Rosiglitazone Stimulates Nitric Oxide Synthesis in Human Aortic Endothelial Cells via AMP-activated Protein Kinase

The thiazolidinedione anti-diabetic drugs increase activation of endothelial nitric-oxide (NO) synthase by phosphorylation at Ser-1177 and increase NO bioavailability, yet the molecular mechanisms that underlie this remain poorly characterized.  

Several protein kinases, including AMP-activated protein kinase, have been demonstrated to phosphorylate endothelial NO synthase at Ser-1177.  

In the current study we determined the role of AMP-activated protein kinase in rosiglitazone-stimulated NO synthesis.  

Stimulation of human aortic endothelial cells with rosiglitazone resulted in the time- and dose-dependent stimulation of AMP-activated protein kinase activity and NO production with concomitant phosphorylation of endothelial NO synthase at Ser-1177.  

Rosiglitazone stimulated an increase in the ADP/ATP ratio in endothelial cells, and LKB1 was essential for rosiglitazone-stimulated AMPK activity in Hela cells.  

Infection of endothelial cells with a virus encoding a dominant negative AMP-activated protein kinase mutant abrogated rosiglitazone-stimulated Ser-1177 phosphorylation and NO production.  

Furthermore, the stimulation of AMP-activated protein kinase and NO synthesis by rosiglitazone was unaffected by the peroxisome proliferator-activated receptor-γ inhibitor GW9662.  

These studies demonstrate that rosiglitazone is able to acutely stimulate NO synthesis in cultured endothelial cells by an AMP-activated protein kinase-dependent mechanism, likely to be mediated by LKB1.

Endothelium-derived nitric oxide (NO), synthesized by endothelial NO synthase (eNOS), is a key regulator of vascular function. Endothelial-derived NO promotes vasodilatation and inhibits platelet aggregation, leukocyte adherence, and vascular smooth muscle proliferation, thereby having a profound influence on blood flow, vascular remodeling, and angiogenesis.

Type 2 diabetes is associated with a greatly increased risk of atheromatous vascular disease, and vascular endothelial dysfunction has been demonstrated in type 2 diabetic patients. There is a growing body of evidence to suggest that the thiazolidinedione class of anti-diabetic drugs improve endothelial function, as assessed by endothelium-dependent vasodilation in patients with type 2 diabetes or insulin resistance.

The hypoglycemic effects of thiazolidinediones are mediated by the transcription factor peroxisome proliferator-activated receptor-γ (PPARγ), but recent work suggests that the improvement of endothelial function by thiazolidinediones is independent of the effect on glycemia. Therefore, the mechanism of action by which thiazolidinediones improve vascular endothelial function remains uncertain.

Recent studies have suggested that prolonged exposure to thiazolidinediones directly improves NO bioavailability in endothelial cells and increases phosphorylation of eNOS at Ser-1177. Phosphorylation of eNOS at Ser-1177 stimulates NO synthesis, and several protein kinases have been demonstrated to phosphorylate eNOS Ser-1177 in endothelial cells, including protein kinase B (also known as Akt) and AMP-activated protein kinase (AMPK).

We have previously shown that AMPK-mediated phosphorylation of eNOS is responsible for rosiglitazone-stimulated NO production in cultured human aortic endothelial cells (HAECs). We demonstrate that rosiglitazone rapidly stimulates eNOS phosphorylation at Ser-1177 and NO expression.
synthesis in an AMPK-dependent, PPARγ-independent manner. We propose that this mechanism underlies, at least in part, the rapid effects of thiazolidinediones on vascular function.

**EXPERIMENTAL PROCEDURES**

**Materials**—Cryopreserved HAECs and cell culture medium were obtained from TCS Cellworks (Botolph Claydon, Buckinghamshire, UK). U937 cells were obtained from Dr. T. Palmer, University of Glasgow, Glasgow, UK. HeLa cells stably expressing wild type LKB1 (LKB1-WT) or kinase-inactive LKB1 (LKB1-KD) have been described elsewhere (24) and were kindly provided by Prof. D. Alessi, University of Dundee, Dundee, UK. Isoform-specific sheep anti-AMPK and anti-LKB1 antibodies have been described elsewhere (25, 26) and were a generous gift from Prof. D. G. Hardie, University of Dundee, Dundee, UK. Rosiglitazone was kindly provided by GlaxoSmithKline (Stevenage, UK). Pioglitazone, troglitazone, and GW9662 were obtained from Axxora UK Ltd. (Nottingham, UK). Tumor necrosis factor α (TNFα) and primers specific to human CD36 and 18 S ribosomal RNA were obtained from Sigma-Aldrich. All other reagents were from sources described previously (19, 27).

**Cell Culture**—HAECs were grown in large vessel endothelial cell medium at 37 °C in 5% CO₂ and used for experiments between passages 3 and 6 as described previously (19, 27). U937 pro-monocytic cells were cultured in RPMI 1640, supplemented with 10% (v/v) fetal calf serum, 100 units/ml penicillin, 0.1 mg/ml streptomycin, and 2 mM L-glutamine at 37 °C in 5% CO₂. HeLa cells stably expressing LKB1-WT or LKB1-KD were cultured as described previously (24).

**Evaluation of NO Production**—Cells cultured in 12-well plates were incubated in serum-free large vessel cell medium for 3–4 h. The cells were then preincubated for 1 h at 37 °C in 0.5 ml/well Krebs Ringer HEPES (KRH) buffer (119 mM NaCl, 20 mM HEPES-NaOH, pH 7.4, 5 mM NaHCO₃, 4.7 mM KCI, 1.3 mM CaCl₂, 1.2 mM MgSO₄, 1 mM NaH₂PO₄, 0.1 mM L-arginine, 5 mM glucose). The medium was removed and replaced with fresh KRH buffer (0.5 ml/well) in the presence of various concentrations of test substances. After incubation for various durations, aliquots of medium were removed and analyzed using a Sievers 280A NO analyzer as described previously (19, 27). The appropriate control experiments were performed in the presence of the eNOS inhibitor, N-(G)-nitro-L-arginine methyl ester (L-NAME, 0.1 mM). Data are presented as L-NAME-sensitive NO synthesis.

**Preparation of Adenoviruses and Infection of HAECs**—Control (Ad.Null) and dominant negative AMPK adenoviruses (Ad.a1DN) were propagated and purified as described previously (19, 27). HAECs were infected with 10 plaque-forming units/cell adenovirus in complete medium and the cells cultured for 48 h prior to experimentation. Under these conditions after infection with a green fluorescent protein (GFP)-expressing virus, the majority (>95%) of HAECs expressed GFP (19).

**Preparation of HAEC Lysates**—Cells were incubated in serum-free medium for 3 h prior to preincubation 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, 5 mM Na₄P₂O₇, 1 mM Na₃VO₄, 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol, 0.1 mM benzamidine, 0.1 mM fluoride, 5 μM soybean trypsin inhibitor, 1% (v/v) Triton X-100, 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.

**Immunoprecipitation and Assay of AMPK**—AMPK was immunoprecipitated from lysates and assayed using the SAMS substrate peptide as described previously (19, 27). Protein concentration was determined by the method of Bradford (28).

**Immuno precipitation and Assay of LKB1**—HAEC lysates (0.1 mg) were added to 5 μg of sheep anti-LKB1 antibody and mixed overnight at 4 °C. Protein G-Sepharose (5 μl of 50% slurry) was added and the volume adjusted to 300 μl with lysis buffer and mixed for 4 h at 4 °C. The mixture was centrifuged (14,000 × g, 30 s, 4 °C) and the pellet washed three times in 50 mM HEPES-NaOH, pH 7.4, 1% (v/v) Triton X-100. Recombinant AMPKα1-(1–312) containing the kinase domain (0.6 μg) constructed and expressed as described previously (29) was incubated with the LKB1 immunoprecipitate in a total volume of 25 μl of assay buffer (50 mM HEPES-NaOH, pH 7.4, 1 mM dithiothreitol, 0.1 mM ATP, 5 mM MgCl₂) at 37 °C with shaking for 30 min. The mixture was centrifuged (14,000 × g, 30 s, 4 °C) and the supernatant subsequently assayed for AMPK activity using the SAMS peptide as described previously (19, 27).

**Monocyte Adhesion Assay**—HAECs were grown to confluence on 24-well tissue culture plates and infected with recombinant AMPK adenoviruses, if desired, for 24 h at 20 plaque-forming units/cell. After treatment as indicated, the medium was aspirated and HAEC monolayers washed thoroughly with serum-free RPMI 1640 and overlaid with 1 × 10⁵ U937 cells/well in serum-free RPMI 1640. The cells were allowed to adhere for 1 h at 37 °C, the medium removed, and monolayers washed three times (1 ml/well serum-free Dulbecco’s modified Eagle’s medium) to remove non-adherent U937 cells. Cells were fixed in 0.5 ml/well 4% (w/v) parafomaldehyde in 5% (w/v) sucrose/phosphate-buffered saline, pH 7.2, and the number of adhered U937 cells per field of confluent HAECs counted on a Zeiss Axiovert 135 microscope with a ×20 objective.

**Nucleotide Extraction and Analysis**—Neutralized perchloric acid extracts were prepared as described previously (30). Nucleotides were separated by HPLC using a variation of the method of Usugi et al. (31). Briefly, nucleotides were separated on a stainless steel column packed with octadecylsilane attached to a Varian Prostar HPLC system equilibrated with 0.1 M triethylammonium phosphate buffer (pH 8) and methanol (96:4, v/v). Elution was monitored at A₂₅₀. The elution positions of ADP and ATP were determined using standard solutions.

**Analysis of mRNA Expression**—HAECs were incubated in serum-free large vessel cell medium for 4 h prior to preincubation for 1 h at 37 °C in KRH buffer. The medium was removed and cells incubated in the presence or absence of 10 μM GW9662 in KRH buffer for 1 h. Rosiglitazone (10 μM) was subsequently added as indicated and HAECs incubated for a further 2 h. Total RNA was prepared from cells using an RNasy kit...
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(Qiagen) according to the manufacturer’s instructions and reverse transcribed to cDNA using Moloney murine leukemia virus reverse transcriptase (Finnzymes, Espoo, Finland). Primers specific for human CD36 (forward, 5'-CTGAGCCGGA-ACTGGGCTGCT-3', and reverse, 5'-GAAGATGGCACCATTGAGCCT-3') and 18S ribosomal RNA (forward, 5'-AAAGGGCTACCACATCCAAG-3', and reverse, 5'-CGCTCCCAAGATCATTCACTAC-3') were used to amplify the cDNA by reverse transcription PCR.

Statistics—Unless stated otherwise, results are expressed as the mean ± S.E. Statistically significant differences were determined using a two-tailed Student’s t test, with p < 0.05 as significant.

RESULTS

We examined the ability of rosiglitazone to modulate NO production and AMPK activity in HAECs. Stimulation of HAECs with rosiglitazone (200 μM) stimulated the rate of NO synthesis within 30 min and reached a maximum 2.1-fold increase at 60 min (Fig. 1A). The increase in the rate of NO synthesis was sustained for 24 h. Under identical conditions, AMPK activity was maximally stimulated 3.5-fold within 30 min and activation was sustained for 24 h (Fig. 1A). Stimulation of NO by rosiglitazone was dose-dependent (Fig. 1B) such that NO synthesis was significantly stimulated by 2 μM rosiglitazone and was stimulated maximally by 20 μM rosiglitazone (2.1-fold).

Stimulation of AMPK activity was also dose-dependent, such that 2 μM rosiglitazone significantly stimulated AMPK activity, reaching a maximum 2.4-fold increase in AMPK activity at 200 μM (Fig. 1B). The principal therapeutic actions of the thiazolidinediones are thought to be alterations in gene expression mediated by PPARγ (12). We therefore determined whether AMPK activation by rosiglitazone was downstream of PPARγ activation. Preincubation of HAECs with the PPARγ inhibitor GW9662 (5 μM) was without effect on both basal or rosiglitazone-stimulated AMPK activity and NO synthesis (Fig. 1B).

Preincubation of HAECs with GW9662 did, however, prevent rosiglitazone (10 μM, 2 h)-stimulated expression of CD36 mRNA, indicating that GW9662 effectively inhibits PPARγ-mediated transcription in HAECs under these conditions (supplemental Fig. S1).

FIGURE 1. Rosiglitazone stimulates AMPK and NO synthesis in HAECs. HAEC AMPK activity and the rate of L-NAME-sensitive NO synthesis in medium were determined after incubation in 200 μM rosiglitazone for the indicated durations (A) or the indicated concentrations of rosiglitazone for 1 h (B) after preincubation with GW9662 (5 μM) for a further 1 h. Data shown represent the mean ± S.E. of NO synthesis or AMPK activity from three independent experiments. *, p < 0.05 relative to value in absence of rosiglitazone. C, under identical conditions, AMPK Thr-172 phosphorylation was determined in HAEC lysates. Representative immunoblots are shown from three independent experiments.

Activation of AMPK requires phosphorylation at Thr-172 by an AMPK kinase. Two AMPK kinases have been isolated to date, LKB1 and Ca2+/calmodulin-dependent kinase kinase (CaMKK) (32–35). It has been proposed that LKB1 activity is constitutive, such that AMP binding to AMPK inhibits dephosphorylation at Thr-172, permitting phosphorylation and activation by LKB1 (36). Using a phospho-Thr-172-specific anti-AMPK antibody, we demonstrated that rosiglitazone also stimulates phosphorylation of AMPK at Thr-172 in a time- and concentration-dependent manner (Fig. 1C), in close agreement with the AMPK assay data (Fig. 1, A and B). We next determined whether LKB1 activity was necessary for rosiglitazone-stimulated AMPK activity. HeLa cells do not express endogenous LKB1; therefore we determined the effect of rosiglitazone on AMPK activity and AMPKThr-172 phosphorylation in HeLa cells stably expressing wild type (LKB1-WT) or kinase-inactive mutant LKB1 (LKB1-KD) (24). Rosiglitazone-stimulated AMPK activity and AMPK Thr-172 phosphorylation were apparent within 15 min in cells expressing LKB1-WT, but no effect of rosiglitazone was apparent in HeLa cells expressing...
Furthermore, we measured the concentrations of AMP, ADP, and ATP in extracts from rosiglitazone-stimulated HAECs by HPLC. Using this method, the AMP concentration was too low to measure accurately, but the ADP/ATP ratio was rapidly and significantly stimulated by 20 \( \mu \text{M} \) rosiglitazone and was stimulated maximally by 200 \( \mu \text{M} \) rosiglitazone (1.26-fold, Fig. 2C). This effect of rosiglitazone was sustained for 24 h as the ADP/ATP ratio increased from 0.12 \( \pm \) 0.01 under basal conditions to 0.185 \( \pm \) 0.005 after 24 h of incubation with 100 \( \mu \text{M} \) rosiglitazone. To determine whether rosiglitazone was able to directly increase LKB1 activity (independent of adenine nucleotide ratios), we determined the activity of LKB1 after stimulation of HAECs with rosiglitazone. Rosiglitazone had no significant effect on LKB1 activity as assessed by its ability to activate recombinant AMPK kinase domain. Basal LKB1 activity was 1.51 \( \pm \) 0.06 nmol \( ^{32}\text{P} \)-incorporated/min/mg protein. In the presence of rosiglitazone, LKB1 activity was 1.62 \( \pm \) 0.51 nmol/min/mg.

We have recently demonstrated that HAECs express CaMKK and that CaMKK mediates vascular endothelial growth factor-stimulated AMPK activation in HAECs (27). Preincubation of cells with the CaMKK inhibitor STO-609 had no significant effect on rosiglitazone-stimulated AMPK activity, AMPK Thr-172 phosphorylation, or phosphorylation of the AMPK substrate acetyl CoA carboxylase (ACC), yet completely inhibited vascular endothelial growth factor-stimulated AMPK activity (Fig. 3).

To determine whether AMPK activation was required for rosiglitazone-stimulated NO synthesis, HAECs were infected with control (Ad.Null) adenoviruses or adenoviruses expressing dominant negative AMPK (Ad.\( \alpha1\text{DN} \)) prior to incubation with rosiglitazone (200 \( \mu \text{M} \)) for 1 or 24 h, and NO production was assessed. HAECs infected with Ad.\( \alpha1\text{DN} \) exhibited significantly attenuated rosiglitazone (1 h)-stimulated NO production (Fig. 4A) compared with control virus-infected cells. In HAECs incubated with rosiglitazone for 24 h there was a non-significant reduction in NO synthesis in cells infected with Ad.\( \alpha1\text{DN} \) compared with control virus-infected cells. Insulin has previously been demonstrated to stimulate NO synthesis by protein kinase B-mediated phosphorylation and activation of
eNOS, independent of AMPK (19, 37). Infection of HAECs had no effect on insulin-stimulated NO synthesis, indicating that the effect of infection with Ad.α1DN did not result in the nonspecific down-regulation of NO synthesis in an AMPK-independent manner (Fig. 4A). Infection with Ad.α1DN markedly attenuated rosiglitazone-stimulated AMPK activity as assessed by ACC phosphorylation and was without effect on eNOS expression (Fig. 4B).

We next determined whether AMPK activity was stimulated by thiazolidinediones other than rosiglitazone. Stimulation of HAECs with troglitazone or pioglitazone (20 μM) for 1 h also stimulated AMPK activity, AMPK Thr-172 phosphorylation, and ACC phosphorylation (Fig. 5). Troglitazone and pioglitazone-stimulated AMPK activity, AMPK Thr-172 phosphorylation, and ACC phosphorylation were unaffected by preincubation with GW9662.

Phosphorylation of eNOS has been demonstrated to be an important determinant of NO production. In particular, phosphorylation at Ser-1177 and dephosphorylation of Thr-495 has been demonstrated to increase eNOS activity (38). We therefore determined the effects of thiazolidinediones on eNOS phosphorylation at these sites in HAECs using phosphorylation site-specific antibodies. Incubation of HAECs with rosiglitazone, pioglitazone, and troglitazone (20 μM) stimulated phosphorylation of eNOS at Ser-1177 (Fig. 5B). In addition, stimulation of HAECs with rosiglitazone, pioglitazone, or troglitazone was without any significant effect on phosphorylation of eNOS at Thr-495 (data not shown). Preincubation of HAECs with GW9662 was without effect on thiazolidinedione-stimulated Ser-1177 phosphorylation. Furthermore, rosiglitazone-stimulated eNOS Ser-1177 phosphorylation was completely inhibited in HAECs infected with Ad.α1DN compared with cells infected with control Ad.Null viruses. In the same lysates, infection with Ad.α1DN abrogated rosiglitazone-stimulated AMPK activity as assessed by ACC phosphorylation (Fig. 6). Endothelial NO synthesis has been demonstrated to cause vasodilatation and inhibit leukocyte adhesion (1). We therefore determined whether the rapid stimulation of NO synthesis by rosiglitazone inhibited the adhesion of pro-monocytic U937 cells to cultured HAECs. Incubation of HAECs with 10 ng/ml TNFα for 6 h stimulated U937 cell adhesion 12-fold. Incubation of HAECs with 100 μM rosiglitazone for 1 h reduced TNFα-stimulated U937 cell adhesion by ~50% (Fig. 7A). This effect of rosiglitazone was completely abrogated by co-incubation with L-NAME. Neither TNFα nor L-NAME had any effect on AMPK Thr-172 phosphorylation or eNOS Ser-1177 phosphorylation in the presence or absence of rosiglitazone (Fig. 7B).
DISCUSSION

The central finding of this report is that rosiglitazone rapidly stimulates NO production and eNOS Ser-1177 phosphorylation in an AMPK-dependent manner in HAECs. In addition, we provide evidence that rosiglitazone stimulates AMPK activity utilizing an LKB1-dependent mechanism. The stimulation of AMPK by rosiglitazone is associated with increased phosphorylation of eNOS-Ser-1177. The stimulation of NO production and phosphorylation of eNOS at Ser-1177 are attenuated by infection of HAECs with Ad.α1DN. These data support the hypothesis that thiazolidinediones increase NO production by an AMPK-dependent mechanism. As AMPK has previously been demonstrated to stimulate NO synthesis via phosphorylation of eNOS at Ser-1177, the complete inhibition of rosiglitazone-stimulated eNOS Ser-1177 phosphorylation by infection with Ad.α1DN provides strong evidence that AMPK-mediated phosphorylation and activation of eNOS underlie rosiglitazone-stimulated NO synthesis. Our observation that troglitazone and pioglitazone also stimulated AMPK activity in HAECs suggests that activation of AMPK is a common mechanism utilized by thiazolidinediones.

Previous studies have demonstrated that incubation of human umbilical vein endothelial cells (HUVECs) with rosiglitazone for 48 h stimulated NO synthesis and phosphorylation of eNOS at Ser-1177 (14). Similarly, troglitazone has been reported to stimulate NO synthesis in less than 3 h and eNOS phosphorylation at the equivalent site (Ser-1179) in bovine aortic endothelial cells (13). In the current study, we show for the first time that rosiglitazone stimulates NO synthesis and eNOS Ser-1177 phosphorylation in HAECs, in agreement with the previous studies in HUVECs and bovine aortic endothelial cells. Furthermore, we demonstrate a mechanism by which the rapid stimulation of NO synthesis and eNOS Ser-1177 phosphorylation is achieved.

In HUVECs, rosiglitazone-stimulated NO synthesis and eNOS Ser-1177 phosphorylation were reported to be completely inhibited in the presence of the PPARγ inhibitor GW9662 (14). In bovine aortic endothelial cells, however, troglitazone-stimulated NO synthesis and eNOS Ser-1179 phosphorylation were only partially inhibited in the presence of the PPARγ inhibitor biphenol A diglycidyl ether (13). In the current study, inhibition of PPARγ with GW9662 abrogated rosiglitazone-stimulated CD36 mRNA expression but was without effect on either the rapid (1 h) stimulation of AMPK activity by thiazolidinediones or NO synthesis by rosiglitazone, suggesting that PPARγ is not an upstream component of rapid thiazolidinedione-stimulated, AMPK-dependent NO synthesis. The likely reason for the differences observed between the current study and those conducted previously may reflect the different cell types utilized but is more likely to be a result of the markedly different durations of rosiglitazone stimulation. In the current study, cells were incubated with rosiglitazone for 1–2 h, whereas Polikandritis et al. (14) stimulated HUVECs with rosiglitazone for 24 h and Cho et al. (13) stimulated with troglitazone for up to 24 h. HAECs stimulated with rosiglitazone for 24 h also exhibited increased AMPK activity, ADP/ATP, and...
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NO synthesis. However, although infection of HAECs with Ad.α1AMPK caused a reduction in NO synthesis in response to incubation with rosiglitazone for 24 h, this effect was not statistically significant. As the principal effect of PPARγ is the regulation of gene transcription, it seems likely that the acute effects observed in the current study are too rapid to be the result of PPARγ-mediated alterations in gene transcription. We cannot, however, rule out the later (12–24 h) effects of rosiglitazone and other thiazolidinediones being the result of altered gene transcription.

Phosphorylation at Thr-172 by upstream AMPK kinases activates AMPK. LKB1 has recently been identified as an upstream kinase for AMPK both in vitro and in vivo (26, 32, 33). Many stimuli of AMPK have been demonstrated to raise the AMP/ATP ratio, and AMP has been demonstrated to inhibit dephosphorylation of AMPK, permitting phosphorylation by LKB1, rather than activation of LKB1 itself (36). No changes in LKB1 activity have yet been demonstrated under conditions that stimulate AMPK phosphorylation, including phenformin, AICAR, muscle contraction, and extreme ischemia in a variety of tissues (39–41). In agreement with these previous studies in other tissues, rosiglitazone was unable to alter LKB1-associated AMPK kinase activity in HAECs. In the current study, rosiglitazone increased the ADP/ATP ratio in HAECs. As increases in the ADP/ATP ratio will increase further the AMP/ATP ratio, these data are in agreement with previous data that indicate rosiglitazone increases the AMP/ATP ratio in H-2Kb muscle cells (20). In addition, rosiglitazone was only able to stimulate AMPK activity in HeLa cells expressing wild type LKB1, indicating that LKB1 is required for rosiglitazone-stimulated AMPK activity in HeLa cells.

It is now apparent that CaMKKβ can act as an alternate upstream kinase to LKB1 that activates AMPK in a Ca²⁺-dependent and AMP-independent manner (34, 35). Furthermore, both thrombin and vascular endothelial growth factor-stimulated AMPK activity has been reported to be mediated by CaMKK in HUVECs and HAECs, respectively (27, 42). Our observation that STO-609 has no effect on rosiglitazone-stimulated AMPK activity indicates that CaMKK is not an upstream kinase responsible for AMPK activation in response to rosiglitazone. Taken together, we propose that rosiglitazone-mediated phosphorylation and activation of AMPK in HAECs is mediated by LKB1 in response to an increase in the cellular AMP/ATP ratio. We cannot, however, rule out the potential involvement of an as yet uncharacterized AMPK kinase that mediates rosiglitazone-stimulated AMPK activity in HAECs.

Endothelial NO synthesis has been demonstrated to inhibit monocyte adhesion to the endothelium, an early, key step in atherogenesis. A previous study has demonstrated a modest reduction in TNFa-stimulated adhesion of U937 cells to HAECs in response to incubation with pioglitazone for 24 h (43). In the current study we have demonstrated that acute (1 h) stimulation with rosiglitazone was sufficient to reduce TNFa-stimulated adhesion of U937 cells to HAECs in a NAME-sensitive manner. These data suggest that AMPK-mediated rosiglitazone-stimulated NO synthesis has rapid functional effects.

In conclusion, a number of studies have demonstrated that thiazolidinediones have the capacity to improve endothelial function in non-diabetic subjects (8, 44) and subjects with type 2 diabetes (7, 9, 10). However, recent analysis has indicated that rosiglitazone increases the risk of myocardial infarction (45), indicating that the molecular mechanisms by which rosiglitazone and other thiazolidinediones have their effects on the cardiovascular system are complex. The beneficial effects of thiazolidinediones have been reported to be independent of glycaemia, inferring a direct effect on vascular tissues (7, 10). The endothelial effects of thiazolidinediones may be relatively rapid; indeed, rosiglitazone has been reported to improve endothelial function within 24 h in healthy human subjects (44), and it is increasingly clear that the thiazolidinediones have non-genomic, PPARγ-independent effects. Furthermore, stimulation of NO synthesis and AMPK activity by rosiglitazone was achieved at concentrations as low as 2 µM. These concentrations are close to those achieved clinically; healthy subjects given 8 mg of rosiglitazone have been demonstrated to exhibit a peak plasma rosiglitazone concentration of 0.7–0.8 µM after 1 h (46).

The findings of the current study provide evidence for an LKB1- and AMPK-dependent mechanism by which thiazolidinediones rapidly contribute to increased NO bioavailability. This mechanism may underlie the rapid effects of thiazolidinediones on endothelial function that are independent of PPARγ-mediated alterations in gene transcription.

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