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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

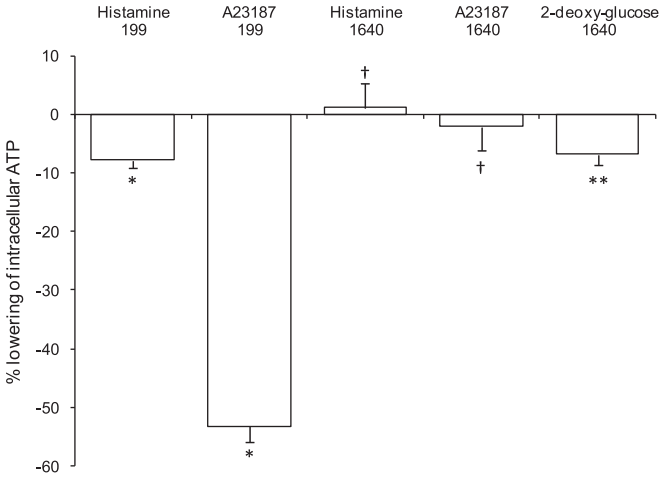

Fig. 1. Effects of histamine or ionophore on ATP levels in different media. Comparison between the effects of 3 minute treatment with histamine (10 μM) and A23187 (0.6 μM) on ATP levels of cells in medium 199 or 1640. Effects of 2-deoxy-glucose treatment (20 mM, 7 min) on ATP levels in cells maintained 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

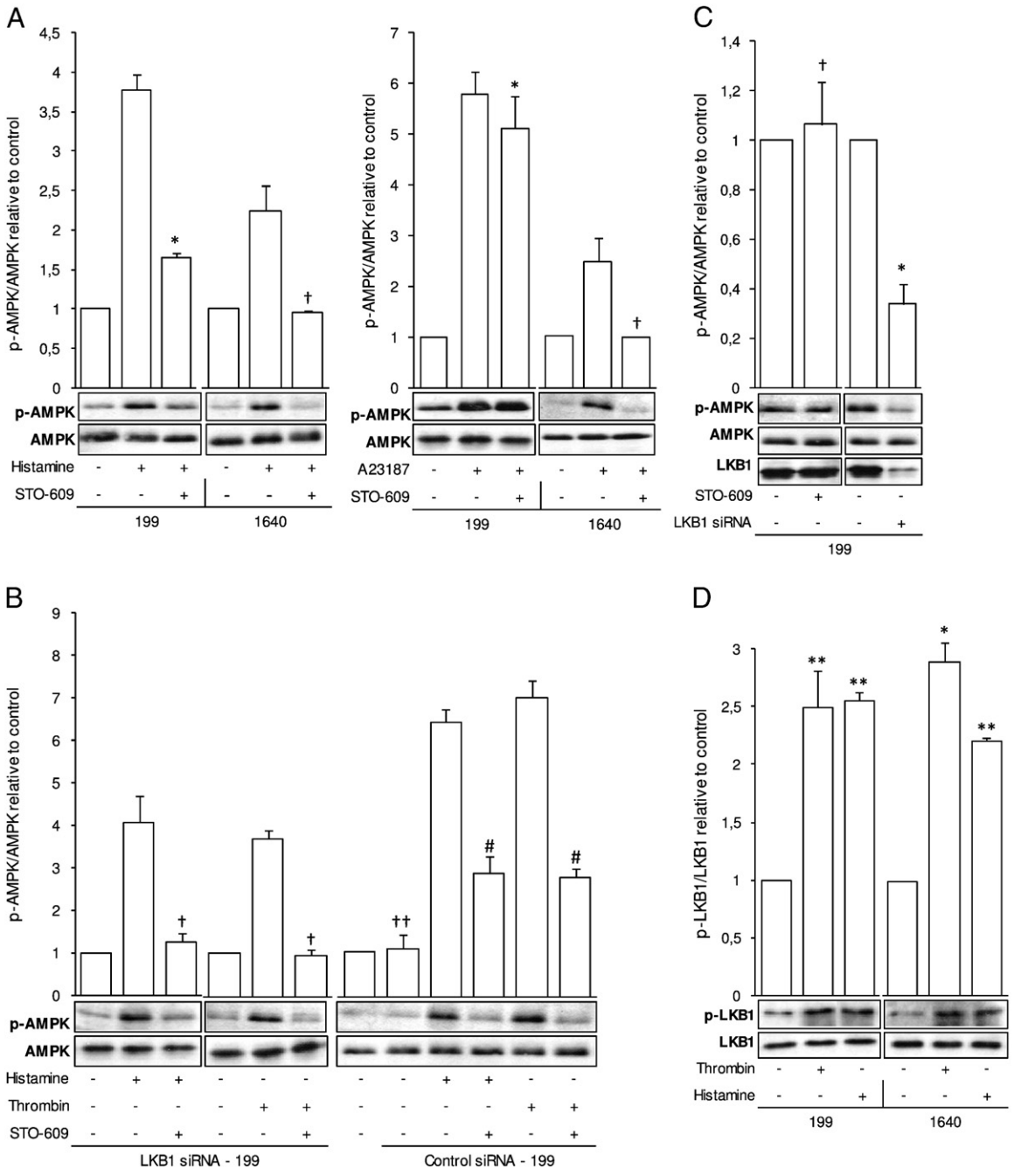

Fig. 2. The role of CaMKK and LKB1 in histamine, thrombin or ionophore mediated phosphorylation of AMPK and in basal phosphorylation of AMPK in different media. (A) The effects of 20 minute pre-treatment with STO-609 (10 μM) on AMPK phosphorylation (Thr172) in medium 199 or 1640 after treatment with 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

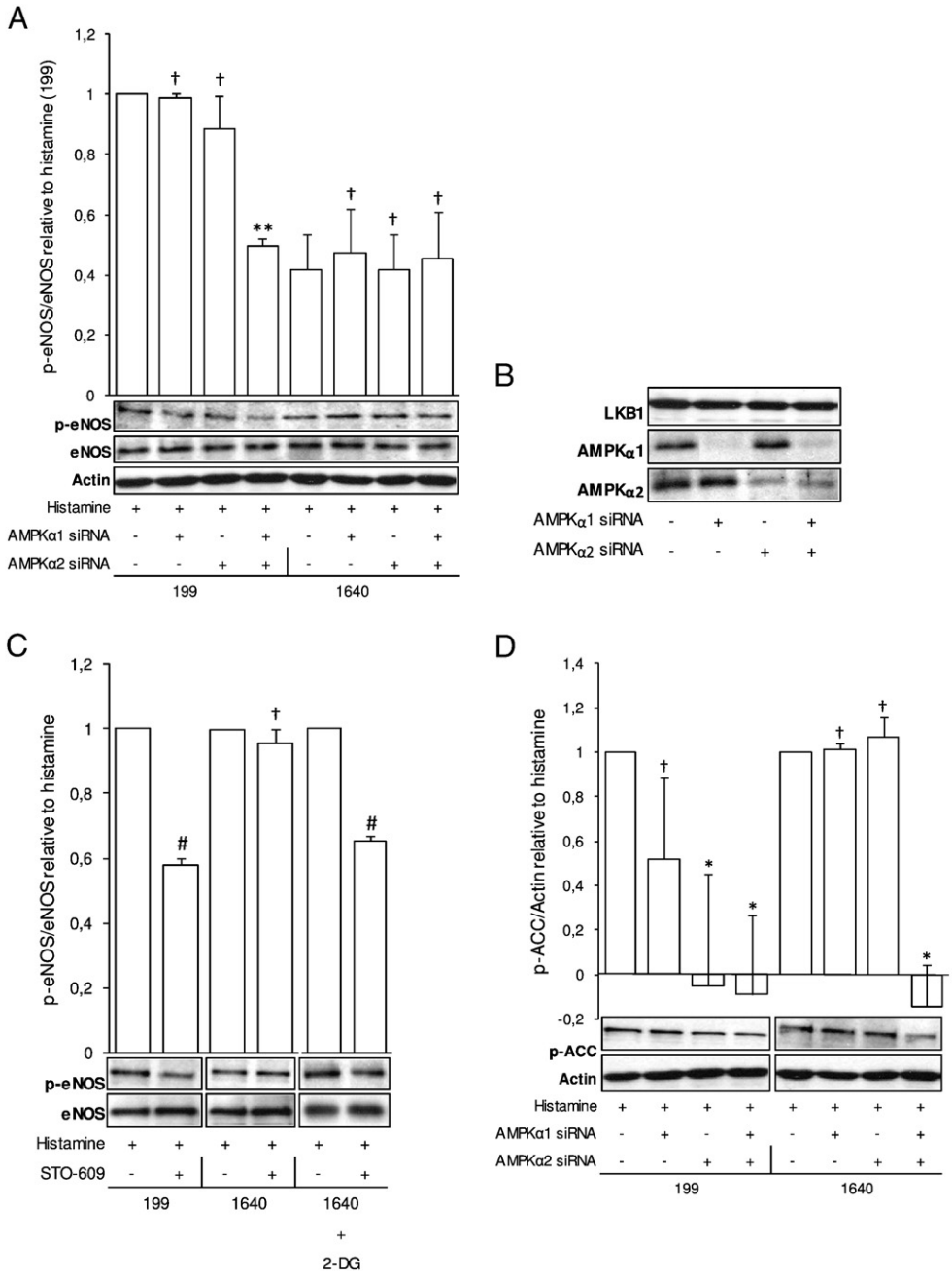

Fig. 3. The role of the two AMPKα isoforms in histamine mediated phosphorylation of eNOS and ACC in different media. (A) The effects of AMPKα1 and/or −α2 siRNA (20 nM) on eNOS phosphorylation (Ser1177) after treatment with histamine (10 μM, 3 min) in medium 199 or 1640. The results are normalized to 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

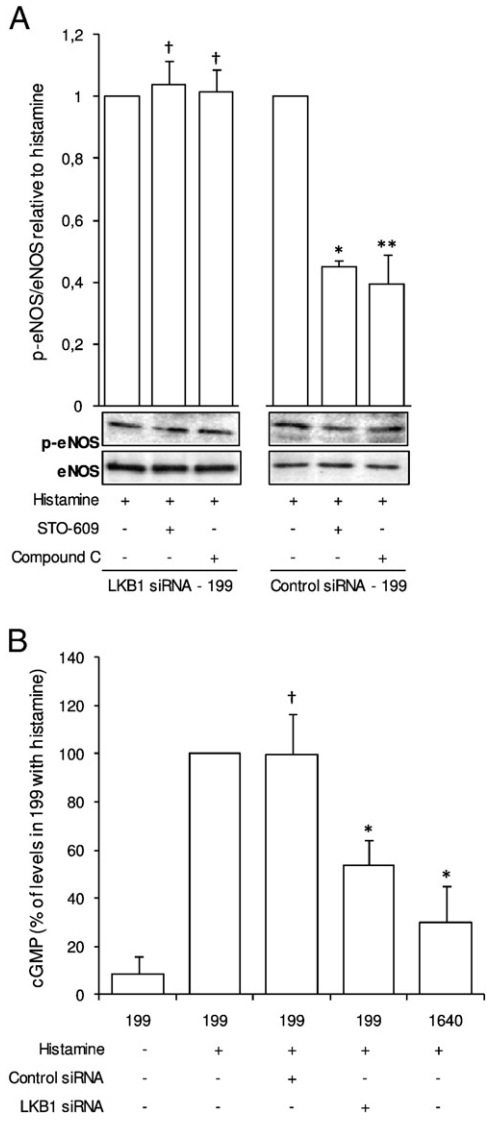

Fig. 4. The role of LKB1 in histamine mediated phosphorylation and activation of eNOS in medium 199. (A) The effects of 20 minute pretreatment with STO-609 (10 μM) or Compound C (10 μM) on histamine (10 μM, 3 min) mediated eNOS phosphorylation (Ser1177) in LKB1 downregulated cells (20 nM) or cells treated 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

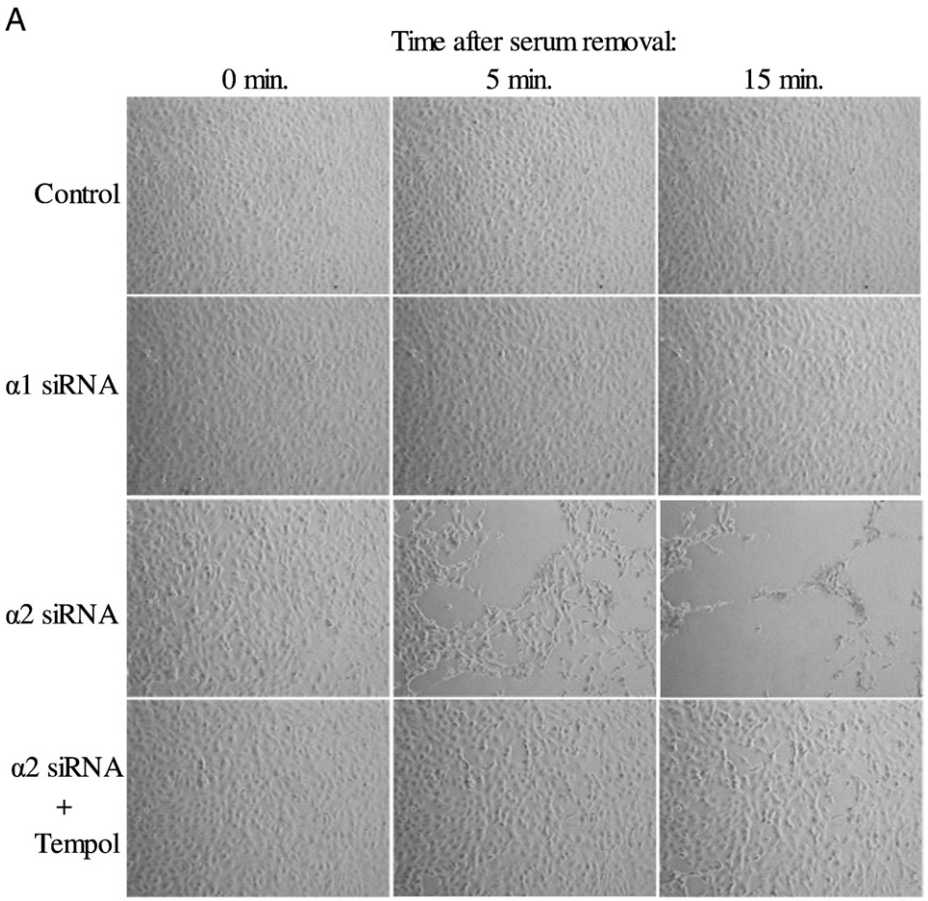

Fig. 5. The role of the two AMPKα isoforms in maintaining endothelial monolayer integrity. (A) A confocal image of HUVEC with or without siRNA for AMPKα1 or −α2 (20 nM, 67 h). The effect of cotreatment with Tempol (10 μM). (B) The effect of siRNA for AMPKα1 and/or −α2 (20 nM, 46 h) on the expression 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).

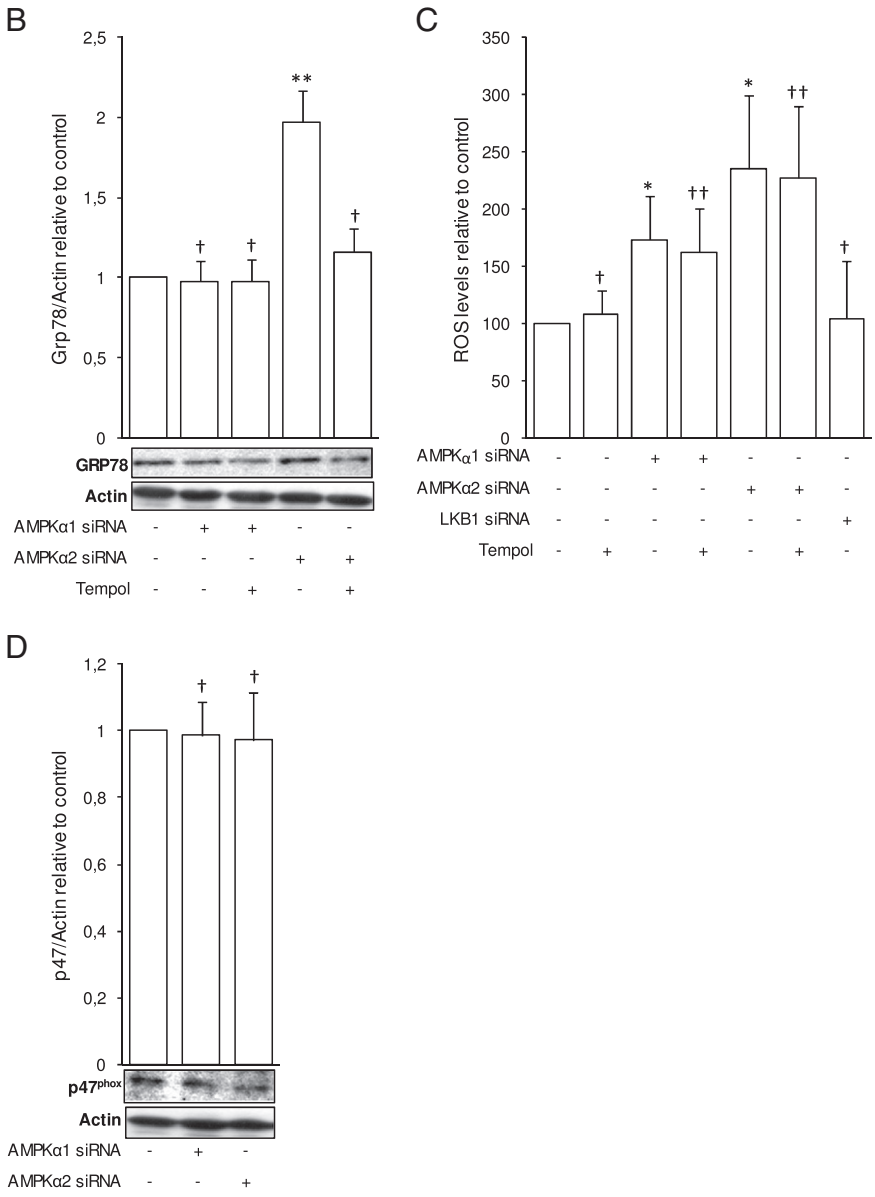

Fig. 5 (*continued*).

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

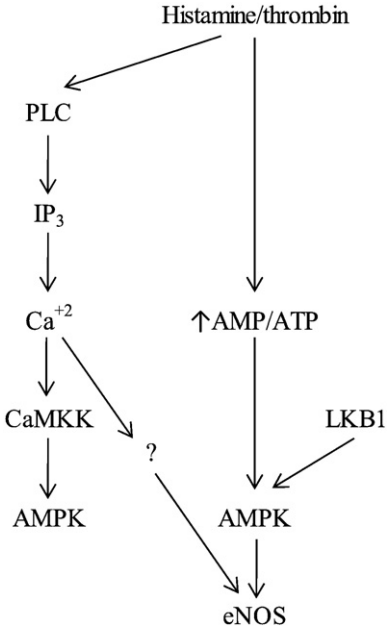

Fig. 6. Diagram showing the proposed pathways after endothelial stimulation with histamine or thrombin. Under conditions where intracellular ATP is lowered by histamine or thrombin, AMPK is activated by both LKB1 and CaMKK and, in turn, mediates eNOS phosphorylation and activation in an LKB1 dependent 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.

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