (not shown).

Stahmann et al. [14] used thrombin as an agonist and showed total inhibition of AMPK phosphorylation by STO-609 in HUVEC. They demonstrated that eNOS phosphorylation by thrombin was not caused by AMPK since inhibition of CaMKK or AMPK or their downregulation by siRNA had no effects on eNOS phosphorylation or NO-synthesis. Neither did they see any changes in intracellular ATP levels after treatment with thrombin. These results are similar to our

Table 1
Comparison of the effect of STO-609 on AMPK and eNOS phosphorylation after thrombin treatment in different media and comparison of the effect of A23187 on cellular ATP levels in different media

	% inhibition of AMPK phosphorylation	% inhibition of eNOS phosphorylation	% ATP lowering
Medium	STO-609	STO-609	
199	72±11	54±14	53±3
199+ phosphatase	100±15	0	9±1
1640	95±6	1±14	2±4
1640+ tween	69±21	35±6	15±6
Williams	99±8	0±16	17±9
Williams+ tween	76±9	41±11	50±12

Data were expressed as percent inhibition of phosphorylation relative to thrombin-stimulated cells without inhibitors and percent lowering of ATP after A23187 treatment relative to unstimulated cells. Data taken from experiments presented in Figs. 1–7.

findings in experiments carried out in culture medium 1640 and Williams or in medium 199 after treatment with apyrase or acid phosphatase. However, in medium 199 or after addition of tween 80 to Williams or 1640, STO-609 only caused partial inhibition of AMPK phosphorylation revealing involvement of a different AMPK kinase, presumably LKB1. Under these circumstances, STO-609 partially inhibited eNOS phosphorylation. Furthermore, as summarized in Table 1, these are the conditions where activation caused a decrease in ATP levels allowing allosteric activation of AMPK as well as phosphorylation by LKB1.

This study did not address the mechanisms by which tween 80 or other constituents of culture medium 199 facilitate the lowering of ATP after thrombin stimulation. Several potentially important upstream events were not studied either such as changes in G-protein coupling and/or interaction with the receptor nor changes in the localization of LKB1 or eNOS.

In conclusion, we show that culture conditions affect endothelial signal transduction pathways. Conditions that subject endothelial cells to a fall in ATP after thrombin stimulation facilitate activation of pathways that are partly dependent on AMPK, cause downstream phosphorylation of eNOS and enhance the magnitude of the response. In contrast, under culture conditions that do not facilitate a fall in ATP after stimulation AMPK activation is exclusively mediated by CaMKK and does not contribute to the phosphorylation of eNOS. In addition to furthering our knowledge on the mechanisms of thrombin mediated stimulation of eNOS, this work has demonstrated how environmental conditions can dictate which signal transduction pathways are activated in endothelial cells and specifically highlighted the importance of cellular energy levels in endothelial signalling.

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Paper IV

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eNOS activation mediated by AMPK after stimulation of endothelial cells with histamine or thrombin is dependent on LKB1

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ABSTRACT

Reports on the role of AMP-activated protein kinase (AMPK) in thrombin-mediated activation of endothelial nitric-oxide synthase (eNOS) in endothelial cells have been conflicting. Previously, we have shown that under culture conditions that allow reduction of ATP-levels after stimulation, activation of AMPK contributes to eNOS phosphorylation and activation in endothelial cells after treatment with thrombin. In this paper we examined the signaling pathways mediating phosphorylation and activation of eNOS after stimulation of cultured human umbilical vein endothelial cells (HUVEC) with histamine and the role of LKB1-AMPK in the signaling. In Morgan's medium 199 intracellular ATP was lowered by treatment with histamine or the ionophore A23187 while in medium RMPI 1640 ATP was unchanged after identical treatment. In medium 199 inhibition of Ca^{+2}/CaM kinase kinase (CaMKK) by STO-609 only partially inhibited AMPK phosphorylation but after gene silencing of LKB1 with siRNA there was a total inhibition of AMPK phosphorylation by STO-609 after treatment with either histamine or thrombin, demonstrating phosphorylation of AMPK by both upstream kinases, LKB1 and CaMKK. Down-regulation of AMPK with siRNA partially inhibited eNOS phosphorylation caused by histamine in cells maintained in medium 199. Downregulation of LKB1 by siRNA inhibited both phosphorylation and activity of eNOS and addition of the AMPK inhibitor Compound C had no further effect on eNOS phosphorylation. When experiments were carried out in medium 1640, STO-609 totally prevented the phosphorylation of AMPK without affecting eNOS phosphorylation. AMPKα2 downregulation resulted in a loss of the integrity of the endothelial monolayer and increased expression of GRP78, indicative of endoplasmic reticular (ER) stress. Downregulation of AMPKα1 had no such effect. The results show that culture conditions affect endothelial signal transduction pathways after histamine stimulation. Under conditions where intracellular ATP is lowered by histamine, AMPK is activated by both LKB1 and CaMKK and, in turn, mediates eNOS phosphorylation in an LKB1 dependent manner. Both AMPKα1 and −α2 are involved in the signaling. Under conditions where intracellular ATP is unchanged after histamine treatment, CaMKK alone activates AMPK and eNOS is phosphorylated and activated independent of AMPK.

1. Introduction

Because of its unique location the endothelial layer is constantly exposed to a variety of stimulatory interactions, physical, humoral and cellular. The transduction of these signals is critical for an appropriate cellular response while derailment of the signaling can be a key feature of a pathologic response or disease [1]. One of the crucial responses to a variety of signals is the production of nitric-oxide (NO) from arginine through the activation of endothelial NO-synthase (eNOS) [2]. While a complex network of pathways, numerous kinases and many phosphorylation sites are involved in the regulation of eNOS activity, phosphorylation at the most thoroughly studied site at Ser1177 is generally found to be a critical requirement for eNOS activation [3]. The best characterized pathway mediating this phosphorylation is the phosphatidylinositol 3-kinase (PI3K)–Akt cascade [4,5]. However, eNOS phosphorylation by AMPK has been observed in a variety of conditions e.g. after treatment with AICAR [6], metformin [7], PPAR agonists [8], adiponectin [9], VEGF [10] or ICAM-1 [11] and under hypoxic conditions [12]. While the role of AMPK as a metabolic masterswitch has been well established in tissues such as the skeletal and cardiac muscle its precise role in endothelial cell metabolism is less well understood [13]. It has been suggested that endothelial cells exist in a permanent state of "metabolic hypoxia"

Abbreviations: ACC, acetyl coenzyme A carboxylase; AMPK, AMP-activated protein kinase; CaMKK, Ca^{+2}/CaM kinase kinase; eNOS, endothelial NO-synthase; ER stress, endoplasmic reticular stress; HUVEC, human umbilical vein endothelial cells; NO, nitric-oxide; PI3K, phosphoinositide 3-kinase; ROS, reactive oxygen species; VEGF, vascular endothelial growth factor

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[14] and that AMPK in endothelial cells may have a more important role in the maintenance of endothelial function and in affecting signaling cascades than as an intracellular fuel gauge [13].

We have previously reported that in primary cultures of human umbilical vein endothelial cells (HUVEC) thrombin stimulates eNOS phosphorylation at Ser 1177 via a pathway that is partly dependent on AMPK [15] but independent of PI3K-Akt [16]. This was demonstrated both by using an inhibitor of AMPK (Compound C) and by gene silencing of both α1 and α2 isoforms of AMPK by siRNA which also caused a reduction in eNOS activity. However, this pathway was only activated under culture conditions that allowed a sharp but brief fall in cellular ATP after thrombin stimulation (Morgan's medium 199). Under culture conditions that prevented or did not allow such a fall in the energy stores of the cells (medium RPMI 1640) AMPK was still activated but exclusively via a different upstream pathway (CaMKK-mediated activation of AMPK) and downstream this activation of AMPK played no role in the activation of eNOS. Thus, after thrombin stimulation of HUVEC we found both AMPK-dependent and AMPK-independent stimulation of eNOS. The mechanism of the AMPK-independent eNOS activation after thrombin treatment, seems to be identical to that reported by Stahmann et al. [17] using similar culture conditions as those not allowing ATP fall after stimulation. However, under those same culture conditions, energy deprivation by 2-deoxy-glucose resulted in an activation of the AMPK-eNOS pathway after stimulation with thrombin [15]. Hypoxia, which presumably causes ATP depletion, has also been shown to cause AMPK-dependent eNOS phosphorylation (activation) [12]. Most recently, however, it has been demonstrated that VEGF activates AMPK via a Ca^{+2}/CaMKKβ-dependent pathway and that the VEGF-stimulated eNOS activation is independent of AMPK even when VEGF treatment is combined with 2-deoxy-glucose to cause energy deprivation [18]. In contrast to thrombin or histamine, VEGF activates eNOS via a PI3K-Akt dependent pathway although AMPK mediated eNOS phosphorylation has also been reported after VEGF treatment [10].

The upstream AMPK kinase mediating the AMPK activation that takes place when ATP falls is not known but has been presumed to be LKB1 [19]. In this paper, using gene silencing by siRNA, we provide direct evidence that LKB1 is necessary for the stimulation of the AMPK-dependent pathway of eNOS activation that partly mediates the NO-response to thrombin stimulation under conditions allowing a fall in cellular ATP levels. Furthermore, we show that this pathway is also activated by histamine, another agonist binding to a G-protein linked receptor on the endothelial surface, as well as by the Ca^{+2}-ionophore A23187 which also mediates reduction in ATP-levels.

We also show that downregulation of the α2 isoform of AMPK resulted in a loss of the integrity of the endothelial monolayer possibly linked to endoplasmic reticular (ER) stress whereas downregulation of the other isoform, α1, had no such effect. Thus, the importance of α2 is further emphasized although the α1 isoform is expressed to a greater extent in HUVEC than the α2 isoform.

2. Materials and methods

2.1. Materials

EBM-2 was purchased from Clonetics. Other cell culture media, Foetal Bovine Serum and Penicillin-Streptomycin were purchased from Gibco Brl, Life Technologies. Tissue culture plates (35 mm) and flasks (50 mL) were from Nunc, Cryotin X (trypsin) from cod was provided by The Science Institute of Iceland. Thrombin, histamine, A23187, Tempol and Trypsin-EDTA Solution were purchased from Sigma. STO-609 and Compound C were from Calbiochem. Hybond ECL Nitrocellulose membrane (6×8 cm), ECL+PLUS Western blotting detection system and Hyperfilm ECL High performance chemiluminescence film were from Amersham Pharmacia Biotech. Cyclic GMP XP Assay kit and antibodies against eNOS, phospho-eNOS (Ser1177), AMPK, phospho-AMPK (Thr172), AMPKα1, AMPKα2, LKB1, phospho-LKB1, GRP78, $p47^{phox}$, Pan-Actin and Anti-Rabbit IgG/HRP-linked came from Cell Signaling Technology. Antibodies against ACC and phospho-ACC (Ser79) were from Upstate. Lipofectamine™ RNAiMAX Reagent and 5-(and 6)-carboxy-2′7′ dichlorodihydofluorescein deacetate (carboxy-H_2DCFDA) were from Invitrogen. Validated siRNA against AMPKα1 (s100), AMPKα2 (s11057) and LKB1 (s13580) came from Ambion as well as a negative control siRNA (Silencer Negative Control #1 with a sequence that does not target any gene product). Eliten kit was purchased from Promega. $gp91^{phox}$ antibody came from Santa Cruz.

2.2. Cell culture

Endothelial cells were cultured from human umbilical veins by a modification of the method of Jaffe et al. as previously reported [20]. The cells were harvested by Cryotin X trypsin digestion and seeded on 35 mm culture dishes in Morgan's medium 199 containing 20% foetal bovine serum and antibiotics (penicillin, 100 units/mL and streptomycin, 100 μg/mL). The culture dishes were incubated at 37 °C in humidified air with 5% CO_2. The medium was changed 24 h after seeding the cells and then every 2–3 days thereafter until the cell culture reached confluence (after ~7 days).

When confluent the cells were washed with the appropriate medium and placed in 1.0 mL serum free medium with or without inhibitors at the indicated concentrations. Agonist was added 10 to 20 min later in a concentration calculated to reach the intended concentration for each experiment and left on for additional 2 to 3 min. The agonists were then removed along with the medium and cellular reactions terminated by adding 250 μL SDS sample buffer. The samples were boiled for 5 min and centrifuged for 10 min at 3000 rpm. The samples were then ready to be either used or stored at −20 °C.

2.3. siRNA transfection

Endothelial cells, grown to approximately 80% confluence in 25 cm^2 tissue culture flasks, were trypsinized and diluted sixfold on to 35 mm culture dishes. 24 h later, the cells were transfected with Lipofectamine™ RNAiMAX transfecting agent containing siRNA for AMPKα1, AMPKα2 or LKB1 (20nM) in an antibiotics-free EBM-2 medium containing 6% serum. Cells were cultured for 44–48 h and protein expression analyzed by Western blotting.

2.4. Electrophoresis and immunoblotting

Samples (8 μL) were resolved by SDS-PAGE (10%). The gels were blotted and the proteins thereby transferred to nitrocellulose. The membranes were hybridized with the indicated antibodies and subsequently with a secondary antibody (Anti-Rabbit IgG/HRP-linked). The immuno-complexes were detected with ECL+PLUS Western blotting detection system and developed onto a film. Equal loading was ascertained by hybridizing membranes with antibodies against unphosphorylated protein. The band intensity was quantified using Kodak 3.5 software.

2.5. Measurement of ATP

ATP was determined by luciferase assay. For validation HPLC was used in which case AMP was also determined (data not shown). For ATP determination using the luciferase assay the medium was removed from the cells and the cells lyzed by adding 0.5 mL of 0.5 N perchloric acid. After 30 min a 10 μL sample was diluted ×100 in water mixed with 80 μL of a luciferase mixture from an Eliten kit by Promega.

2.6. Cell morphology

Cells were treated with siRNA for AMPKα1 or −α2 as described in Section 2.3 with or without the indicated inhibitor. After 67 h of treatment with siRNA the cells were washed with medium 199 without serum and placed in that medium for additional 30 min, again with or without an inhibitor. Cell morphology was monitored with confocal microscopy and photographic images obtained at the indicated intervals using Leica DFC310 FX.

2.7. Determination of ROS levels

For determination of ROS levels, cells were washed twice with PBS and incubated with 10 μM carboxy-H_2DCFDA which is taken up by the cells and cleaved by intracellular esterases and transformed to a fluorescent dye when oxidized. After 15 min the cells were trypsinized and fluorescence analyzed by flow cytometry.

2.8. cGMP measurements

HUVEC monolayers were washed with Morgan's medium 199 or medium1640 and placed in 1.0 mL Morgan's medium 199 or medium 1640 containing 0.25 mM IBMX. The cells were incubated for 17 min. at 37 °C. Histamine was then added to give a final concentration of 10 μM and the cells further incubated for 3 min. The reaction was stopped by removing the medium and adding 0.5 mL of 0.1 M HCl. Intracellular cGMP was determined using an enzyme immunoassay kit in accordance with the manufacturer's instructions.

2.9. Statistical analysis

Values are expressed as average ± S.D. Unpaired, two-tailed Student's *t*-test was performed for comparisons between groups. The level of significance was set at $p<0.05$. Software used was GraphPad Prism 5.00.

3. Results

3.1. The effects of histamine or A23187 on intracellular ATP in different media

To investigate the upstream mechanisms of AMPK activation after treatment with histamine or the Ca^{+2}-ionophore A23187, we measured the levels of intracellular ATP after stimulation (Fig. 1). In medium 199, treatment with histamine (10 μM, 3 min.) lowered intracellular ATP by 8 ± 1%. Treatment with the ionophore A23187 (0.6 μM, 3 min.) lowered intracellular ATP by 53 ± 3%. In medium 1640, the effects of histamine or A23187 on ATP was negligible (1 ± 4% increase and 2 ± 4% lowering, respectively). After 2-deoxy-glucose (20 mM, 7 min.) had been added to medium 1640 intracellular ATP was lowered by 7 ± 2%.

3.2. The effects of histamine or A23187 on AMPK phosphorylation — role of the upstream kinases LKB1 and CaMKK

In medium 199, both histamine and A23187 caused phosphorylation of AMPK that was partly inhibited by the CaMKK inhibitor STO-609, histamine stimulation by 76 ± 4% and A23187 stimulation by 14 ± 5% (Fig. 2A). In culture medium 1640, the phosphorylation of AMPK caused by these agonists was totally inhibited by STO-609 (105 ± 3% for histamine, 102 ± 2% for A23187) (Fig. 2A). After LKB1 downregulation there was a complete inhibition of histamine or thrombin mediated phosphorylation of AMPK by STO-609 in cells maintained in culture medium 199 (95 ± 5% and 102 ± 4%, respectively) (Fig. 2B). Similar response was found after stimulation with the Ca^{+2}-ionophore A23187 (data not shown). In contrast, in cells treated with control siRNA, AMPK phosphorylation after histamine or thrombin was partly inhibited by

Fig. 1. Effects of histamine or ionophore on ATP levels in different media. Comparison between the effects of 3 minute treatment with histamine (10 μM) and A23187 (0.6 μM) on ATP levels of cells in medium 199 or 1640. Effects of 2-deoxy-glucose treatment (20 mM, 7 min) on ATP levels in cells maintained in medium 1640. Results are expressed as % lowering of ATP level relative to control (unstimulated cells) and show the average ± S.D. of at least three independent experiments each done in duplicate. (†ns/*$p<0.001$/**$p<0.05$ vs control).

STO-609 (67 ± 2% and 72 ± 3%, respectively). As shown in Fig. 2C, LKB1 downregulation greatly reduced LKB1 expression without affecting AMPK expression (Fig. 2C).

To test the role of the two upstream AMPK kinases in the basal phosphorylation of AMPK, we measured AMPK phosphorylation in unstimulated cells treated with STO-609 or siRNA for LKB1 (Fig. 2C). In medium 199, downregulation of LKB1 lowered the basal phosphorylation of AMPK by 66 ± 8% whereas pretreatment with STO-609 had no effect. In medium 1640, AMPK phosphorylation was unaffected by these manipulations (not shown). Treatment of cells with control siRNA had no effect on basal AMPK phosphorylation (Fig. 2B) and LKB1expression was unaffected by treatment with AMPKα1 and/or −α2 siRNA (Fig. 3B).

To test the role of LKB1 phosphorylation in the response to histamine and thrombin we compared the phosphorylation of LKB1 at Ser428 in medium 199 and in medium 1640. As shown in Fig. 2D, both agonists caused similar phosphorylation of LKB1 at Ser428 in the two media.

3.3. The involvement of AMPK in eNOS phosphorylation after histamine

Histamine caused phosphorylation of eNOS in both media. However, in medium 199, simultaneous downregulation of AMPKα1 and −α2 by siRNA partially inhibited the phosphorylation of eNOS after treatment with histamine (51 ± 3% inhibition) whereas the same treatment had no effect in medium 1640. Downregulation of either AMPKα1 or −α2 separately had a nonsignificant effect in both media (Fig. 3A). Western blots showing specific siRNA knockdown of each isoform as well as an unchanged expression of LKB1 is demonstrated in Fig. 3B. Pretreatment with STO-609 (an inhibitor of AMPK as well as CaMKK [21]) partially inhibited histamine stimulated eNOS phosphorylation in medium 199 (42 ± 2% inhibition) compared to no effects in medium 1640 (4 ± 4% inhibition) (Fig. 3C). However, when cellular ATP was lowered by pretreatment with 2-deoxy-glucose, STO-609 inhibited histamine stimulated eNOS phosphorylation by 35 ± 2% in medium 1640, further supporting the partial contribution of AMPK in eNOS phosphorylation after histamine stimulation in cells where intracellular ATP is lowered. Interestingly, phosphorylation of ACC after histamine treatment (Fig. 3D) was largely inhibited in medium 199 after downregulation of AMPKα2 by siRNA (105 ± 51%), compared to the insignificant effect of AMPKα1

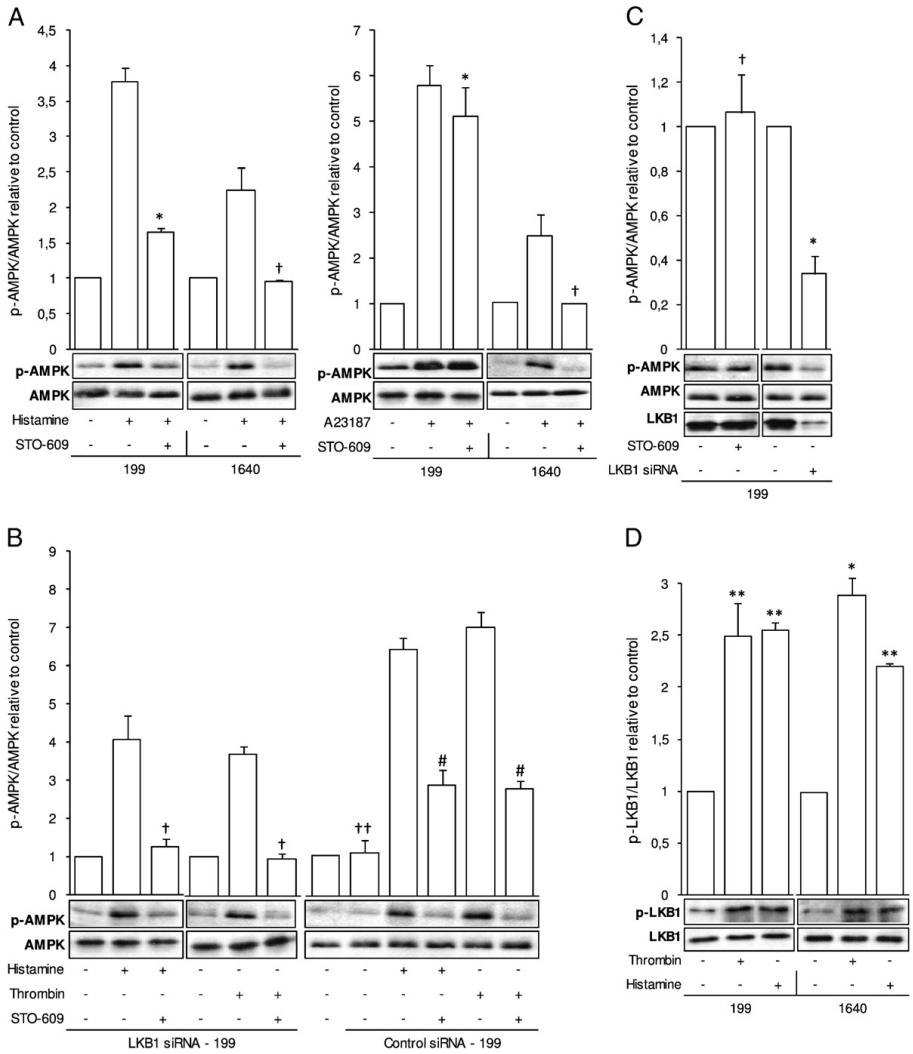

Fig. 2. The role of CaMKK and LKB1 in histamine, thrombin or ionophore mediated phosphorylation of AMPK and in basal phosphorylation of AMPK in different media. (A) The effects of 20 minute pre-treatment with STO-609 (10 μM) on AMPK phosphorylation (Thr172) in medium 199 or 1640 after treatment with histamine (10 μM, 2 min) or A23187 (0.6 μM, 2 min). (B) The effects of STO-609 on AMPK phosphorylation in cells in medium 199, pretreated with siRNA for LKB1 (20 nM) or control siRNA (20 nM) and then stimulated with histamine or thrombin (1 U/mL, 2 min). (C) The effects of STO-609 or LKB1 downregulation by siRNA on basal phosphorylation of AMPK in medium 199. Basal phosphorylation of AMPK in cells treated with control siRNA is shown in 2B. (D) Thrombin or histamine mediated phosphorylation of LKB1 (Ser428) in cells in either medium 199 or 1640. Confluent endothelial cells were stimulated as indicated, lysates were electrophorized and blotted as described in "Methods" and detected using antibodies against AMPK, Thr172 phosphorylated AMPK, LKB1 or Ser428 phosphorylated LKB1. The results are expressed as a ratio of phospho-AMPK:total AMPK or phospho-LKB1:total LKB1 relative to control and show the average ± S.D. of at least three independent experiments. (†ns/#$p<0.001$/*$p<0.01$/**$p<0.05$ vs untreated cells, ††ns vs cells without siRNA).

downregulation. These different effects between the two AMPK isoforms were not detected in medium 1640 where downregulation of either AMPKα1 or −α2 separately had little or no effects on ACC phosphorylation after histamine (−1 ± 3% and −7 ± 9% inhibition respectively). Downregulation of both AMPK isoforms simultaneously caused a total inhibition of ACC phosphorylation after histamine treatment both in medium 199 and 1640 (109 ± 36% and 115 ± 19% inhibition, respectively). Negative control siRNA had no effect on ACC-phosphorylation (not shown).

3.4. The role of LKB1 in the phosphorylation and activation of eNOS

As demonstrated previously, eNOS phosphorylation after histamine stimulation is partly dependent on AMPK under conditions where intracellular ATP is lowered by histamine treatment (Fig. 3A). AMPK phosphorylation is then mediated by both LKB1 and CaMKK (Fig. 2B). Downregulation of LKB1 reduced histamine induced eNOS phosphorylation by 64 ± 5% whereas control siRNA had no effect (not shown). Also, in cells treated with siRNA for LKB1, pretreatment with

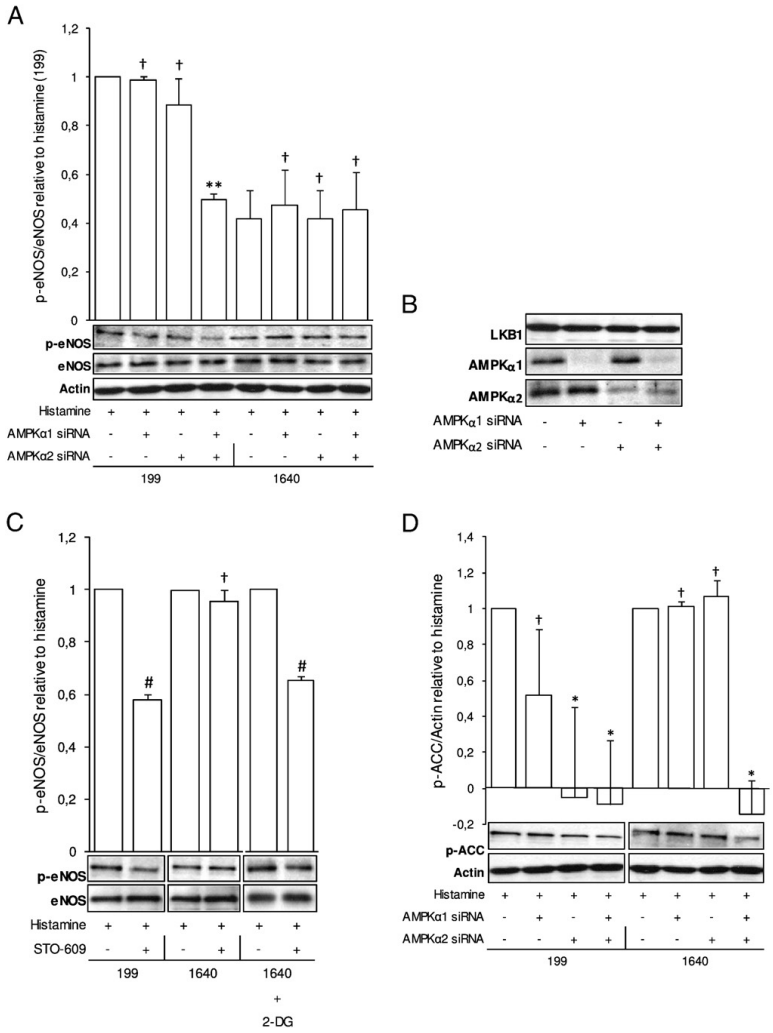

Fig. 3. The role of the two AMPKα isoforms in histamine mediated phosphorylation of eNOS and ACC in different media. (A) The effects of AMPKα1 and/or −α2 siRNA (20 nM) on eNOS phosphorylation (Ser1177) after treatment with histamine (10 μM, 3 min) in medium 199 or 1640. The results are normalized to the effects of histamine in cells maintained in 199 without siRNA treatment. (B) The effect of siRNA for AMPKα1 and/or −α2 on the expression of both AMPKα isoforms and LKB1 in HUVEC. (C) The effects of 20 minute pre-treatment with STO-609 (10 μM) on eNOS phosphorylation caused by histamine in medium 199, 1640 or 1640 with added 2-deoxy-glucose (2-DG) (20 mM, 7 min). The results are normalized to the effects of histamine alone in each medium. (D) The effects of AMPKα1 and/or −α2 siRNA (20 nM) on ACC phosphorylation (Ser79) after treatment with histamine (10 μM, 3 min) in medium 199 or 1640. The results are normalized to the effects of histamine in cells without siRNA treatment in both media. Cells were treated as described in "Methods" and results in A, C and D expressed as a ratio of phospho-eNOS:total eNOS or phospho-ACC:Actin relative to cells stimulated with histamine. Phosphorylation of control cells has been subtracted from all values causing some ratios to fall below zero. Results show the average ± S.D. of at least three independent experiments. (†ns/*$p<0.05$/**$p<0.01$/#$p<0.001$ vs cells treated with histamine alone).

STO-609 or the AMPK inhibitor Compound C had no further effect on eNOS phosphorylation by histamine in medium 199 (Fig. 4A). Treatment with control siRNA did not change the inhibitory effects of STO-609 or Compound C on eNOS phosphorylation after histamine (55 ± 2% and 61 ± 10% inhibition, respectively) (Fig. 4A). Thus, the results show that the histamine induced phosphorylation of eNOS mediated by AMPK is dependent on LKB1. Finally, to test for involvement of LKB1 in NO-production, we determined cGMP levels after histamine treatment. In cells treated with LKB1 specific siRNA, there was a 46 ± 10% reduction in the accumulation of cGMP after histamine stimulation, similar to the response in cells maintained in medium 1640 (Fig. 4B).

3.5. The role of AMPKα2 in maintaining endothelial monolayer integrity

Although AMPKα1 is expressed to a much greater extent in endothelial cells than the α2 isoform [15,22], the importance of the latter is not only manifested in the effects of AMPKα2 downregulation by siRNA on ACC phosphorylation but also by marked effects of AMPKα2 downregulation by siRNA on the morphology of the cell

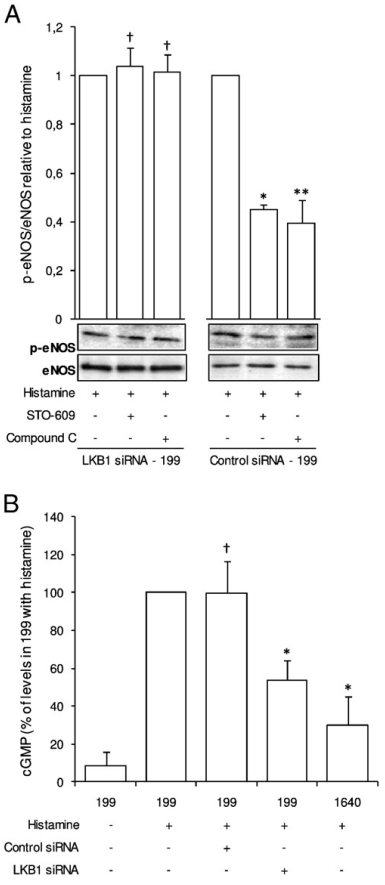

Fig. 4. The role of LKB1 in histamine mediated phosphorylation and activation of eNOS in medium 199. (A) The effects of 20 minute pretreatment with STO-609 (10 μM) or Compound C (10 μM) on histamine (10 μM, 3 min) mediated eNOS phosphorylation (Ser1177) in LKB1 downregulated cells (20 nM) or cells treated with control siRNA (20 nM). Cells were treated as described in "Methods". Results are normalized to the response to histamine alone either in cells pretreated with LKB1 or control siRNA and show the average ± S.D. of at least three independent experiments. (†ns/*$p<0.001$/**$p<0.01$ vs cells treated with histamine alone). (B) Cells were stimulated by histamine (10 μM, 10 min) in medium 199 or 1640. LKB1 was downregulated in medium 199 using LKB1 specific siRNA (20 nM). Intracellular cGMP was determined using an enzyme immunoassay kit as described in "Methods". The results are expressed as percent of the response to histamine in medium 199 without siRNA pre-treatment and show the average ± S.D. of four independent experiments each done in duplicate. (†ns/*$p<0.01$ vs cells treated with histamine in medium 199).

culture. HUVEC were treated with siRNA for AMPKα1 or −α2 for 67 h and then transferred to serum free media for up to 30 min. This resulted in deterioration of monolayer integrity in cells lacking AMPKα2, with shrinkage of the cells and loss of contact both between cells and with the culture substrate (Fig. 5A). Cells lacking the α1 isoform showed no morphological changes nor did control cells (not treated with siRNA). Treatment of the AMPKα2 downregulated cells with the SOD-mimetic Tempol markedly inhibited these morphologic changes. Downregulation of LKB1 did not affect the morphology (not shown). As shown in Fig. 5B the expression of the chaperone GRP78, an indicator of ER stress, was greatly enhanced after downregulation of AMPKα2 whereas downregulation of AMPKα1 had no such effect. Treatment with Tempol prevented the increase of GRP78 expression in cells treated with AMPKα2 siRNA. Downregulation of either AMPKα1 or AMPKα2 but not LKB1 increased the level of reactive oxygen species (ROS) as measured by DCF fluorescence (Fig. 5C). The increase in ROS levels in AMPKα downregulated cells was not affected by treatment with Tempol. AMPKα1 or −α2 downregulation had no effect on the expression of the $p47^{phox}$ subunit of NADPH-oxidase (Fig. 5D) nor the expression of the $gp91^{phox}$ subunit (data not shown).

4. Discussion

The heterotrimeric serine/threonin protein kinase AMPK maintains the balance between cellular ATP production and consumption and has been described as a "metabolic masterswitch" [23]. In endothelial cells, AMPK is activated by a variety of stimuli only some of which cause changes in the energy level of the cell [13]. While several studies have described phosphorylation of Ser1177 on eNOS by AMPK [6–12], other studies have disassociated AMPK activation from eNOS phosphorylation and activation [13,24].

In this study we found that in endothelial cells stimulated by histamine, culture conditions dictate which signal transduction pathways are activated. Under conditions where intracellular ATP is lowered after histamine stimulation, AMPK is activated by two upstream kinases, LKB1 and CaMKK, and in turn, mediates eNOS phosphorylation. Under conditions not facilitating ATP-lowering after histamine treatment AMPK is still activated, but only via the CaMKK pathway, and downstream, this AMPK-activation played no role in the phosphorylation of eNOS and there was much less NO-production. We have previously demonstrated a similar response in cells treated with thrombin [15]. It has been presumed that LKB1 is the upstream AMPK kinase mediating AMPK activation when ATP falls after stimulation. In this paper, using the gene silencing of LKB1 by siRNA, we directly demonstrate how LKB1 contributes to the AMPK dependent pathway of eNOS phosphorylation after treatment with the G-protein activating agonists histamine and thrombin as well as by the Ca^{+2}-ionophore A23187.

The activation of AMPK requires the phosphorylation of threonine 172 (Thr172) within the α catalytic subunit mediated by one or more upstream kinases (AMPKK) [23]. In mammals, two kinases have been identified as physiological kinases upstream of AMPK. These are LKB1 [19] and CaMKK [25] although Tak1 (transforming growth factor-beta-activated kinase) has been shown to phosphorylate and activate AMPK in a cell free system [26]. Additionally, in epithelial cells, TAK1 has been shown to activate AMPK independently of LKB1 or CaMKK [27]. The phosphorylation of AMPK Thr172 is reversible and the phosphatase PP2Cα is suggested to play a major role in the regulation of AMPK activity [28].

Recently, it has been demonstrated that AMP does not directly activate LKB1 [29] or CaMKK [25] or promote phosphorylation of AMPK by these upstream kinases [28]. A new model proposes two distinct mechanisms for AMP-mediated activation of AMPK, i.e. by a direct allosteric activation and by protection from phosphatases [28,30]. This model assumes that AMPK is phosphorylated by a Ca^{+2}-activated pathway dependent on CaMKK, and an AMP-dependent pathway, mediated by LKB1. The phosphorylating and activating effects of the constitutively active LKB1 on Thr172 would, according to this model, increase when the level of intracellular AMP rises and inhibits Thr172 dephosphorylation by phosphatases [28]. Conversely, since CaMKK requires a Ca^{+2} signal for activation which in turn causes phosphorylation of AMPK, this pathway is not dependent on AMP, as demonstrated by Stahmann and others [17,31]. Our findings that the basal phosphorylation of AMPK in endothelial cells is mediated by LKB1 without a contribution of CaMKK (Fig. 2C) is in concordance with this model.

The contribution of AMPK in mediating thrombin induced phosphorylation and activation of eNOS was originally discovered when it was realized that thrombin reduced basal phosphorylation of

Akt and inhibited EGF mediated phosphorylation of Akt while stimulating eNOS phosphorylation [32]. These experiments were carried out on HUVEC maintained in culture medium 199. Subsequently, Stahmann and coworkers showed total inhibition of thrombin mediated phosphorylation of AMPK by inhibiting the upstream kinase CaMKK by STO-609 [17]. Furthermore, in their system, they convincingly showed that eNOS phosphorylation by thrombin was not mediated by AMPK since inhibition of CaMKK or AMPK or their downregulation by siRNA had no effects on eNOS phosphorylation or NO-production, seemingly contradicting our previous findings. When we repeated our experiments in culture medium 1640 or Williams medium our results were identical to those of Stahmann and coworkers. The reconciliation came through the demonstration that thrombin stimulation of endothelial cells differentially affected cellular ATP levels, depending on the culture medium used. In culture medium 199 there is a fall in ATP after stimulation by thrombin [15] and, as shown in this paper, also after histamine as well as the ionophore A23187. In culture medium 1640 there is no such fall in ATP after stimulation with any of these agonists. As we previously showed with thrombin [15] and in this paper with histamine, AMPK is partly activated independent of CaMKK and contributes to phosphorylation of eNOS only under conditions that allow or facilitate an agonist induced fall in cellular ATP (medium 199 or medium 1640 with added 2-deoxy-glucose). Furthermore, by using gene silencing of LKB1 we show that the upstream AMPK kinase in this pathway is indeed LKB1. Only when AMPK is activated by this LKB1 dependent pathway does it contribute to the phosphorylation of eNOS (Fig. 4A). After LKB1 downregulation there is marked reduction in NO-production after histamine stimulation approaching the level observed in cells maintained in medium 1640. In medium 1640 ATP is not lowered after histamine stimulation and the LKB1-AMPK-eNOS pathway is not activated. In LKB1 downregulated cells neither STO-609 (an inhibitor of CaMKK and AMPK [21]) nor Compound C (an inhibitor of AMPK) had any inhibitory effects on histamine mediated eNOS phosphorylation demonstrating the dependence of the pathway on LKB1.

In view of marked differences between the composition of the two media, Morgan's 199 and RPMI 1640, we examined several possibilities that could explain the different responses to stimulation in the different media [15]. These included the presence of purines (ATP, UTP, adenosine, xanthine and hypoxanthine), cholesterol and vitamins in medium 199 and their absence in medium 1640. However, when the contribution of these ingredients had been excluded we found a clear difference in ATP levels within cells after stimulation depending on the presence of the non-ionic detergent tween 80 in the medium. Adding tween 80 to medium 1640 in the miniscule amounts (20 μg/mL) that are present in medium 199 resulted in a significant lowering of intracellular ATP after ionophore treatment and to a similar activation of downstream pathways after thrombin treatment (AMPK, ACC, eNOS phosphorylation) as in cells stimulated in medium 199 [15]. Addition of triton X-100, another non-ionic detergent, to medium 1640, had similar effects as the addition of tween 80 (data

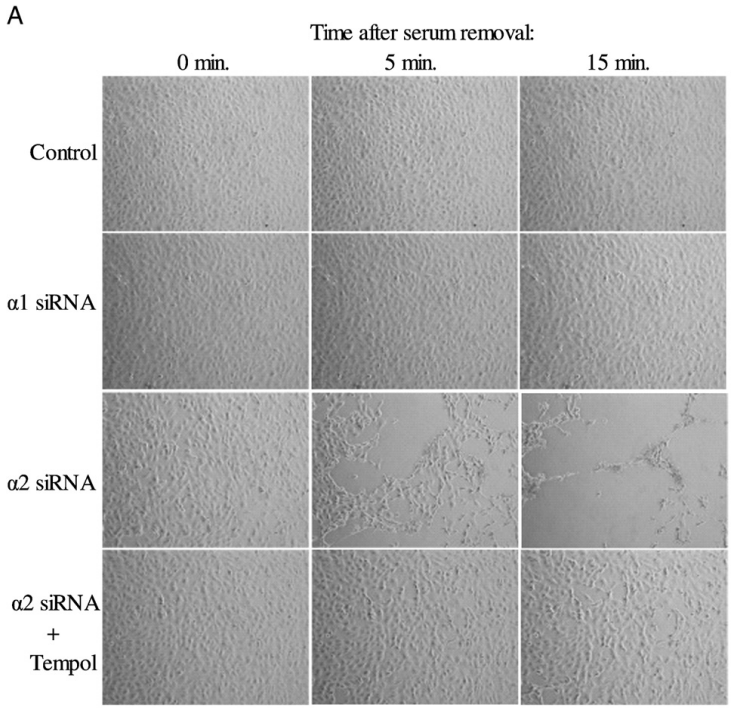

Fig. 5. The role of the two AMPKα isoforms in maintaining endothelial monolayer integrity. (A) A confocal image of HUVEC with or without siRNA for AMPKα1 or −α2 (20 nM, 67 h). The effect of cotreatment with Tempol (10 μM). (B) The effect of siRNA for AMPKα1 and/or −α2 (20 nM, 46 h) on the expression of GRP78 and the effects of Tempol (10 μM). (C) The effects of LKB1, AMPKα1 and/or −α2 siRNA (20 nM, 46 h) on ROS levels in HUVEC. The effect of cotreatment with Tempol (10 μM). (D) The effects of AMPKα1 and/or −α2 siRNA (20 nM, 46 h) on the expression of $p47^{phox}$ in HUVEC. Cells were treated as described in Methods and results expressed as a ratio of GRP78:Actin or $p47^{phox}$:Actin relative to control and show the average ± S.D. of at least three independent experiments. The level of ROS was measured as described in Methods and the results expressed as average ± S.D. of seven independent experiments each done in duplicate. (†ns/*$p<0.01$/**$p<0.05$ vs control, ††ns cells treated with AMPKα siRNA and Tempol vs cells treated with siRNA alone).

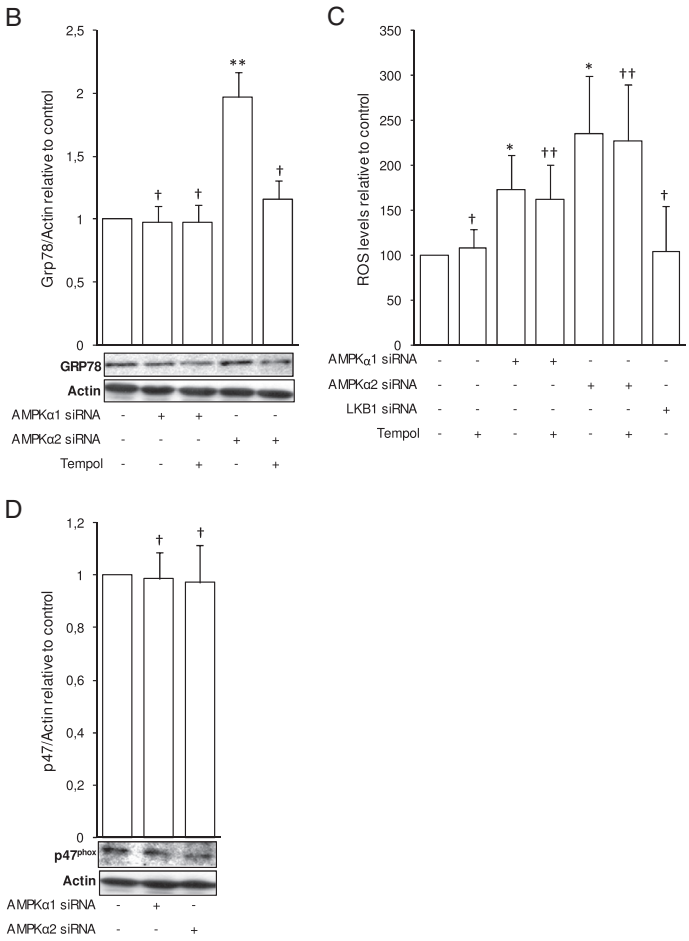

Fig. 5 (*continued*).

not shown), suggesting that the detergent properties contribute to the ATP fall after stimulation of HUVEC with ionophore, histamine or thrombin although not affecting ATP in unstimulated cells.

Recently it was reported that although VEGF treatment of HUVEC caused activation of AMPK as well as phosphorylation of eNOS at Ser1177, the phosphorylation was not dependent on AMPK activity [18]. Furthermore, AMPK did not phosphorylate eNOS even if the cells were cotreated with 2-deoxy-glucose to lower ATP levels. These results are in contrast to those previously reported by Reihill et al. [10] who, based on the effects of dominant negative AMPK and wortmannin, concluded that in human aortic endothelial cells both AMPK and Akt contributed to eNOS phosphorylation and activation after VEGF treatment. However, in contrast to our results with histamine or thrombin, the AMPK contribution of eNOS phosphorylation in the VEGF treated cells was mediated only by CaMKK and not by LKB1 as it was totally prevented by CaMKK siRNA. Martinelli et al. recently reported that ICAM-1, which does not activate PI3K-Akt, also causes AMPK-dependent eNOS phosphorylation via CaMKK in microvascular endothelial cells [11].

It has been suggested that phosphorylation of LKB1 at Ser428 facilitates activation of AMPK [33,34]. However, in melanocytes, this phosphorylation was shown to prevent activation of AMPK by LKB1 [35,36]. In our study, we found that the treatment of HUVEC with histamine or thrombin caused similar phosphorylation of LKB1 at Ser428 in medium 199 and medium 1640 (Fig. 2D). Thus, differences in LKB1 phosphorylation after stimulation cannot be invoked as an explanation for the differences in eNOS phosphorylation in the two media.

Although the α1 isoform is expressed to a greater extent in endothelial cells than the α2 isoform [6,22] and it has even been questioned whether the latter is expressed in endothelial cells at all [37], we previously found that both isoforms are present in primary cultures of HUVEC and that both contribute to the phosphorylation of eNOS [15]. Furthermore, endothelial cells cultured from AMPKα2 knockout mice have been found to produce much less NO after stimulation by Ca^{+2} ionophore than cells cultured from wild type mice [38].

Recently, Dong et al. found that the downregulation of AMPKα2 caused ER stress in HUVEC and although the α1 isoform was

expressed at much higher levels than the α2 isoform, downregulation of AMPKα2 caused greater ER stress [22]. Although we find that both isoforms of AMPKα have to be downregulated to prevent eNOS phosphorylation after either histamine or thrombin stimulation, we observe that in medium 199 downregulation of α2 alone is sufficient to prevent ACC phosphorylation. Furthermore, in our study the stress of serum removal produced almost instant morphologic changes in confluent monolayers of endothelial cells with downregulated AMPKα2 but not in cells with downregulated AMPKα1 or LKB1. These changes, involving shrinkage of cells with loss of intercellular contacts and contact between cells and substrate, were markedly inhibited or delayed by the SOD-mimetic Tempol. Downregulation of AMPKα2, but not α1, also caused ER stress as measured by an increase in GRP78 expression [39] and this increase was prevented by Tempol. Downregulation of AMPKα2 caused an increase in ROS levels as quantitated by DCF fluorescence. However, in contrast to the morphological changes and ER stress, the effect on ROS levels was also observed in AMPKα1 downregulated cells and was not prevented by Tempol. Colombo and Moncada have recently shown that α1 AMPK is a regulator of the antioxidant status of endothelial cells [40]. They observed that silencing of AMPKα1 in HUVEC caused a decrease in the expression of genes involved in antioxidant defences, including MnSOD, catalase and thioredoxin and an accumulation of ROS.

Regulation of antioxidant status by AMPK is also suggested by increased levels of antioxidant enzymes by agents such as metformin and AICAR which cause an increase in AMPK activity [41]. An increase in ROS level could also result from an increase in production and Wang et al. found increased expression of various components of NADPH oxidase in AMPKα2 downregulated cells [42]. We, however, did not observe any increase in the expression of $gp91^{phox}$ or $p47^{phox}$ in our experiments. More work is needed to clarify the role of AMPKα2 in maintaining endothelial monolayer integrity.

The demonstration of an LKB1-AMPK-eNOS signaling pathway in endothelial cells adds one more element of complexity to eNOS regulation (Fig. 6). It is of interest that environmental conditions dictate which pathway is activated in response to external stimulation

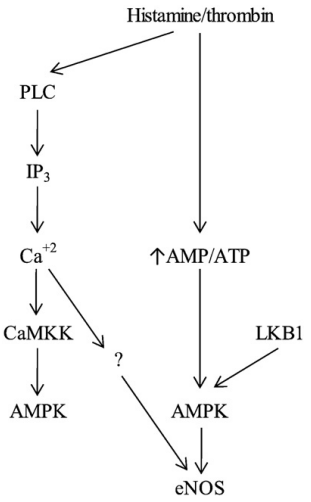

Fig. 6. Diagram showing the proposed pathways after endothelial stimulation with histamine or thrombin. Under conditions where intracellular ATP is lowered by histamine or thrombin, AMPK is activated by both LKB1 and CaMKK and, in turn, mediates eNOS phosphorylation and activation in an LKB1 dependent manner. Both AMPKα1 and −α2 are involved in the signaling. Under conditions where intracellular ATP is unchanged after histamine or thrombin treatment, CaMKK alone activates AMPK and eNOS is phosphorylated and activated independent of AMPK.

but even more so is the key role played by the cellular ATP level. A fall in the cellular energy level is a well known consequence of pathologic conditions such as ischemia, hypoxia and infection. AMPK-mediated vasodilation through NO would be one more example of how AMPK is involved in the provision of energy at many levels of biological organization [43].

In conclusion, we have shown that culture conditions affect endothelial signal transduction pathways. Under conditions where intracellular ATP is lowered by histamine, AMPK is activated by both LKB1 and CaMKK and, in turn, mediates eNOS phosphorylation and activation that is LKB1 dependent. Both the α1 and α2 isoforms of AMPK are involved. Conversely, under conditions where intracellular ATP is unchanged after histamine treatment, there is less NO-production, activation of AMPK is only dependent on CaMKK and eNOS is phosphorylated and activated independently of AMPK. Furthermore, we also show that AMPKα2 is necessary for maintaining integrity of the endothelial monolayer under the stress of serum removal, and that downregulation of AMPKα2 but not AMPKα1 results in an increased expression of GRP78 which is indicative of ER stress.

Acknowledgements

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