Phosphorylation of LKB1 at Serine 428 by Protein Kinase C-ζ Is Required for Metformin-Enhanced Activation of the AMP-Activated Protein Kinase in Endothelial Cells

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Abstract

Background—Metformin, one of most commonly used antidiabetes drugs, is reported to exert its therapeutic effects by activating AMP-activated protein kinase (AMPK); however, the mechanism by which metformin activates AMPK is poorly defined. The objective of the present study was to determine how metformin activates AMPK in endothelial cells.

Methods and Results—Exposure of human umbilical vein endothelial cells or bovine aortic endothelial cells to metformin significantly increased AMPK activity and the phosphorylation of both AMPK at Thr172 and LKB1 at Ser428, an AMPK kinase, which was paralleled by increased activation of protein kinase C (PKC)-ζ, as evidenced by increased activity, phosphorylation (Thr410/40ζ), and nuclear translocation of PKC-ζ. Consistently, either pharmacological or genetic inhibition of PKC-ζ ablated metformin-enhanced phosphorylation of both AMPK-Thr172 and LKB1-Ser428, suggesting that PKC-ζ might act as an upstream kinase for LKB1. Furthermore, adenoviral overexpression of LKB1 kinase-dead mutants abolished but LKB1 wild-type overexpression enhanced the effects of metformin on AMPK in bovine aortic endothelial cells. In addition, metformin increased the phosphorylation and nuclear export of LKB1 into the cytosols as well as the association of AMPK with LKB1 in bovine aortic endothelial cells. Similarly, overexpression of LKB1 wild-type but not LKB1 S428A mutants (serine replaced by alanine) restored the effects of metformin on AMPK in LKB1-deficient HeLa-S3 cells, suggesting that Ser428 phosphorylation of LKB1 is required for metformin-enhanced AMPK activation. Moreover, LKB1 S428A, like kinase-dead LKB1 D194A, abolished metformin-enhanced LKB1 translocation as well as the association of LKB1 with AMPK in HeLa-S3 cells. Finally, inhibition of PKC-ζ abolished metformin-enhanced coimmunoprecipitation of LKB1 with both AMPKα1 and AMPKα2.

Conclusions—We conclude that PKC-ζ phosphorylates LKB1 at Ser428, resulting in LKB1 nuclear export and hence AMPK activation.

Keywords

diabetes mellitus; endothelium; metabolism; molecular biology

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Disclosures
None.

Metformin is one of the most widely used antidiabetic agents for type 2 diabetes mellitus. In the UK Prospective Diabetic Study, metformin significantly reduced the incidence of death and cardiovascular events in obese type 2 diabetic patients compared with patients treated with other glucose-lowering reagents but with the same glycemic control,[1] suggesting that metformin may have unique protective effects against diabetes-related cardiovascular complications. In addition, metformin is shown to improve vascular endothelial function in diabetic humans.[2–7] Although it has been in clinical use for decades, the precise mechanism of action of metformin has yet to be established. Recent studies[8,9] demonstrate that metformin activates AMP-activated protein kinase (AMPK) in vitro and in vivo. Furthermore, we[10,11] found that clinically relevant concentrations of metformin activated AMPK in cultured endothelial cells and aortas in vivo and ascribed the beneficial effects of metformin on vascular function to AMPK activation. In addition, activation of AMPK by metformin is required for decreased glucose production and increased fatty acid oxidation in hepatocytes[9] and for increased glucose uptake in skeletal muscle.[8]

AMPK is a serine/threonine kinase and an evolutionary conserved regulator of the cellular response to low energy.[12,13] It is activated when nutrient supply is limited, ATP generation is impaired, or cellular energy demand is increased.[13,14] AMPK has a critical role in many metabolic processes, and, once activated, AMPK phosphorylates a number of proteins that result in increased glucose uptake and metabolism as well as fatty acid oxidation. In addition, AMPK simultaneously inhibits hepatic lipogenesis, cholesterol synthesis, and glucose production[15] and is also responsible for increased fatty acid oxidation in response to the adipocyte-derived hormones leptin[16,17] and adiponectin.[18] Phosphorylation of Thr172 in the activation loop of the catalytic α subunit is a prerequisite of significant kinase activity, and an increase in AMP/ATP ratios further allosterically stimulates the enzyme, resulting in 1000-fold activation.

Recently, at least 2 upstream kinases, LKB1 and calcium/calmodulin-dependent kinase kinase (CaMKK)-β, have been identified as AMPK kinases.[19–24] LKB1, a 50-kDa serine/threonine kinase, was originally identified as the product of the gene mutated in the autosomal dominantly inherited Peutz-Jeghers cancer syndrome.[25] In resting cells, LKB1 is reported to be predominantly located in the nucleus,[26–28] and, like AMPK, LKB1 forms a heterotrimeric complex with regulatory proteins termed STRAD and MO25, which are required for its activation and cytosolic localization.[29] LKB1 has been shown to mediate Thr172 phosphorylation of AMPK both in vitro and in intact cells.[19,21,23]

The mechanism by which metformin activates AMPK remains controversial, although earlier studies[30,31] demonstrate that metformin might activate AMPK by decreasing cellular energy by acting as an inhibitor of complex I of the respiratory chain. However, several recent studies[32,33] argue against this notion because in these studies metformin activates AMPK without affecting the AMP/ATP ratio. Interestingly, metformin neither activates AMPK in a cell-free assay nor influences the phosphorylation of AMPK by LKB1 in vitro. Furthermore, it was found that in cell lines such as HeLa-S3 cells and A549 cells, which lack LKB1 expression, or in LKB1$^{-/-}$ mouse embryonic fibroblasts, AMPK could not be activated by a variety of agonists and stresses, including metformin.[19,23] Mice lacking LKB1 expression have significantly inhibited AMPK activity and blunted increases in glucose transport caused by electrically stimulated muscle contraction and the AMPK-activating compound 5-aminoimidazole-4-carboxamide 1-β-D-ribofuranos-ide (AICAR) and phenformin.[34] Importantly, with the use of mice that have LKB1 deleted in liver, it has been reported that metformin required LKB1 in the liver to lower blood glucose.[35] Although these studies have established the critical role of LKB1 in AMPK activation, none of these studies have elucidated the upstream signaling that controls LKB1-dependent AMPK activation. In the present study,

we have found that metformin activates AMPK by activating PKC-ζ–dependent LKB1 phosphorylation at Ser428, a site that is required for LKB1 nuclear export into the cytosol and its association with AMPK.

Methods

A full description of methods, including methods such as protein kinase assays for PKC-ζ, AMPK, and LKB1, immunocytochemical staining of LKB1, and preparation of subcellular fractions, can be found in the online-only Data Supplement.

Cell Culture and Treatment

Human umbilical vein endothelial cells (HUVEC) were cultured in medium 200 supplemented with low-serum growth supplements. Bovine aortic endothelial cells (BAEC) were maintained in endothelial basal medium with 2% serum and growth factors before use. After they reached confluence, BAEC were serum deprived overnight before the experiments. HeLa-S3 and A549 cells were grown in F-12K medium supplemented with 10% serum. All culture media were supplemented with penicillin (100 U/mL) and streptomycin (100 μg/mL). The cells were treated with 1 mmol/L metformin for 1 hour after adenoviral infection or pretreatment with protein kinase inhibitor.

Plasmid Transfection and Adenovirus Infection

Site-directed mutagenesis of LKB1 was performed as described previously.[36] Mutations were confirmed by DNA sequencing, and plasmid DNA was extracted on a large scale with the use of Qiagen EndoFree plasmid maxi kit (catalogue No. 12362) and transfected into HeLa-S3 with the use of the Lipofectamine 2000 kit from Invitrogen (catalogue No. 11668-019), according to the instructions provided by the supplier. Twenty-four hours after transfection, the cells were treated with metformin (1 mmol/L) or vehicle for 1 hour. Both LacZ expression vector and untreated cells were used as control.

BAEC or HUVEC were infected with adenovirus expressing a wild-type PKC-ζ (PKC-ζ-WT) or a dominant negative mutant PKC-ζ (PKC-ζ-DN). A replication-defective adenoviral vector expressing green fluorescence protein (ad-GFP) was used as control. The AMPK-DN adenoviral vector was constructed from AMPK-α2 bearing a mutation altering lysine 45 to arginine (K45R) as described previously.[10,11] Cells were infected with the adenovirus at a multiplicity of infection of 100 in serum-free medium overnight. Cells were then washed and incubated in fresh medium without serum for an additional 18 to 24 hours before experimentation. Under these conditions, infection efficiency was typically >75% as determined by GFP expression.

Statistical Analysis

Values are expressed as mean±SE. All of the data were analyzed with a 1-way ANOVA followed by Bonferroni post hoc analyses, except for those obtained from the time course, which were analyzed with repeated-measures ANOVA. A *P* value of <0.05 is considered statistically significant.

The authors had full access to and take full responsibility for the integrity of the data. All authors have read and agree to the manuscript as written.

Results

Metformin Induces AMPK and Acetyl Coenzyme A Carboxylase Phosphorylation

Because AMPK activation requires the phosphorylation of Thr172 in the activation loop of $\alpha1$ and $\alpha2$ subunits,[37,38] AMPK activity was determined in Western blots by monitoring the phosphorylation of both AMPK at Thr172 and its best-characterized downstream kinase, acetyl coenzyme A carboxylase (ACC) at Ser79, by using specific antibodies. AMPK activation was further confirmed by AMPK activity assayed as ^{32}P incorporation into SAMS (HMRSAMSGLHLVKRR) peptides. Confluent HUVEC and BAEC were treated with metformin (1 mmol/L) for 1 hour. As shown in Figure 1A, metformin markedly increased the detection of AMPK-Thr172 and ACC at Ser79 in both HUVEC and BAEC, respectively. Because metformin activated AMPK in both BAEC and HUVEC at a similar potency, we performed most of the experiments in BAEC.

Inhibition of PKC-ζ Attenuates Metformin-Enhanced AMPK-Thr172 Phosphorylation

We have previously shown that inhibition of PKC-ζ attenuates ONOO⁻-enhanced AMPK activation in BAEC.[36] To establish PKC-ζ as a mediator for metformin-induced activation of AMPK, we first determined whether PKC-ζ–specific pseudosubstrate peptide (PKC-ζ-PS), a synthetic peptide that selectively inhibits PKC-ζ without affecting other PKC isoforms, altered the effects of metformin on AMPK-Thr172 and ACC-Ser79 phosphorylation. As depicted in Figure 1B, PKC-ζ-PS significantly ablated metformin-enhanced phosphorylation of both AMPK-Thr172 (n=5; $P<0.01$) (Figure 1B and 1C) and ACC-Ser79 (Figure 1B). The inhibitory effect of PKC-ζ-PS was also confirmed by the suppression of AMPK activity. As depicted in Figure 1D, PKC-ζ-PS dose-dependently suppressed metformin-enhanced AMPK activity (Figure 1D), as assayed by the SAMS peptides.

Additional evidence for PKC-ζ–dependent AMPK activation was obtained from genetic inhibition of PKC-ζ. As depicted in Figure 1E and 1F, adenoviral overexpression of either GFP, PKC-ζ-DN, or PKC-ζ-WT did not alter the basal levels of AMPK in BAEC. However, overexpression of PKC-ζ-DN, but not GFP, abolished the effects of metformin on both AMPK and ACC, whereas overexpression of PKC-ζ-WT significantly enhanced metformin-enhanced phosphorylation of both AMPK-Thr172 and ACC-Ser79 (Figure 1E and 1F) and AMPK activity (Figure 1G).

Activation of PKC-ζ by Metformin

We next determined whether metformin activated PKC-ζ in BAEC. The phosphorylation of PKC-ζ at Thr410/403 by upstream kinases such as phosphoinositide 3-kinase (PI-3 kinase)/ PDK-1 axis and translocation of PKC-ζ from the cytosol into cytoplasmic membrane are considered critical steps in the activation of PKC-ζ. Thus, PKC-ζ phosphorylation was monitored in total cell lysates in Western blots by using specific antibodies. As shown in Figure 2A, metformin increased PKC-ζ Thr410/403 phosphorylation without altering PKC-ζ expression. Inhibition of PKC-ζ with PKC-ζ-PS abolished metformin-induced PKC-ζ phosphorylation (Figure 2A), indicating a specific inhibition by PKC-ζ-PS.

We next assayed PKC-ζ activity by using ^{32}P incorporation in PKC-ζ–specific peptides. As expected, overexpression of PKC-ζ-DN, but not GFP, attenuated PKC-ζ activity, whereas overexpression of PKC-ζ-WT increased PKC-ζ activity in BAEC (Figure 2B). In agreement with increased PKC-ζ phosphorylation at Thr410/403 (Figure 2A), metformin significantly increased PKC-ζ activity in BAEC or BAEC infected with GFP. Overexpression of PKC-ζ-DN abolished metformin-enhanced PKC-ζ activation, whereas PKC-ζ-WT increased PKC-ζ activity (Figure 2B). These results implied that metformin activated PKC-ζ.

The translocation of PKC-ζ is considered a critical step in PKC-ζ activation. Exposure of BAEC to metformin significantly increased the presence of PKC-ζ in membrane fractions but lowered the amount of PKC-ζ in the cytosol (Figure 2C and 2D). In parallel, metformin also increased the translocation of PKC-ζ from the cytosol into the nuclei (Figure 2E and 2F). The purity of these subcellular fractions was confirmed by using antibodies against specific protein marker enzymes[39],[40] of the cytosol (lactate dehydrogenase), plasma membrane (alkaline phosphatase), or nucleus (histone H2AX), respectively. The nuclear histone H2AX was detected only in the nuclear fraction but not in cytosolic or membrane fractions (Figure 2G). Lactate dehydrogenase was detected only in the cytosolic fraction, whereas alkaline phosphatase was found only in the membrane fraction (Figure 2G). Thus, metformin caused cellular redistribution of PKC-ζ from the cytosol to nuclei and membranes.

Protein Kinase A and p90 Ribosomal S6 Kinase Are Not Required in Metformin-Enhanced AMPK Activation

Because both protein kinase A (PKA) and p90 ribosomal S6 kinase (RSK) are reported to phosphorylate LKB1 at Ser428, we next determined whether PKA or RSK was involved in metformin-enhanced phosphorylation of AMPK-Thr172 or LKB1-Ser428. As depicted in Figure 3A and 3B, administration of either H89 (10 μmol/L), a selective PKA inhibitor, or SL0101 (10 μmol/L), a selective RSK inhibitor, did not alter metformin-enhanced phosphorylation of AMPK-Thr172 or LKB1-Ser428 (Figure 3A and 3B). In contrast to increased PKC-ζ phosphorylation, metformin affected neither PKA catalytic subunit (PKAc) phosphorylation at Thr197 nor RSK3 phosphorylation at Thr356/Ser360 (Figure 3C), suggesting that metformin had no effect on PKA or RSK. Further evidence comes from coimmunoprecipitation experiments. As shown in Figure 3D, LKB1 was coimmunoprecipitated with PKCζ but not with either PKA or RSK. Furthermore, metformin markedly increased the coimmunoprecipitation of LKB1 with PKC-ζ (Figure 3D), which was sensitive to PKC-ζ inhibition (Figure 3D), suggesting that PKC-ζ activity was required for the association of PKC-ζ with LKB1. Indeed, alteration of PKC-ζ activity by overexpression of either PKC-ζ-DN or PKC-ζ-WT did not alter the phosphorylation of PKAc or RSK (Figure 3E), suggesting that the effect of PKC-ζ was independent of PKA or RSK in BAEC. Taken together, these results suggest that metformin-enhanced phosphorylation of LKB1 and AMPK was PKC-ζ dependent but independent of PKA or RSK.

Metformin-Enhanced AMPK Activation Requires LKB1

At least 2 upstream kinases, including LKB1 and CaMKK-β, have been identified as AMPK kinases.[19–24] We next determined whether metformin required LKB1 to activate AMPK in BAEC. As depicted in Figure 4A and 4B, overexpression of the kinase-dead LKB1 mutant LKB1-D194A abolished metformin-enhanced phosphorylation of both AMPK-Thr172 and ACC-Ser79 in BAEC. In contrast, overexpression of LKB1-WT enhanced the effects of metformin on the phosphorylation of both AMPK and ACC. Because overexpression of GFP did not alter the effect of metformin, these results suggested that LKB1 was required for metformin-enhanced AMPK activation in endothelial cells.

Metformin-Enhanced LKB1-Ser428 Phosphorylation Is PKC-ζ Dependent

We have previously demonstrated that ONOO$^-$ enhances the phosphorylation of LKB1 at Ser428 via PKC-ζ.[36] We next determined whether metformin altered the phosphorylation of LKB1 at Ser428. As depicted in Figure 4C and 4D, metformin significantly increased the phosphorylation of LKB1 at Ser428. Overexpression of PKC-ζ-DN, which ablated metformin-enhanced AMPK activation (Figure 1E through 1G), abolished metformin-enhanced LKB1-Ser428 phosphorylation (Figure 4C and 4D). Conversely, overexpression of GFP or PKC-ζ-WT did not suppress metformin-enhanced LKB1-Ser428 phosphorylation (Figure 4C and 4D).

However, alteration of PKC activity by overexpression of PKC-ζ-DN or PKC-ζ-WT did not alter LKB1 activity (Figure 4E). In addition, consistent with our previous results,[34,36,41,42] metformin did not alter LKB1 activity in the cells infected with either PKC-ζ-DN or PKC-ζ-WT (Figure 4E). Thus, changes of AMPK-Thr172 phosphorylation cannot be attributed to altered kinase activity of LKB1.

Metformin-Enhanced PKC-ζ Phosphorylation Occurs Before the Phosphorylation of Both LKB1 and AMPK

We next determined the time course of metformin-enhanced phosphorylation of PKC-ζ, LKB1, and AMPK. As shown in Figure 4F and 4G, metformin significantly increased the PKC-ζ phosphorylation within 1 minute. The phosphorylation of LKB1-Ser428 was significantly elevated ≈10 minutes after metformin treatment, whereas a significant increase of AMPK-Thr172 phosphorylation was observed at 30 minutes and reached a peak at 60 minutes (Figure 4F and 4G). These data suggest a chronology of events, with metformin rapidly activating first PKC (Thr410/403), then LKB1 (Ser428), and finally AMPK (Thr172).

Metformin Triggers LKB1 Translocation From Nucleus Into Cytoplasm

Earlier works[27,43–45] had indicated that LKB1 is localized mainly in the nucleus, whereas AMPK is localized mainly in the cytoplasm. LKB1 must be exported from the nucleus into cytosols where AMPK is located. We first used immunohistochemical staining to assay whether metformin altered the subcellular localization of LKB1 in HUVEC because all commercially available antibodies against LKB1 could not be used in BAEC. As expected, LKB1 was found mainly in the nucleus of nonstimulated HUVEC (Figure 5A). In metformin-treated HUVEC, LKB1 was detected mainly in the cytosols. Metformin-enhanced LKB1 nuclear export was further confirmed by Western blot analysis of LKB1 in subcellular fractions. As shown in Figure 5B and 5C, metformin significantly increased the protein amount of LKB1 in the cytosol, whereas it was markedly reduced in the nucleus of HUVEC (Figure 5A through 5C). Similarly, LKB1 was predominantly located in the nuclear fractions in BAEC. Exposure of BAEC to metformin reduced the amount of LKB1 in the nuclear fraction, whereas it increased the amount of LKB1 in the cytosols (data not shown). Interestingly, the signals corresponding to LKB1-Ser428 phosphorylation were significantly increased in both cytosol and nuclei of metformin-treated BAEC, whereas the reduced nuclear LKB1 protein content after metformin treatment (Figure 5A through 5C) corresponded to a relatively high LKB1-Ser428 signal (Figure 5D and 5E). These data suggest that LKB1 phosphorylation might occur in the nucleus, resulting in LKB1 translocation from nuclear to cytoplasmic locations.

Mutation of LKB1 Serine 428 With Alanine Prevents Metformin-Enhanced LKB1 Translocation

We next determined how metformin increased LKB1 translocation. With the use of site-directed mutagenesis techniques, Ser428 of LKB1 was mutated into alanine (LKB1-S428A, loss of function). In addition, Asp194 of LKB1, which is essential for LKB1 activity, was also replaced with alanine, resulting in a kinase-dead LKB1 mutant (LKB1-D194A). These plasmids were transfected into LKB1-deficient A549 cells. After transfection, the subcellular distributions of LKB1 were detected by using a mouse anti-His Tag antibody. As expected, A549 transfected with Lac-Z did not react with an anti-His Tag antibody (data not shown). Like LKB1 in HUVEC, LKB1 was found mainly in the nucleus of A549 transfected with either LKB1 WT, S428A mutants, or D194A mutants (Figure 6A). Metformin markedly increased LKB1 staining in the cytosol of A549 transfected with LKB1-WT but not in A549 transfected with either LKB1-D194A or LKB1-S428A mutants (Figure 6A). Western blot analysis of LKB1 in the subcellular fractions confirmed that LKB1-WT or LKB1 mutants were all nuclear proteins and that metformin significantly increased the amount of cytosolic LKB1 in A549

cells transfected with LKB1-WT but not LKB1 mutants (Figure 6B). These results suggest that the Ser428 phosphorylation of LKB1 as well as LKB1 activity was required for metformin-enhanced nuclear export.

Mutation of Ser428 Into Alanine Abolishes Metformin-Enhanced AMPK Activation

Because LKB1-Ser428 mutation altered its subcellular localization, we next determined whether Ser428 mutation of LKB1 also altered metformin-enhanced AMPK activation. As shown in Figure 7A and 7B, metformin did not activate AMPK in either HeLa-S3 or HeLa-S3 transfected with either LacZ or the D194A mutant of LKB1, confirming the essential role of LKB1 in metformin-enhanced AMPK activation. Conversely, transfection of LKB1-WT significantly increased the phosphorylation of AMPK and ACC in HeLa-S3 cells transfected with WT LKB1 (Figure 7A and 7B). Interestingly, the point mutation of LKB1-Ser428 into alanine, like the kinase-inactive mutant D194A, abolished metformin-enhanced phosphorylation of AMPK. Similarly, overexpression of LKB1-S428A and LKB1-D194A mutants ablated peroxynitrite (ONOO⁻, 100 μmol/L)-enhanced phosphorylation of AMPK, whereas the mutation of Ser428 into aspartic acid (LKB1-S428D), which mimics phosphorylation of Ser428, increased AMPK phosphorylation as LKB1-WT did (Figure 7C).

We next assayed the activity of mutant LKB1 with Ser428 (Ser431 in mouse) altered to alanine in comparison to WT LKB1, using an artificial peptide substrate. As shown in Figure 7D, LKB1-Ser431A mutants exhibited a 10% reduction of LKB1 activity compared with LKB1 WT.

We next determined whether LKB1-Ser428 phosphorylation increased LKB1 activity. Mouse LKB1-WT or LKB1-S431A (equivalent to human 428) mutants were coexpressed with both MO25α and STRADα in bacteria. Partially purified LKB1 WT or LKB1-S431A complex was coincubated with recombinant PKC-ζ. PKC-ζ–dependent LKB1 phosphorylation and LKB1 activity were assayed by using LKB1tide or p53. PKC-ζ increased ^{32}P incorporation in LKB1 WT paralleled with a 10% increase in LKB1 activity but had less effect on LKB1-S431A mutants. Only LKB1, but not STRADα or MO25α, was phosphorylated by PKC-ζ as determined by ^{32}P autoradiographic gels when PKC-ζ was incubated with LKB1-MO25α-STRADα complex in vitro (data not shown). Because LKB1 S431A mutants expressed a 10% reduction of LKB1 activity, whereas PKC-ζ caused a 10% increase of LKB1 activity, these results suggest that the phosphorylation of LKB1 at Ser428/431 might increase LKB1 activity by 10%. However, such rather small increases of LKB1 activity would only partly explain the strongly elevated AMPK activity after metformin treatment, therefore emphasizing the importance of the nucleocytoplasmic redistribution of LKB1 that is expected to increase AMPK activation in the cytosol.

Mutation of Ser428 Into Alanine Abolishes Metformin-Enhanced Association of LKB1 With AMPK

We have previously shown that ONOO⁻ activates AMPK by increasing the association of LKB1 and AMPK.[36] In addition, we found that PKC-ζ coimmunoprecipitated with LKB1 and metformin increased the association of LKB1 with PKC-ζ. We next determined whether phosphorylation of LKB1-Ser428 by PKC-ζ contributed to metformin-enhanced association of AMPK with LKB1 and therefore whether LKB1 was immunoprecipitated from BAEC and stained with AMPK in Western blots. As shown in Figure 7E, metformin showed significantly increased coimmunoprecipitation of LKB1 with AMPKα1 or AMPKα2. Importantly, inhibition of PKC-ζ blunted metformin-enhanced association of LKB1 with AMPK (Figure 7E), suggesting that PKC-ζ enhances the association of LKB1 with AMPK.

We next investigated whether mutations of LKB1-Ser428 into alanine altered the interaction of AMPK and LKB1 in HeLa-S3 cells. Metformin increased the association of AMPK and LKB1 in HeLa-S3 cells transfected with LKB1 WT but not in HeLa-S3 transfected with LacZ or LKB1 mutants (Figure 7F), implying that LKB1-Ser428 phosphorylation is required for metformin-enhanced AMPK activation.

Discussion

In the present study, we have established that LKB1-Ser428 phosphorylation by atypical PKC-ζ plays an essential role in metformin-enhanced AMPK activation. LKB1-Ser428 phosphorylation is required for both its nuclear export of LKB1 into cytosols, in which it forms an active complex with STRAD and MO25 and consequent AMPK phosphorylation. Because various stimuli increased the phosphorylation of LKB1 at Ser428, it is tempting to speculate that LKB1-Ser428 phosphorylation might be a common pathway required for AMPK activation.

LKB1 localization depends on its kinase activity, import into the nucleus, and retention within cytoplasm.[27,29] It has been reported that cytoplasmic localization of LKB1 is critical for its normal function.[27,29] Several residues including Ser31, Thr336, Thr366, and Ser 428 of LKB1 have been identified as phosphorylation sites, although their physiological functions are largely unknown. In the present study, we have provided evidence that metformin induced AMPK activation by phosphorylating LKB1 at Ser428, which results in translocation of LKB1 from the nucleus to the cytosol and increased association of LKB1 with AMPK. The key findings can be summarized as follows: metformin increases LKB1 phosphorylation at Ser428, induces LKB1 translocation from nucleus to cytoplasm, and enhances coimmunoprecipitation of LKB1 with AMPK. Mutation of Ser428 into alanine prevented translocation of LKB1 from the nucleus to the cytoplasm and abolished metformin-enhanced association of LKB1 with AMPK, thus abolishing AMPK activation. The LKB1-S428A mutation did not affect normal nuclear localization of LKB1 but prevented LKB1 translocation to the cytoplasm after metformin treatment, suggesting that phosphorylation of Ser428 might be essential for LKB1 translocation from the nucleus to the cytoplasm. Thus, Ser428 phosphorylation located in the C-terminus of LKB1 might play a crucial role in regulating AMPK activation. In agreement with this notion, Forcet et al[46] demonstrated that C-terminal mutations impair LKB1-mediated activation of AMPK and downstream signaling but do not affect LKB1 autokinase activity or abolish LKB1-induced growth arrest. Our data indicate that mutation of Ser428 of LKB1 to alanine impaired its ability to export from nucleus and subsequent association with AMPK after metformin treatment (Figure 7F), suggesting that phosphorylation of Ser428 might contribute to its binding to AMPK and that mutations of this site into alanine uncouple this process, thereby resulting in reduced enzyme-substrate recognition. These results are in contrast with the previous report that mutation of Ser431 to either alanine to abolish phosphorylation or to glutamate to mimic phosphorylation did not significantly affect the cellular localization or catalytic activity of LKB1 in vitro.[47] The reasons for the discrepancy are unknown and might be related to the species differences (human versus mouse) or cell types. In vivo, LKB1 forms a heterotrimeric complex with 2 proteins termed STRAD and MO25.[29,48] STRAD is an LKB1-specific adaptor and substrate; when binding to the kinase domain of LKB1, STRAD enhances LKB1 activity >100-fold and anchors LKB1 in the cytosol. MO25 stabilizes the binding of STRAD to LKB1 and cooperates with STRAD to localize LKB1 in the cytoplasm of cells.[49] In addition, LKB1 can also be localized in the cytoplasm of cells through an interaction with a cytosolic protein called LIP1 (LKB1 interacting protein-1).[50] It is reported that in muscle-specific LKB1 knockout mice, AICAR, phenformin, or muscle contraction did not increase AMPKα2 activity in the skeletal muscle, indicating that LKB1 is the major upstream kinase of AMPK.[34] However, these 3 treatments failed to increase LKB1 activity.[41] In our study, we found that metformin increased AMPK phosphorylation and activity.

Although it did not change LKB1 activity to a great extent, metformin markedly enhanced LKB1 phosphorylation at Ser428; mutation of Ser428 into alanine prevented translocation of LKB1 from nucleus to cytoplasm and abolished metformin-enhanced association of AMPK with LKB1, thus abrogating AMPK activation. Theses results demonstrate that the phosphorylation of LKB1 at Ser428 plays a crucial role in AMPK activation by altering its cellular location or by enabling it to interact with regulatory proteins or protein substrates. In addition, the active form of LKB1 might be as important as Ser428 phosphorylation because the mutation of LKB1 D194 into alanine prevented the translocation of the enzyme. Because LKB1 S431A mutants expressed a 10% reduction of LKB1 activity, whereas PKC-ζ caused a 10% increase of LKB1 activity, metformin-enhanced AMPK activity is likely via the nucleocytoplasmic redistribution rather than an increase of LKB1 activity as a result of LKB1-Ser428 phosphorylation. Moreover, it remains to be determined whether other sites are required for LKB1 translocation.

We have previously reported[10,11,51,52] that activation of PI-3 kinase by metformin or ONOO⁻ might enhance AMPK activity by increasing the association of LKB1 with AMPK. Indeed, inhibition of c-Src or PI-3 kinase activity by pharmacological inhibition or dominant negative mutants abolishes metformin- or ONOO⁻-enhanced AMPK activation in endothelial cells.[10,11,53,54] In the present study, we not only confirm but also extend these observations by demonstrating that PKC-ζ, a member of the PI-3 kinase AGC family, plays a critical role in metformin-induced AMPK activation. Indeed, it has been shown that some AMPK agonists, such as AICAR and dinitrophenol, increase PKC-ζ activity in isolated extensor digitorum longus muscle and L6 myotubes.[55] In type 2 diabetic patients, metformin increases PKC-ζ activity, accompanied by increased AMPK activity.[56] We have also demonstrated that PKC-ζ phosphorylates LKB1 at Ser428 in both in vitro assays and in cultured cells, including endothelial cells, pericytes, adipocytes, and cultured vascular smooth muscle cells.[36] Furthermore, inhibition of PKC-ζ attenuates AMPK activation by other stimuli (Z.X., unpublished data, 2008). Because other kinases, including cAMP-dependent PKA and p90 RSK, are reported to phosphorylate LKB1 at Ser428, the Ser428 phosphorylation of LKB1 might be a common pathway for AMPK activation triggered by signaling pathways other than the c-Src/PI-3 kinase axis (Figure 8).

To summarize, we provide evidence that PKC-ζ plays a critical role in regulating AMPK activity. Our data suggest that metformin-induced AMPK activation requires LKB1 and demonstrate that phosphorylation of Ser428 of LKB1 could affect cellular location of LKB1 and its interaction with AMPK. We conclude that PKC-ζ can regulate AMPK activity by increasing LKB1 phosphorylation, resulting in LKB1 nuclear export and consequent AMPK Thr172 phosphorylation by LKB1. In addition, the Ser428 phosphorylation of LKB1 might be a common pathway for AMPK activation triggered by signal pathways, including PI-3 kinase/PKC-ζ axis, PKA, and RSK.

CLINICAL PERSPECTIVE

Metformin is one of most commonly used drugs for the treatment of type II diabetes mellitus. Unlike traditionally used glucose-lowering reagents, such as sulfonylureas or insulin, metformin improves cardiovascular functions and reduces cardiovascular risks in diabetes. Earlier studies from us and others found that clinically relevant concentrations of metformin activated the AMP-activated protein kinase (AMPK); these studies ascribed the beneficial effects of metformin on vascular function to AMPK activation. The present study was designed to elucidate the mechanism by which metformin activates AMPK. Exposure of cultured endothelial cells to metformin significantly increased the phosphorylation of both AMPK at Thr172 and LKB1 at Ser428, an AMPK kinase, which was paralleled by increased activation of protein kinase C (PKC)-ζ, as evidenced by increased activity, phosphorylation,

and nuclear translocation of PKC-ζ. Consistently, either pharmacological or genetic inhibition of PKC-ζ ablated metformin-enhanced phosphorylation of both AMPK at Thr172 and LKB1 at Ser428. Our data suggest that the Ser428 phosphorylation of LKB1 by PKC-ζ is required for metformin-triggered AMPK activation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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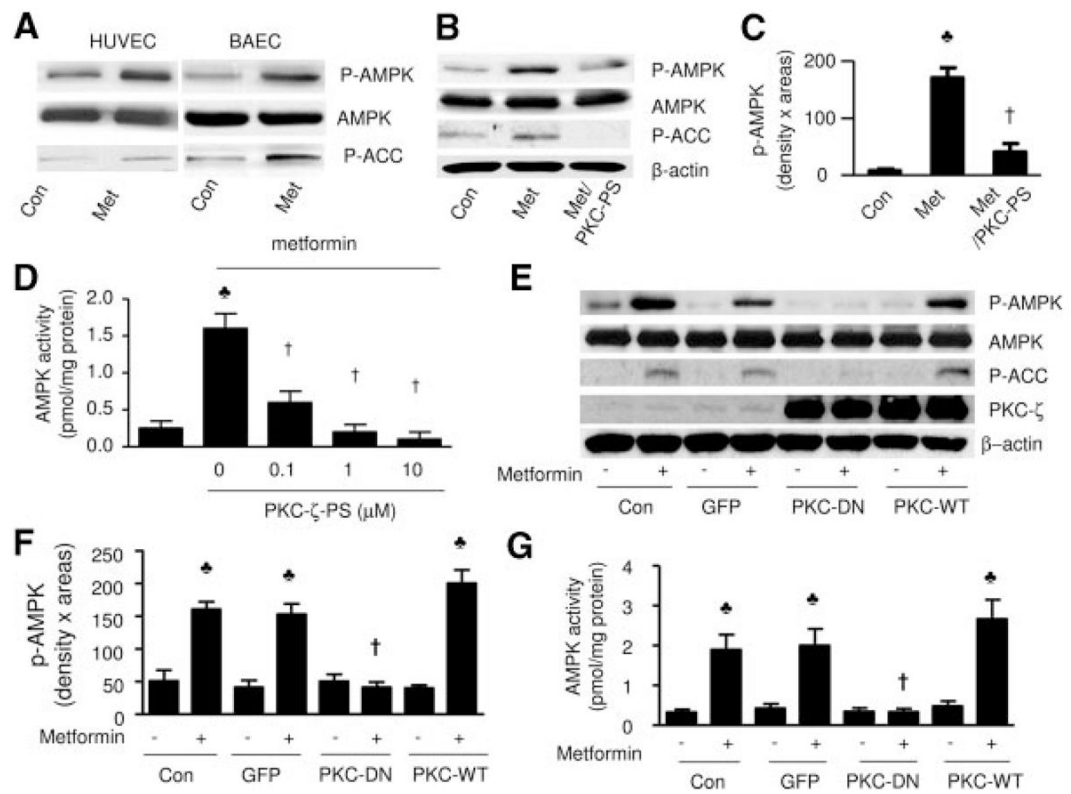
Figure 1.
Inhibition of PKC-ζ attenuates metformin-enhanced AMPK activity. Confluent BAEC or HUVEC, with or without adenovirus infection, were preincubated with PKC-ζ-PS for 30 minutes before being exposed to metformin. After treatment, the cells were lysaed and extracted. Both phosphorylated AMPK-Thr172 and ACC-Ser79 were detected in Western blots by using specific antibodies, as described in Methods. A, Metformin (Met) activates AMPK in BAEC and HUVEC. The blot is a representative of 6 blots obtained from 6 individual experiments. Con indicates control. B and C, Selective inhibition of PKC-ζ attenuated metformin-enhanced AMPK activity (n=6). ♣$P<0.05$ (control [Con] vs metformin treated), †$P<0.05$ (metformin vs metformin plus PKC-ζ-PS). D, PKC-ζ-PS dose-dependently inhibits metformin-enhanced AMPK activity in BAEC (n=5). ♣$P<0.05$ (control vs metformin), †$P<0.05$ (metformin vs metformin plus PKC-ζ-PS). E and F, Genetic inhibition of PKC-ζ with PKC-ζ-DN, but not the adenovi-rus encoding GFP, attenuated metformin-enhanced phosphorylation of AMPK-Thr172 and ACC-Ser79 in BAEC. The blot is representative of 5 blots from 5 individual experiments (n=5). ♣$P<0.05$ (compared with control), †$P<0.05$ (metformin-treated vs metformin plus adenovirus). G, AMPK activity was assayed as described in Methods (n=4). ♣$P<0.05$ (compared with control), †$P<0.05$ (metformin-treated vs metformin plus adenovirus).
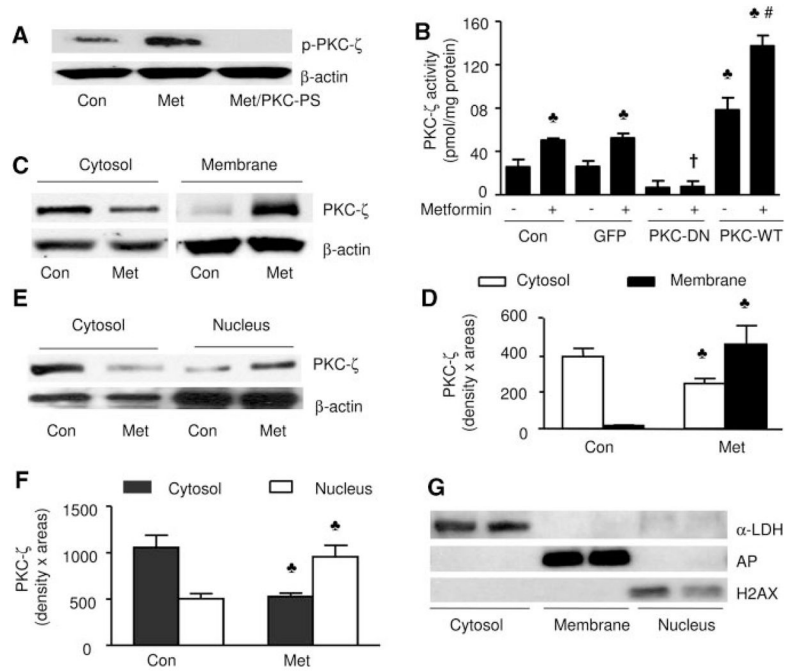
Figure 2.
Metformin increases PKC-ζ phosphorylation and the translocation of PKC-ζ from cytosol to the membrane. Confluent BAEC were exposed to metformin (1 mmol/L, 1 hour), and the translocation of PKC-ζ and PKC-ζ phosphorylation was assayed as described in Methods. A, Metformin (Met) increased the phosphorylation of PKC-ζ in BAEC. The blot is a representative of 3 blots obtained from 3 independent experiments. Con indicates control. B, PKC-ζ activity was determined as described in Methods (n=4). ♣$P<0.05$ (compared with control [Con]), †$P<0.05$ (metformin-treated vs metformin plus adenovirus), #$P<0.05$ (PKC-ζ-WT vs metformin plus WT adenovirus). C and D, Metformin increases the translocation of PKC-ζ to the membrane (n=5). ♣$P<0.05$ (control vs metformin-treated). E and F, Metformin increases the translocation of PKC-ζ from cytosol into the nucleus. The blot is a representative of 3 blots from 3 individual experiments (n=5). ♣$P<0.05$ (control vs metformin-treated). G, Analysis of the purity of subcellular fractions. The subcellular fractions were prepared as described in Methods. Marker enzymes were detected by Western blot with the use of specific antibodies.
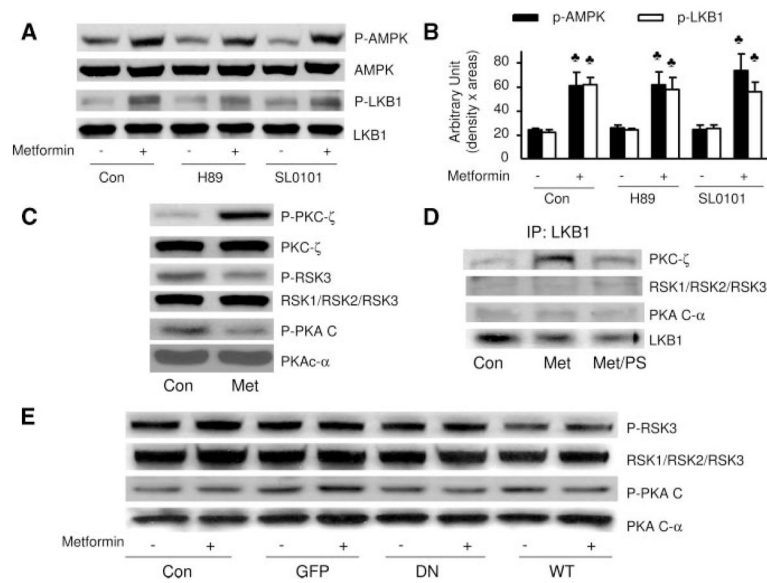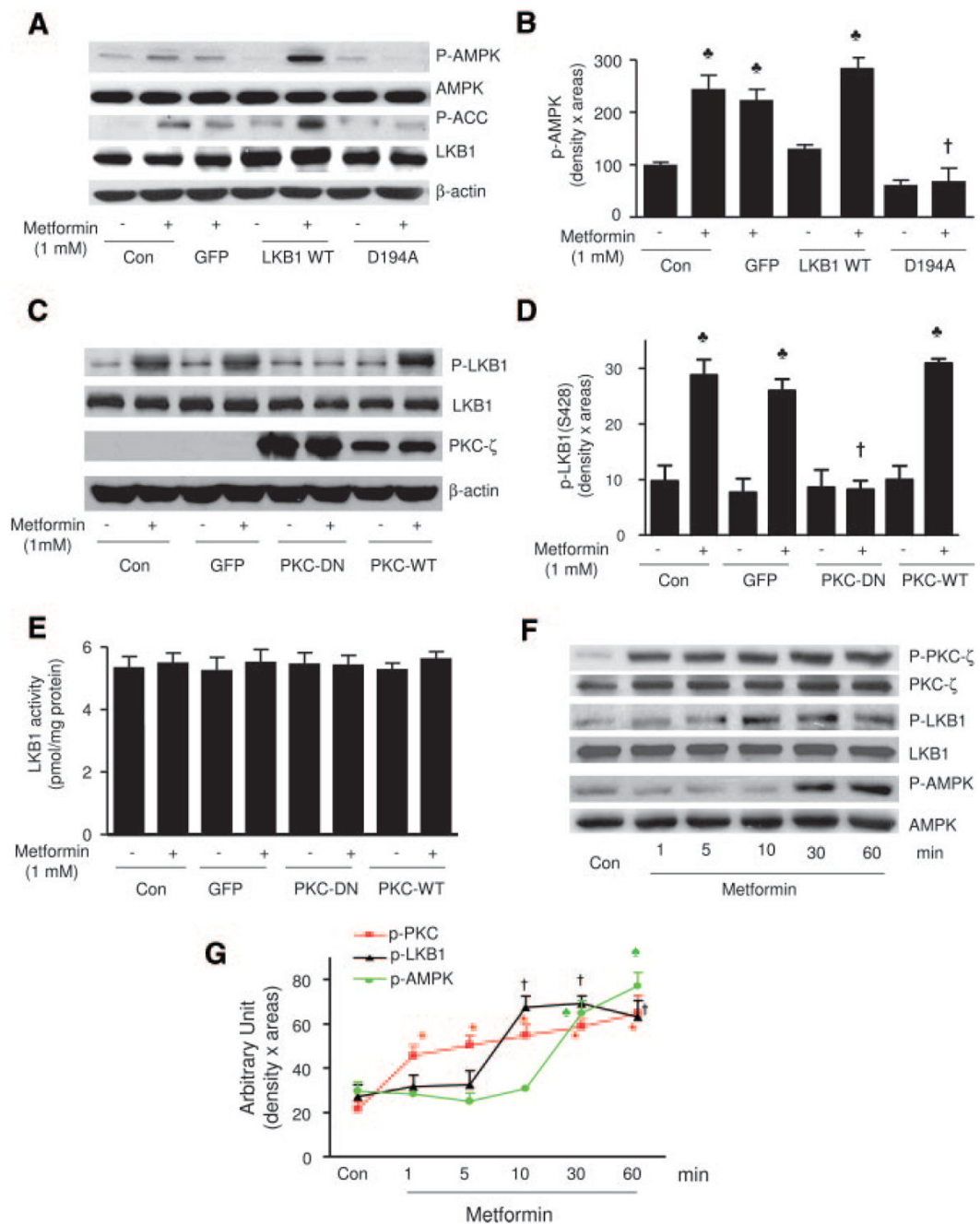
Figure 3.
Neither PKA nor RSK is involved in metformin-enhanced AMPK activation in BAEC. A and B, Confluent BAEC were pretreated with protein kinase inhibitors for 30 minutes followed by treatment with metformin (1 mmol/L) for 1 hour, the cells were lysed, and phosphorylation of AMPK and LKB1 was detected by Western blot. The blot is a representative of blots from 3 different experiments. ♣P<0.05 compared with respective control (Con). C, BAEC were treated with metformin (Met) (1 mmol/L) for 1 hour, cell lysate was prepared, and the phosphorylation of PKC-ζ, PKAc, and RSK3 was detected by Western blot with the use of specific antibodies. The blot is a representative of 3 blots obtained from 3 individual experiments. D, LKB1 was immunoprecipitated, and PKC-ζ, PKAc, and RSK3 were detected by Western blotting. The blot is representative of blots from 3 different experiments. E, Confluent BAECs were transfected with PKC-ζ-DN and PKC-ζ-WT adenovirus for 48 hours and treated with metformin for 1 hour. The cells were lysed and analyzed by Western blotting. The blot is a representative of 3 blots obtained from 3 individual experiments.

Figure 4.
PKC-ζ–dependent LKB1 phosphorylation of LKB1 at serine 428. A and B, Metformin-activated AMPK is LKB1 dependent in BAEC. Adenoviral overexpression of the kinase-dead LKB1 mutant blocks metformin-induced AMPK activation, whereas LKB1 WT overexpression enhanced the effect of LKB1 in BAEC. The blot is a representative of 5 blots from 5 independent experiments. C and D, Metformin-enhanced LKB1 phosphorylation at serine 428 is PKC-ζ dependent. The blot is a representative of 5 blots from 5 independent experiments (n=5). ♣$P<0.05$ (control [Con] vs metformin), †$P<0.05$ (metformin vs metformin plus PKC-ζ adenovirus). E, LKB1 activity was measured as described in Methods (n=4). F and G, Time course of PKC-ζ, LKB1, and AMPK phosphorylation. BAEC were treated with

metformin (1 mmol/L), and the phosphorylation of PKC-ζ, LKB1, and AMPK was detected at the indicated time by Western blot analysis. The blot is a representative of 3 blots from 3 individual experiments. *♣†$P<0.05$ compared with respective controls.
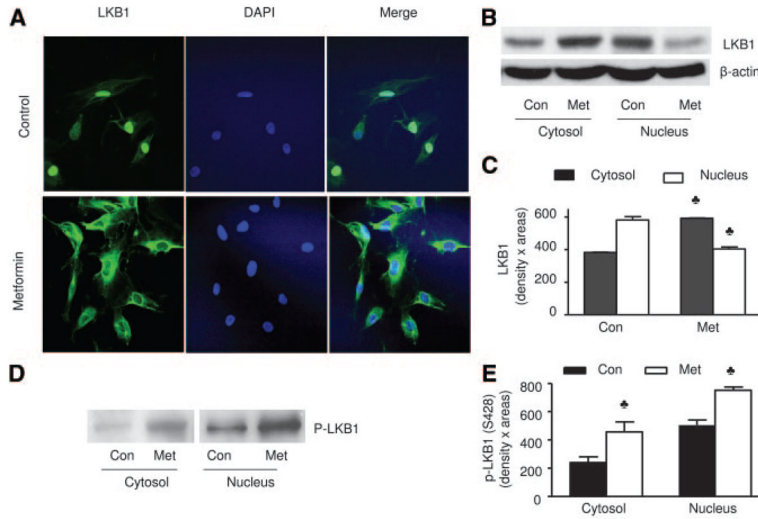
Figure 5.
Metformin increases the translocation of LKB1 from nucleus into cytosol in HUVEC. A, Immunocytochemical staining of translocation of LKB1 from nucleus to the cytosols caused by metformin in HUVEC. B and C, Metformin (Met) also increases the amount of LKB1 in the cytosol while it decreases LKB1 in the nuclei in HUVEC. The blot is a representative of 5 blots obtained from 5 independent experiments. Con indicates control. D and E, Metformin increases the serine 428 phosphorylation of LKB1 in both nucleus and cytosol. The blot is a representative of 3 blots obtained from 3 independent experiments.
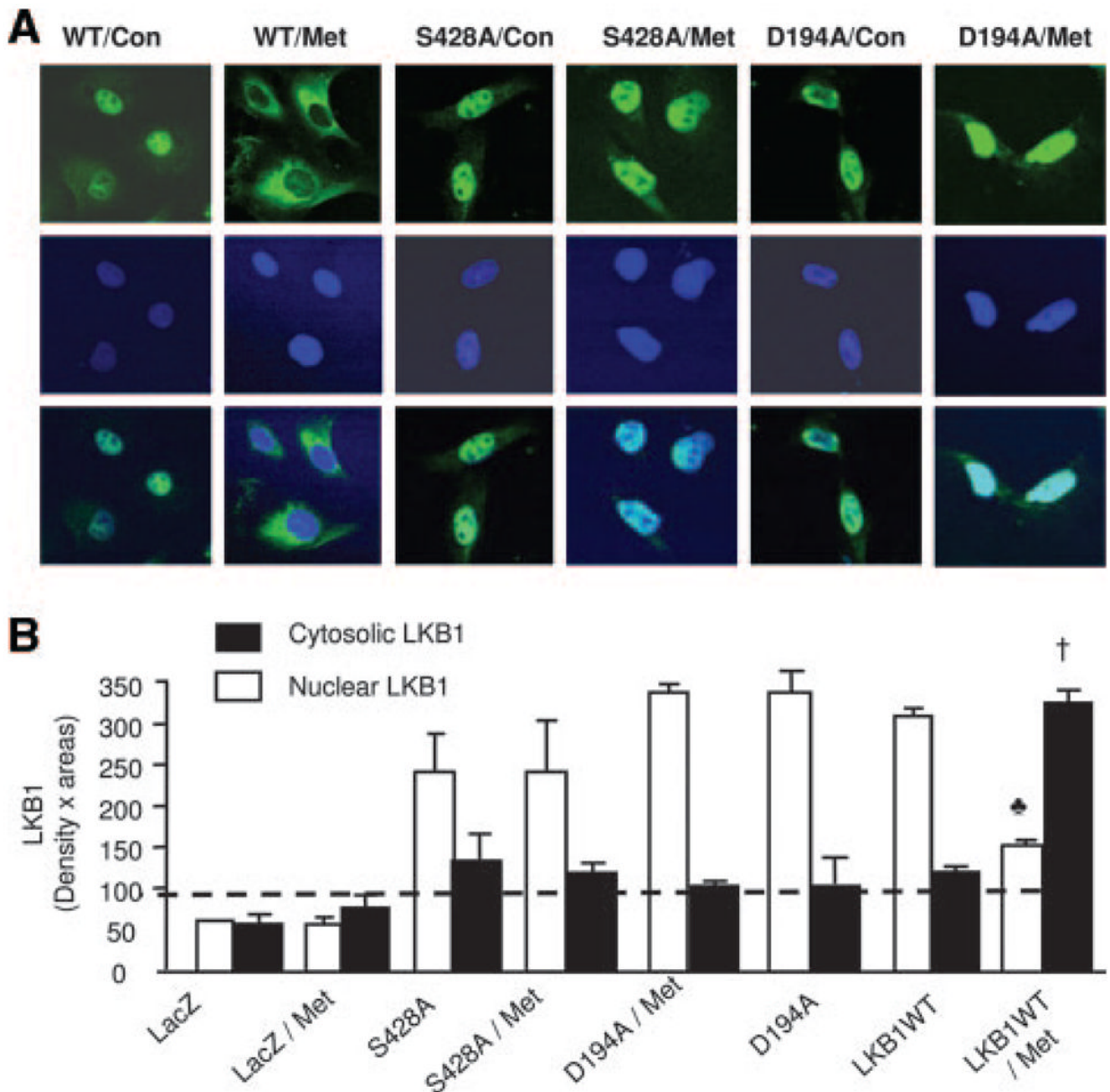
Figure 6.
The phosphorylation of LKB1-Ser428 is required for metformin-enhanced translocation of LKB1 and AMPK activation. A, Immunocytochemical staining of metformin (Met)-enhanced LKB1 translocation in A549 cells. The cells were transfected with indicated plasmids, as described in Methods. LKB1 was detected by using a mouse anti-His Tag antibody. The image with Lac-Z was omitted because A549 cells transfected with Lac-Z did not react with the antibody. Con indicates control. B, Western blot detection of LKB1 in A549 cells. The blot is a representative of 5 blots from 5 independent experiments (n=5). ♣$P<0.05$ (WT vs WT plus metformin), †$P<0.05$ (WT plus metformin vs WT or S428A plus metformin).
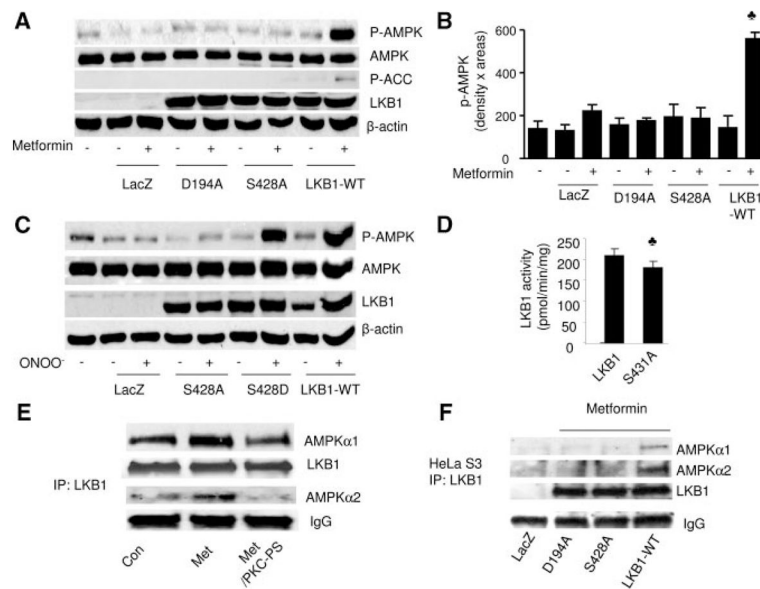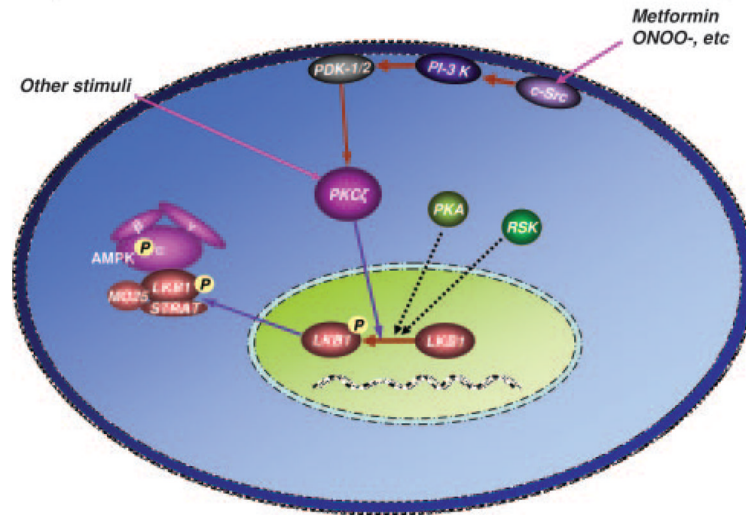
Figure 7.
Ser428 phosphorylation of LKB1 is required for metformin-enhanced AMPK activation and coimmunoprecipitation of LKB1 with AMPK. A and B, Metformin activates AMPK in HeLa-S3 overexpressing LKB1-WT but not LKB1-Ser428A mutants. After being transfected with LKB1-WT or kinase-dead LKB1 mutants or the LKB1-Ser428A mutant, HeLa-S3 cells were exposed to metformin (1 mmol/L, 1 hour). AMPK activation was monitored in Western blots with the use of specific antibodies. The blot is a representative of 5 blots from 5 independent experiments. C, Ser428 phosphorylation of LKB1 is required for ONOO⁻-enhanced AMPK activation. After being transfected with LKB1-WT or LKB1-Ser428A (Ser428 was mutated into alanine, loss of function) or the LKB1-Ser428D (Ser428 replaced by aspartic acid, phosphorylation mimicking), HeLa-S3 cells were exposed to ONOO⁻ (100 μmol/L). AMPK activation was monitored by Western blotting with the use of specific antibodies. The blot is a representative of 5 blots from 5 independent experiments. D, LKB1 activity in LKB1 WT or LKB1-Ser431A mutants. E, Metformin increases the association of LKB1 and AMPK. LKB1 was immunoprecipitated from BAEC, and AMPK was detected in Western blots. Inhibition of PKC-ζ attenuated metformin-enhanced association of LKB1 with AMPK. The blot is a representative of 5 blots from 5 independent experiments. F, Mutation of Ser428 of LKB1 into alanine (S428A) abolishes metformin-enhanced coimmunoprecipitation of LKB1 with AMPK. Metformin increased the coimmunoprecipitation of LKB1 with LKB1 WT but not with LKB1 mutant. The blot is a representative of 3 blots from 3 individual experiments.

Proposed mechanism for metformin-enhanced AMPK activity

Figure 8.
LKB1 nuclear export is required for metformin-enhanced AMPK activation. In the resting state, LKB1 is mainly located in the nucleus, whereas LKB1 activity required that adaptor proteins MO25 and STRAD as well as AMPK-α1 (the major isoform of AMPK catalytic units in endothelial cells) be located in the cytosol. Metformin (or ONOO$^-$) activates PI-3 kinase, resulting in PDK-1/2 phosphorylation. PDK-1/2 phosphorylates PKC-ζ, resulting in the translocation of the latter into nucleus as well as cytoplasmic membrane. In the nucleus, activated PKC-ζ phosphorylates LKB1 at serine 428 and likely other sites. Phosphorylated LKB1 is exported from the nucleus via an unknown mechanism into the cytosol. LKB1 is subsequently bound to the preexisting adaptors proteins (STRAD and MO25), which recruit and phosphorylate AMPK at Thr172. AMPK is likely also activated by other stimuli, which either activate PKC-ζ or increase LKB1-Ser428 phosphorylation (PKA or RSK90). Overall, our results suggest that the PI-3 kinase-PDK1/2-PKC-ζ pathway plays a critical role in metformin-enhanced AMPK activation.