Recent studies have indicated that endothelial nitric-oxide synthase (eNOS) is regulated by reversible phosphorylation in intact endothelial cells. AMP-activated protein kinase (AMPK) has previously been demonstrated to phosphorylate and activate eNOS at Ser-1177 in vitro, yet the function of AMPK in endothelium is poorly characterized. We therefore determined whether activation of AMPK with 5′-aminoimidazole-4-carboxamide ribonucleoside (AICAR) stimulated NO production in human aortic endothelial cells. AICAR caused the time- and dose-dependent stimulation of AMPK activity, with a concomitant increase in eNOS Ser-1177 phosphorylation and NO production. AMPK was associated with immunoprecipitates of eNOS, yet this was unaffected by increasing concentrations of AICAR. AICAR also caused the time- and dose-dependent stimulation of protein kinase B phosphorylation. To confirm that the effects of AICAR were indeed mediated by AMPK, we utilized adenovirus-mediated expression of a dominant negative AMPK mutant. Expression of dominant negative AMPK attenuated AICAR-stimulated AMPK activity, eNOS Ser-1177 phosphorylation and NO production and was without effect on AICAR-stimulated protein kinase B Ser-473 phosphorylation or NO production stimulated by insulin or A23187. These data suggest that AICAR-stimulated NO production is mediated by AMPK as a consequence of increased Ser-1177 phosphorylation of eNOS. We propose that stimuli that result in the acute activation of AMPK in endothelial cells stimulate NO production, at least in part due to phosphorylation and activation of eNOS. Regulation of endothelial AMPK therefore provides an additional mechanism by which local vascular tone may be controlled.

Mammalian AMP-activated protein kinase (AMPK) is a heterotrimeric member of a protein kinase family highly conserved.

* This work was supported by a British Heart Foundation intermediate fellowship (to I. P. S.) and project grant (to J. R. P., J. M. C. C., and G. W. G.), a Tenovus-Scotland project grant (to I. P. S.), Biotechnology and Biological Sciences Research Council Grant 17/REI18463 (to G. W. G.), and a Joint Equipment Initiative grant (industrial partner Menarini Pharmaceuticals) (to J. R. P., J. M. C. C., and G. W. G.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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‡The abbreviations used are: AMPK, 5′-AMP-activated protein kinase; AICAR, 5′-aminoimidazole-4-carboxamide ribonucleoside; DAF-2, 4,5-diaminofluorescein; DAF-2DA, DAF-2-diacetate; eNOS, endothelial nitric-oxide synthase; HAECS, human aortic endothelial cells; HUVECs, human umbilical vein endothelial cells; i-NAME, Nω-nitro-i-arginine methyl ester; PEG, poly(ethylene glycol) 6000; PKB, protein kinase B; DN, dominant negative; Pfu, plaque-forming unit; KKH, Krebs Ringer HEPES.
endothelial cells subjected to fluid shear-stress, vascular endothelial growth factor, insulin-like growth factor-I, and insulin (15–16, 18–20). Expression of dominant negative PKB in endothelial cells inhibits NO production and eNOS Ser-1177 phosphorylation in response to vascular endothelial growth factor and insulin (16–17, 21) but not fluid shear stress (21). Fluid shear stress-stimulated eNOS phosphorylation is wortmannin-sensitive (15, 18–19), yet the kinase responsible remains uncertain. Although ischemia has been demonstrated to stimulate AMPK activity, eNOS activity, and eNOS phosphorylation at Ser-1177 in rat heart (14), a number of important questions remain. Ischemia has been demonstrated to deplete cellular ATP and affect a host of signaling pathways (22–24),

| Immunoprecipitating antibody | Basal | 2 mM AICAR |
|-----------------------------|-------|------------|
| AMPKα1                      | 0.30 ± 0.06 | 0.78 ± 0.15 |
| AMPKα2                      | 0.02 ± 0.01 | 0.06 ± 0.04 |
| AMPKα1 and AMPKα2           | 0.36 ± 0.08 | 0.87 ± 0.11 |

**FIG. 1.** Effects of AICAR and metformin on HAEC AMPK activity. A, cells were incubated in control medium (□), 0.5 mM AICAR (■), or 2 mM metformin (○) for various times and lysates prepared. B, cells were incubated with the indicated concentrations of AICAR for 30 min, and lysates were prepared. Total AMPK was immunoprecipitated from HAEC lysates (100 μg) with a mixture of anti-AMPKα1 and -α2 antibodies and assayed for AMPK activity. The results are expressed as the mean ± S.D. AMPK activity for three independent experiments. One unit of AMPK activity is that required to incorporate 1 nmol of 32P into the AMARA substrate peptide/min. *, p < 0.05 relative to the value in the absence of AICAR; †, p < 0.01 relative to the value in the absence of AICAR.

**FIG. 2.** AICAR stimulates NO production in HAECs. A, cells cultured in 24-well dishes were preincubated in KRH buffer in the presence or absence of 0.1 mM l-NAME. The media were removed and replaced with fresh KRH buffer in the presence or absence of the indicated compounds. After 1 h, media were removed and assayed for NO2 content as described under “Experimental Procedures.” The data shown represent the mean ± S.D. of l-NAME-sensitive NO synthesis from six independent experiments. *, p < 0.05 relative to value in absence of AICAR. †, p < 0.01 relative to value in absence of AICAR. B, cells cultured on coverslips were loaded with the NO-sensitive dye, DAF-2DA, before incubation with the indicated concentrations of AICAR for 30 min. Some coverslips were coincubated with 0.1 mM l-NAME. Confocal images were obtained, and the DAF-2 fluorescence was quantified. The results are expressed as the mean ± S.D. fluorescence for 10 random images from each of triplicate coverslips. *, p < 0.05 relative to the value in the absence of AICAR. †, p < 0.01 relative to the value in the absence of AICAR.

**TABLE I**

The effect of 2 mM AICAR on catalytic α subunit isoform-specific AMPK activity in HAECs

Lysates (100 μg) prepared from cells incubated for 30 min in the presence or absence of 2 mM AICAR were immunoprecipitated using either anti-AMPKα1, anti-AMPKα2, or both. The immunoprecipitates were subsequently assayed for AMPK activity. The results are expressed as AMPK activity (units/mg or protein) for two separate experiments performed in triplicate. One unit of AMPK activity is that required to incorporate 1 nmol of 32P into the AMARA substrate peptide/min.
and it has yet to be demonstrated that activation of AMPK regulates eNOS activity directly in intact endothelial cells in the absence of disturbed adenine nucleotide ratios. Additionally, there have, to date, been no studies of the effects of AMPK activation on NO synthesis in human cells. There is substantial interest in the AMPK cascade as a possible therapeutic target for the treatment of metabolic disorders such as diabetes (4, 25–26). Administration of AICAR in mice and rats has highlighted the therapeutic potential of AMPK activation in glucose homeostasis (27–30) and has been reported to lower blood pressure in hypertensive, obese Zucker rats (31), yet the mechanism involved has yet to be characterized. It is, therefore, important that the effects of AMPK activation in tissues such as endothelium, which would be subjected to such agents in vivo, are fully investigated. Incubation of human umbilical vein endothelial cells (HUVECs) with AICAR has been demonstrated to stimulate AMPK (32–34). It should be pointed out, however, that the specificity of AICAR remains uncertain. Therefore, any implicated role of AMPK should be confirmed using another method of manipulating AMPK activity such as the use of dominant negative AMPK in this study.

In this study we have expanded the studies of Chen et al. (14) and have investigated the effects of activation of AMPK cascade on NO synthesis in human aortic endothelial cells (HAECs) using the AMPK activator, AICAR. We report that acute incubation of HAECs with AICAR stimulates AMPK

![Fig. 3. Effect of AICAR on AMPK association with eNOS. HAEC lysates were prepared from cells incubated with various concentrations of AICAR for 30 min. eNOS was immunoprecipitated from 200 µg of lysate, and the immunoprecipitated proteins were resolved by SDS-PAGE, transferred to nitrocellulose, and probed with rabbit anti-eNOS or sheep anti-AMPKα1 antibodies. Representative immunoblots are shown, repeated with similar results on three different samples of lysates.](image)

![Fig. 4. Effect of AICAR on eNOS Ser-1177 phosphorylation. HAEC lysates were prepared from cells incubated with 2 mM AICAR for various durations or various concentrations of AICAR for 30 min. Lysates (5 µg) were resolved by SDS-PAGE, transferred to nitrocellulose, and probed with rabbit anti-eNOS or anti-phospho-Ser-1177 antibodies. A, representative immunoblots are shown, repeated with similar results on three different samples of lysates. B and C, quantification of Ser-1177 phosphorylation from three independent experiments using National Institutes of Health (NIH) Image software. *, p < 0.05 relative to the value in the absence of AICAR. †, p < 0.01 relative to the value in the absence of AICAR.](image)
activity, Ser-1177 phosphorylation, and NO production, effects inhibited by the expression of a dominant-negative AMPK mutant. We propose that stimuli that result in the acute activation of AMPK activity in endothelial cells stimulate NO production, at least in part due to phosphorylation and activation of eNOS. Therefore, regulation of endothelial AMPK may provide a mechanism that controls NO production and thereby local vascular tone.

**EXPERIMENTAL PROCEDURES**

**Materials**—Cryopreserved HAECs and cell culture media were obtained from TCS Cellworks (Botolph Claydon, Bucks, UK). AICAR, metformin, soybean trypsin inhibitor, benzamidine, AMP, ATP, and rabbit anti-eNOS antibody were supplied by Sigma. [γ-32P]ATP and horseradish peroxidase-conjugated secondary antibodies were from Amersham Biosciences. L-NAME, A23187, DAF-2DA, PD90859, and mouse anti-eNOS antibodies were from Calbiochem. Insulin (Actrapid) was obtained from Novo-Nordisk (Copenhagen, Denmark). Protein G-Sepharose was from Amersham Biosciences. Sheep anti-PKB antibody was supplied by Upstate Biotechnology, Inc. (Lake Placid, NY). Rabbit anti-p42/44MAPK, anti-phospho-p42/44MAPK, anti-phospho-eNOS Ser-1177, and anti-phospho-PKB Ser-473 antibodies were obtained from Cell Signaling Technology (Beverly, MA). AMARA peptide (AMARAASAAALARR) was synthesized by Dr. G. Bloomberg, University of Bristol, UK. Isoform-specific sheep anti-AMPK antibodies have been described elsewhere (6, 35) and were a generous gift from Prof. D. G. Hardie, University of Dundee, Dundee, UK. All other reagents were from sources described previously (36).

**HAEC Cell Culture**—HAECs were grown in large vessel endothelial cell medium at 37 °C in 5% CO₂ and passaged when at 80% confluence. Cells were used for experiments between passages 3 and 6.

**Evaluation of Nitric Oxide Release Using a Sievers NO Meter**—Cells cultured in 24-well plates were preincubated for 1 h at 37 °C in 0.5 ml well Krebs Ringer HEPES (KRH) buffer (119 mM NaCl, 20 mM Na-HEPES, pH 7.4, 5 mM NaHCO₃, 4.7 mM KCl, 1.3 mM CaCl₂, 1.2 mM MgSO₄, 1 mM KH₂PO₄, 100 μM l-arginine, 5 mM glucose) at 37 °C. The medium was removed and replaced with 0.2 ml of fresh KRH buffer in the presence or absence of AICAR for 1 h. The medium was removed and refluxed in glacial acetic acid containing NaI. Under these conditions, NO₂⁻, a stable breakdown product of NO, is quantitatively reduced to NO. NO-specific chemiluminescence was then analyzed using a Sievers 280A NO meter. Values were corrected for NO₂⁻ present in media in the absence of cells, and the appropriate control experiments were performed in the presence of the eNOS inhibitor, L-NAME. NO production (adjusted for L-NAME-insensitive production) is expressed per hour per well.

**Evaluation of Nitric Oxide Release by DAF-2 Fluorescence**—To further determine NO production in HAECs, a membrane-permeable fluorescent indicator DAF-2DA was used that allows a quantitative determination of NO production (37). Cells grown on coverslips in 6-well plates were preincubated for 30 min at 37 °C in 1 ml/well KRH buffer in the presence or absence of 0.1 mM t-NAME. DAF-2DA (5 μM final) was added, and the coverslips were incubated for a further 15 min at 37 °C.

**Fig. 5. Effect of AICAR on PKB Ser-473 phosphorylation.** HAEC lysates were prepared from cells incubated with 2 mM AICAR for various durations or various concentrations of AICAR for 30 min. Lysates (5 μg) were resolved by SDS-PAGE, transferred to nitrocellulose, and probed with sheep anti-PKB or rabbit anti-phospho-Ser-473 antibodies. A, representative immunoblots are shown, repeated with similar results on three different samples of lysates. B and C, quantification of Ser-473 phosphorylation from three independent experiments using NIH Image software. *, p < 0.05 relative to the value in the absence of AICAR. †, p < 0.01 relative to the value in the absence of AICAR.
The medium was removed and replaced with 1 ml of fresh KRH buffer in the presence or absence of L-NAME and test substances. After incubation for 30 min at 37°C, the medium was removed, and the cells fixed in 4% (w/v) paraformaldehyde in phosphate-buffered saline, washed, and mounted on slides. Specimens were observed under a Zeiss LSM5 Pascal laser-scanning confocal microscope with a 40×1.1003 objective, excitation wavelength of 488 nm, and a LP515-nm filter. Triplicate coverslips were prepared for each experimental condition, and 10 random images of HAECs were collected from each and quantified using MetaMorph (Universal Imaging, West Chester, PA) software.

**Preparation of HAEC Lysates**—Cells grown in 100-mm diameter cell culture dishes were preincubated for 1 h at 37°C in 5 ml of KRH buffer. The medium was replaced with 5 ml of fresh KRH buffer containing test substances and incubated for various durations at 37°C. The medium was removed, and 0.5 ml of ice-cold lysis buffer (50 mM Tris-HCl, pH 7.4, at 4°C, 50 mM NaF, 2 mM Na3PO4, 0.1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol, 0.1 mM benzamidine, 0.1 mM phenylmethylsulfonyl fluoride, 5 g/ml soybean trypsin inhibitor, 1% (w/v) Thesit, 250 mM mannitol) added. The cell extract was scraped off and transferred to a microcentrifuge tube. Extracts were vortex-mixed and centrifuged (14,000×g, 3 min, 4°C). Supernatants were snap-frozen in liquid N2 and stored at −80°C before use. Sequential PEG precipitation was used to prepare 2.5–6.25% PEG precipitates from some lysates, which were snap-frozen in liquid N2 and stored at −80°C before AMPK assay (38).

**Adenoviruses**—Dominant negative AMPK adenovirus (Ad.a1-DN) was constructed from AMPKα1 containing a mutation altering Asp-157 to alanine (D157A) as described previously (6). Recombinant adenoviruses were propagated in HEK293 cells, purified by cesium chloride density centrifugation, and stored as described previously (6).

**Infection of HAECs**—HAECs were infected with 10 Pfu/cell adenovirus in complete medium. After 2 h, 1 further volume of complete medium was added, and the cells were cultured for 48 h before experimentation. Preliminary studies revealed that within 48 h of infection with a green fluorescent protein-expressing virus, the majority (>95%) of HAECs expressed green fluorescent protein.

**Immunoprecipitation of eNOS**—HAEC lysate (200 μg) was added to 20 μl of 25% (v/v) protein G-Sepharose prebound to 5 μg of mouse anti-eNOS, and the volume was made up to 300 μl with lysis buffer and mixed for 2 h at 4°C on a rotating mixer. The mixture was then centrifuged (14,000×g, 30 s, 4°C), and the pellet was washed 3 times with 1 ml of lysis buffer at 4°C.

**Immunoprecipitation and Assay of AMPK**—AMPK was immunoprecipitated from lysates and assayed using the AMARA substrate peptide as described previously (36). An AMARA peptide kinase could also be detected in PEG precipitates resuspended in HEPES-Brij buffer (50 mM HEPES, pH 7.4, 1 mM dithiothreitol, 0.02% (v/v) Brij-35) as described previously (38). Protein concentration was determined by the method of Bradford (39).

**Statistics**—Unless stated otherwise, results are expressed as the means ± S.D. Statistically significant differences were determined using a 2-tailed independent-samples Student’s t test, with p < 0.05 as significant using Statview software.

**RESULTS**

The expression of two distinct isoforms of the catalytic α subunit of AMPK (termed α1 and α2) has been demonstrated in

![Fig. 6. Effect of AICAR on p42/44MAPK phosphorylation.](image-url)
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Fig. 7. The expression and activity of AMPK in HAECs infected with adenovirus-expressing AMPK mutant constructs. HAECs were infected with 10 Pfu/cell of the viruses indicated 48 h before experimentation. A, lysates (5 μg) prepared from cells incubated in the presence or absence of 2 mM AICAR for 30 min, were resolved by SDS-PAGE, transferred to nitrocellulose, and probed with sheep anti-AMPKα1 antibodies. Representative immunoblots are shown, repeated with similar results on three different samples of lysates. B, 2.5-6.25% PEG precipitates were prepared from cells incubated in the presence (filled bars) or absence of 2 mM AICAR (open bars) for 30 min and assayed for AMPK activity. The results are expressed as AMPK activity for three separate experiments performed in triplicate. *, p < 0.05 relative to mock-infected cells.

A number of tissues (3, 40). To determine the effects of AICAR and metformin on AMPK activity in HAECs, total AMPK was assayed in HAEC lysates prepared from cells incubated for various duration in 0.5 mM AICAR or 2 mM metformin after immunoprecipitation with a mixture of anti-α1 and anti-α2 antibodies. Treatment of HAECs with AICAR elicited a transient activation of AMPK. The effect reached a maximum 2.3-fold stimulation at 30 min, which was sustained for a further 30 min (Fig. 1A). However, treatment of HAECs with metformin had no significant effect on AMPK activity over the time course used in this study (Fig. 1A). Activation of AMPK by AICAR was dose-dependent (Fig. 1B) such that AMPK was stimulated maximally (~3-fold) by 2 mM AICAR, a concentration at which all further experiments were performed. To determine the relative activities of AMPK complexes containing either catalytic subunit isoform in HAECs, AMPK was immunoprecipitated using anti-α1 or -α2 antibodies and assayed for AMPK activity under basal and AICAR-stimulated conditions. The majority (~90%) of the AMPK activity in both basal and AICAR-stimulated conditions was in complexes containing the α1 catalytic subunit isoform (Table 1).

Next we examined the ability of AICAR to modulate NO production in HAECs. AICAR-stimulated NO production was measured using a Sievers 280A NO meter. AICAR stimulated L-NAME-sensitive NO production in a dose-dependent manner and was stimulated maximally by 2 mM AICAR (Fig. 2A). AICAR-stimulated NO production was only marginally less than that elicited by A23187, which directly increases intracellular Ca2+ concentrations or insulin, which has been demonstrated to stimulate NO production via PKB-mediated phosphorylation and activation of eNOS at Ser-1177 (17, 20) (Fig. 2A). To confirm the stimulation of NO production by AICAR, HAECs were loaded with DAF-2, a dye that upon binding to an oxidized species of NO results in fluorescence. Quantification of confocal images of DAF-2 fluorescence demonstrated the dose-dependent stimulation of NO production in cells stimulated with AICAR for 30 min (Fig. 2B). AICAR-stimulated DAF-2 fluorescence was completely inhibited in cells pretreated with the NOS inhibitor L-NAME (Fig. 2B).

AICAR has been demonstrated to be associated with eNOS immunoprecipitated from rat heart homogenates and also to phosphorylate eNOS at Ser-1177 in vitro (14). As shown in Fig. 3, AMPKα1 was present in eNOS immunoprecipitates, yet increasing concentrations of AICAR had no effect on the level of AMPK associated with eNOS.

We next used an anti-phospho-Ser-1177 antibody to determine the phosphorylation of eNOS at Ser-1177 in HAEC lysates from cells stimulated with AICAR (Fig. 4A). Quantitative analysis of Western blots demonstrated that AICAR stimulated the time- and dose-dependent phosphorylation of eNOS at Ser-1177, maximal at 30 min and 0.5 mM AICAR (Fig. 4, B and C). AICAR has previously been demonstrated to enhance insulin-stimulated phosphorylation of PKB at Ser-473 in HUVECs cultured in 30 mM glucose (33). Phosphorylation of PKB at Ser-473 is associated with activation, and PKB has previously been demonstrated to activate eNOS by phosphorylation at Ser-1177 (15–16, 18–19). We, therefore, used an anti-phospho-Ser-473 antibody to determine the activity of PKB in HAEC lysates from cells stimulated with AICAR (Fig. 5A). Quantitative analysis of Western blots illustrated that AICAR stimulated the time- and dose-dependent phosphorylation of PKB at Ser-473, maximal at 120 min with 5 mM AICAR (Fig. 5, B and...
C). It has been suggested that activation of AMPK can activate the p42/44MAPK cascade in isolated skeletal muscles (41). In contrast, AICAR has been demonstrated to inhibit insulin-like growth factor-I- and palmitate-stimulated p42/44MAPK activation in NIH-3T3 cells and astrocytes, respectively (42, 43). We therefore used an anti-phospho-MAPK antibody to determine the activity of p42/44MAPK in HAEC lysates from cells stimulated with AICAR (Fig. 6A). Quantitative analysis of Western blots illustrated that AICAR inhibited phosphorylation of p42/44MAPK, maximal by 60 min with 2 mM AICAR (Fig. 6, B and C). p42/44MAPK has been demonstrated to phosphorylate and inactivate eNOS in vitro, yet the site(s) of phosphorylation remained uncharacterized (44). However, treatment of HAECs with the MEK inhibitor PD90859 at 10 μM, a concentration that completely inhibits p42/44MAPK phosphorylation (data not shown), did not stimulate basal NO synthesis (data not shown).

To determine whether the effects of AICAR were mediated by AMPK itself or increased PKB activity, we infected HAECs with dominant-negative (Ad.a1-DN) AMPK using an adenoviral vector. After infection with control (Ad.null) or Ad.a1-DN viruses, HAEC lysates were analyzed by Western blotting for expression of mutant and endogenous AMPKα1 subunits. Expression of a1-DN protein was detected in HAEC lysates from cells infected with Ad.a1-DN. Expression of a1-DN significantly attenuated AICAR-stimulated AMPK activity (Fig. 7B). Infection of HAECs with Ad.null had no effect on AMPKα1 expression or total AMPK activity compared with mock-infected cells (Fig. 7, A and B). Analysis of NO production demonstrated that expression of a1-DN significantly attenuated AICAR-stimulated NO production in HAECs (Fig. 8).

Analysis of DAF-2 fluorescence in HAECs infected with control or a1-DN viruses confirmed the inhibition of AICAR-stimulated NO production by expression of a1-DN (data not shown). Expression of a1-DN had no effect on insulin- or A23187-stimulated NO production (Fig. 8). We next used anti-phospho-Ser-1177 and anti-phospho-Ser-473 antibodies to determine the phosphorylation of eNOS at Ser-1177 and PKB at Ser-473 in HAEC lysates from cells expressing a1-DN in the presence or absence of AICAR (Fig. 9A). Quantitative analysis of Western blots illustrated that AICAR-stimulated Ser-1177 phosphorylation was significantly attenuated in cells expressing a1-DN (Fig. 9B). Infection of cells with a control (null) virus had no effect on AICAR-stimulated Ser-1177 phosphorylation (Fig. 9B). Similarly, infection of HAECs with either virus had no significant effect on either eNOS or PKB expression (Fig. 9A) or AICAR-stimulated PKB Ser-473 phosphorylation (Fig. 9C). Infection of HAECs with a1-DN had no effect on insulin-stimulated Ser-1177 phosphorylation of eNOS or insulin-stimulated phosphorylation of PKB at Ser-473 (Fig. 10).

DISCUSSION

The central finding of this paper is that direct stimulation of human aortic endothelial cells with AICAR stimulates NO production. The stimulation of NO production is associated with increased phosphorylation of eNOS at Ser-1177 and is inhibited by infection of HAECs with an adenovirus expressing a dominant negative mutant AMPK. These data support the hypothesis that the effects of AICAR are mediated by AMPK. The dose dependence of AICAR-stimulated AMPK activation demonstrated in this study is in broad agreement with a previous study in HUVECs, yet the time course of AMPK activation is more transient (32). This difference may reflect differences in the metabolism of AICAR and accumulation of ZMP (5′-aminoimidazole-4-carboxamide ribonucleotide) between endothelial cells from the different vascular sources. However, incubation of HAECs with the antidiabetic drug, metformin, which has been demonstrated to activate AMPK in hepatocytes (8), had no effect on HAECs in this study even at concentrations as high as 2 mM. The mechanism by which metformin stimulates AMPK has recently been proposed to be independent of changes in AMP/ATP (9, 12). It may, therefore, be possible that a component of the as yet undefined mechanism by which metformin stimulates AMPK is absent in HAECs. Alternatively, longer incubation periods with metformin may be required to see any effect on AMPK activity. The principal AMPK catalytic subunit isoform contributing to AMPK activity in HAECs was the α1 isoform. AMPKα1 is the major catalytic subunit isoform in the majority of tissues examined to date, with the exception of liver, cardiac muscle, and skeletal muscle (3). In agreement with previous studies in rat heart and bovine aortic endothelial cells, we have demonstrated that the catalytic subunit of AMPK is associated with eNOS as assessed by co-immunoprecipitation (45). Indeed, stimulation of bovine aortic endothelial cells with peroxynitrite has been demonstrated to stimulate both AMPK activity and association with eNOS as assessed by co-immunoprecipitation (45). However, in the current study, concentrations of AICAR that markedly stimulated AMPK activity had no effect on the association of AMPK with eNOS. This difference may represent a species-specific effect or reflect the different natures of the stimuli used.

The time and dose dependence of AICAR-stimulated eNOS phosphorylation at Ser-1177 and the dose-dependence of NO production correlated with AICAR-stimulated AMPK activation, supporting the hypothesis that phosphorylation and activation of eNOS was a result of AMPK activation. However, AICAR also stimulated PKB Ser-473 phosphorylation (an indicator of PKB activity). AICAR was previously demonstrated to
increase insulin-stimulated PKB Ser-473 phosphorylation in HUVECs cultured in 30 mM glucose (33). PKB-mediated phosphorylation and activation of eNOS at Ser-1177 has previously been demonstrated in intact cells in response to vascular endothelial growth factor and insulin (17, 18, 21). Similarly, it has been suggested that AMPK activation by AICAR can either stimulate or inhibit the p42/44MAPK pathway (41–43). p42/44MAPK has been demonstrated to phosphorylate and inhibit eNOS in vitro at an as yet uncharacterized site(s) (44). In this study AICAR inhibited basal p42/44MAPK activity, assessed by Western blots of p42/44MAPK phosphorylation. However, the effects of AICAR are unlikely to be a result of reduced p42/44MAPK-mediated inhibitory phosphorylation of eNOS as inhibition of p42/44MAPK phosphorylation and activation by the MEK inhibitor, PD90859, did not stimulate NO synthesis in this study. To determine whether AICAR-stimulated NO production was a result of PKB- or AMPK-mediated phosphorylation of Ser-1177, we utilized adenovirus-mediated expression of dominant negative AMPK in HAECs. Expression of dominant negative AMPK inhibited AICAR-stimulated AMPK activity, Ser-1177 phosphorylation, and NO production, providing evidence that AMPK mediates the effect of AICAR. However, expression of the dominant negative mutant did not significantly inhibit basal AMPK activity, Ser-1177 phosphorylation, or NO production such that the kinase(s) responsible for basal Ser-1177 phosphorylation remain uncertain.

In addition, expression of dominant negative AMPK had no effect on AICAR-stimulated PKB phosphorylation, suggesting

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**Fig. 9.** The effects of infection with adenovirus expressing AMPK mutant constructs on AICAR-stimulated eNOS and PKB phosphorylation. HAECs were infected with 10 PFU/cell of the viruses indicated 48 h before experimentation. Subsequently, HAEC lysates were prepared from cells incubated in the presence (filled bars) or absence of 2 mM AICAR (open bars) for 30 min. Lysates (5 μg) were resolved by SDS-PAGE, transferred to nitrocellulose, and probed with the antibodies indicated. A, representative immunoblots are shown, repeated with similar results on three different samples of lysates. B, quantification of Ser-1177 phosphorylation from three independent experiments using NIH Image software. *, p < 0.05 relative to the value in the absence of AICAR. C, quantification of Ser-473 phosphorylation from three independent experiments using NIH image software. *, p < 0.05 relative to the value in the absence of AICAR.
that PKB phosphorylation is not downstream of AMPK activation. Importantly, insulin-stimulated NO production and Ser-1177 phosphorylation, which is thought to be mediated by PKB, was unaffected by expression of dominant negative AMPK. The magnitude of insulin-stimulated Ser-1177 phosphorylation was comparable with that elicited by AICAR, yet insulin stimulated PKB phosphorylation at Ser-473 5–6-fold, compared with the 2.5–3-fold stimulation of Ser-473 phosphorylation by AICAR. These data argue that the effect of the dominant negative mutant specifically inhibits AMPK and does not have a global effect on phosphorylation at Ser-1177 by sequestering the substrate, eNOS.

As a consequence, AICAR-stimulated phosphorylation of eNOS at Ser-1177 is unlikely to be mediated by PKB. Furthermore, AICAR stimulates PKB in the absence of stimulated NO production in HAECs expressing dominant negative AMPKa1, suggesting that stimulation of PKB is not sufficient to stimulate Ser-1177 phosphorylation in endothelial cells. Indeed, previous studies have proposed that PKB stimulation is necessary but not sufficient for insulin-stimulated NO synthesis in endo-

**FIG. 10.** The effects of infection with adenovirus-expressing AMPK mutant constructs on insulin-stimulated eNOS and PKB phosphorylation. HAECs were infected with 10 Pfu/cell of the viruses indicated 48 h before experimentation. Subsequently, HAEC lysates were prepared from cells incubated in the presence or absence of 0.1 μM insulin for 5 min as indicated. Lysates (5 μg) were resolved by SDS-PAGE, transferred to nitrocellulose, and probed with the antibodies indicated. A, representative immunoblots are shown, repeated with similar results on three different samples of lysates. B, quantification of Ser-1177 and Ser-473 phosphorylation from three independent experiments using NIH image software. *, p < 0.05 relative to the value in the absence of insulin. †, p < 0.01 relative to the value in the absence of insulin.
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AMPK may be activated during periods of oxidative stress in the heart that it produces superoxide rather than nitric oxide. Therefore, aortic endothelial cells (45). Peroxynitrite uncouples eNOS such agonists is mediated by activation of AMPK. Interestingly, the effects of AICAR, yet this does not exclude an indirect effect of insulin-stimulated whole-body glucose utilization (26). No data concerning the role of AMPK in the endothelial cell NO production.

Stimulation of AMPK has been proposed to occur in response to a number of physiological stimuli in vivo. An acute bout of moderate intensity exercise was demonstrated to stimulate AMPK in human skeletal muscle (47, 48). In addition, ischemia in heart and glucose deprivation in both pancreatic β-cells andHUVECs have both been demonstrated to stimulate AMPK activity (14, 29, 49, 50). Other physiological stimuli that have been proposed to stimulate AMPK include β-adrenergic stimulation in adipose (51), leptin in skeletal muscle (10), and adiponectin in muscle (11). The antidiabetic drugs metformin and rosiglitazone have also recently been demonstrated to stimulate AMPK activation in human skeletal muscle (47, 48). In addition, ischemia–reperfusion activates AMPK in the heart (49). The association of AMPK with eNOS demonstrated in this and other studies would indicate that AMPK is able to phosphorylate eNOS directly. It has been suggested that AMPK activation can stimulate the p38 MAPK cascade and atypical PKC (46). Further studies are required to determine whether a kinase downstream of AMPK phosphorylates eNOS at Ser-1177 and the sites, if any, in addition to Ser-1177 that are phosphorylated in HAEcs in response to AICAR.

These studies demonstrate that AICAR stimulates NO production in isolated endothelial cells via AMPK. Administration of AICAR has been reported to lower blood pressure in hypertensive, obese Zucker rats (31). The current study demonstrates that AICAR has direct effects on NO synthesis in cultured endothelial cells, which may explain the hypotensive effects of AICAR, yet this does not exclude an indirect effect of AICAR in the regulation of blood pressure in whole organisms. Further studies are required to elucidate whether AMPK activation regulates vascular tone in isolated blood vessels. The substantial interest in the AMPK cascade as a possible therapeutic target for the treatment of metabolic disorders such as diabetes warrants further investigation of AMPK activation in tissues such as endothelium. Recent studies in AMPKnull mice illustrates that extramuscular AMPKnull is required for insulin-stimulated whole-body glucose utilization (26). No data concerning the regulation of nitric oxide or vascular tone is currently available in this knockout model, however. Because AMPKnull is the predominant isoform in HAEcs, the development of AMPKnull knockout mice is required to provide further information concerning the role of AMPK in the endothelial cell NO production.
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J. Biol. Chem. 2003, 278:31629-31639.
doi: 10.1074/jbc.M212831200 originally published online June 4, 2003

Access the most updated version of this article at doi: 10.1074/jbc.M212831200

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