Activation of Protein Kinase Cζ by Peroxynitrite Regulates LKB1-dependent AMP-activated Protein Kinase in Cultured Endothelial Cells* 

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We previously reported the phosphoinositide 3-kinase-dependent activation of the 5′-AMP-activated kinase (AMPK) by peroxynitrite (ONOO−) and hypoxia-reoxygenation in cultured endothelial cells. Here we show the molecular mechanism of activation of this pathway. Exposure of bovine aortic endothelial cells to ONOO− significantly increased the phosphorylation of both Thr172 of AMPK and Ser1179 of endothelial nitric-oxide synthase, a known downstream enzyme of AMPK. In addition, activation of AMPK by ONOO− was accompanied by increased phosphorylation of protein kinase Cζ (PKCζ) (Thr410/403) and translocation of cytosolic PKCζ into the membrane. Further, inhibition of PKCζ abrogated ONOO−-induced AMPK-Thr172 phosphorylation as that of endothelial nitric-oxide synthase. Furthermore, overexpression of a constitutively active PKCζ mutant enhanced the phosphorylation of AMPK-Thr172, suggesting that PKCζ is upstream of AMPK activation. In contrast, ONOO−-activated PKCζ in LKB1-deficient HeLa-S3 but affected neither AMPK-Thr172 nor AMPK activity. These data suggest that LKB1 is required for PKCζ-enhanced AMPK activation. In vitro, recombinant PKCζ phosphorylated LKB1 at Ser428, resulting in phosphorylation of AMPK at Thr172. Further, direct mutation of Ser428 of LKB1 into alanine, like the kinase-inactive LKB1 mutant, abolished ONOO−-induced AMPK activation. In several cell types originating from human, rat, and mouse, inhibition of PKCζ significantly attenuated the phosphorylation of both LKB1-Ser428 and AMPK-Thr172 that were enhanced by ONOO−. Taken together, we conclude that PKCζ can regulate AMPK activity by increasing the Ser428 phosphorylation of LKB1, resulting in association of LKB1 with AMPK and consequent AMPK Thr172 phosphorylation by LKB1.

The AMP-activated protein kinase (AMPK) is a serine/threonine kinase and a member of the Snf1/AMPK protein kinase family (1–3). Its activity is stimulated by an increase in intracellular AMP-to-ATP ratio in response to stresses such as exercise (4–6), hypoxia (7, 8), oxidative stress (9, 10), and glucose deprivation (11). AMPK activation switches on catabolic pathways that produce ATP and switches off anabolic pathways that consume ATP. The activation of AMPK leads to phosphorylation of a number of proteins that result in increased glucose uptake and metabolism as well as fatty acid oxidation and simultaneously in inhibition of hepatic lipogenesis, cholesterol synthesis, and glucose production (reviewed in Refs. 12–14). AMPK is also responsible for increased fatty acid oxidation in response to the adipocyte-derived hormones leptin (15) and adiponectin (16). Because AMPK activation could have beneficial metabolic consequences for diabetic patients, AMPK has emerged as a potential target for the treatment of obesity and type II diabetes (reviewed in Refs. 3 and 17). It has been demonstrated that two classes of anti-diabetic drugs, metformin (18, 19) and thiazolidinediones (20), can act at least in part through activation of AMPK in liver and muscle.

AMPK is an obligatory heterotrimer containing catalytic α subunit and regulatory β and γ subunits, each of which occur in at least two isoforms. Activation of AMPK absolutely requires its phosphorylation at Thr172 in the activation loop of α1 and α2 subunits by one or more upstream kinases (AMPKks) (21, 22).

The major breakthrough in identifying the first AMPKK came from research on the regulation of the AMPK ortholog Snf-1 in Saccharomyces cerevisiae (23, 24). The T-loop residue of Snf-1 was phosphorylated by a group of three related protein kinases bearing homology to mammalian LKB1, which was subsequently identified by several laboratories as being the major upstream kinase for AMPK (25–27). LKB1 was found to co-purify with liver AMPK and to phosphorylate recombinant AMPK complexes. In addition, AMPK could not be activated in mammalian cells that lacked LKB1 expression or in cells that were treated with Hsp90 inhibitors, which decrease LKB1 expression (28, 29). Finally, LKB1 turned out to phosphorylate the T-loop of all 12 human kinases that are phylogenetically related to AMPK (AMPK subfamily) (30). However, paradoxically, neither the activity of LKB1 itself nor that of AMPK-related kinases was regulated directly by the stimuli known to activate AMPK, like e.g. AMP, AICAR, or muscle contraction (31, 32). Thus, the question remained regarding how AMPK stimuli can lead to LKB1-dependent AMPK activation.

We had previously reported that peroxynitrite (ONOO−), a potent phosphoinositide; PKA, protein kinase A; PKG, protein kinase G; PKC, protein kinase C; PMA, phorbol 12-myristate 13-acetate; PS, pseudosubstrate; AMPK, AMPK upstream kinase; ACC, acetyl-CoA carboxylase; AICAR, aminomimidazole-4-carboxamide-1-β-D-ribose.
oxidant formed by the combination of superoxide anions (O₂⁻) and NO at a diffusion-controlled rate, activated AMPK independent of the cellular AMP/ATP ratios (19, 33, 34). We further demonstrated that short periods of hypoxia-reoxygenation (H/R)-activated AMPK in a ONOO⁻-dependent manner, which was also independent of the AMP/ATP ratios, but was sensitive to PI 3-kinase inhibition with either pharmacological inhibitors or overexpression of PKD1 dominant negative mutants (PDK1-DN) (34). PI 3-kinase-dependent AMPK activation has since been observed by others in insulin-stimulated platelets (35) and adiponectin-stimulated endothelial cells (36). Paradoxically, activation of PI 3-kinase with insulin or growth factors that stimulate the PI 3-kinase/Akt/PDK1 pathway either did not affect AMPK in most cell types (37) or rather caused AMPK inhibition in some cases (34, 38). Therefore, PI 3-kinase/PDK1 will very likely not serve as a direct upstream kinase for LKB1, and the mechanism for AMPK activation remains to be established.

In the present study, we have established a central role of atypical protein kinase Cζ (PKCζ), a protein kinase of the AGC family, as a key regulator in LKB1-dependent AMPK activation. We found that inhibition of PKCζ with pharmacological and genetic inhibitors effectively blocked AMPK activation caused by ONOO⁻. This AMPK activation pathway was LKB1-dependent, involved LKB1 phosphorylation at Ser428 within the C-terminal part of LKB1, and led to the association of LKB1 with AMPK. The central role of PKCζ in the LKB1-AMPK axis is further supported by the fact that inhibition of PKCζ with either pharmacological (PKCζ-PS) or genetic (PKCζ-DN) inhibitors blunted AMPK activation caused by (ONOO⁻). Finally, in vitro, recombinant PKCζ phosphorylated both LKB1 at Ser428. We conclude that PKCζ-dependent and LKB1-mediated AMPK activation might play important roles in regulating not only cellular energy metabolism but also signaling pathways that control cell growth, differentiation, and survival.

EXPERIMENTAL PROCEDURES

Materials

Bovine aortic endothelial cells (BAEC) and cell culture media were obtained from Clonetics Inc. (Walkersville, MD). BAEC were maintained in endothelial basal medium with 2% serum and growth factors before use. HeLa-S3 cells were obtained from ATCC (Manassas, VA) and were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% serum. All culture media were added with penicillin (100 units/ml) and streptomycin (100 μg/ml) (32). [32P]ATP was obtained from PerkinElmer Life Sciences. The SAMS peptide and recombinant LKB1 were purchased from Upstate Biotechnology, Inc. (Lake Placid, NY). Antibodies against phospho-AMPK (Thr172), AMPK-α, phospho-eNOS (Ser1177), phosphor-LKB1 (Ser428, Ser334, and Thr189), LKB1 (for Western blot), PKCζ, phospho-PKCζ (Thr410-415), PKCα/β, and phospho-PKCα/β (Thr586,641) were obtained from Cell Signaling Inc. (Beverly, MA). Antibody against eNOS was obtained from the BD Bioscience. Protein A/G-agarose beads, antibodies against PKCζ and LKB1 (used for immunoprecipitation assay, catalogue number Sc-5638, D-19) were from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). ONOO⁻, SIN-1, recombinant PKCζ, PKC βII, Go6983, H89, KT5823, calphostin C, wortmannin, LY294002, and arcaryriabulin A (2,3-bis(1H-indol-3-yl)maleimide) were obtained from Calbiochem. Cell-permeable myristoylated both PKCζ-PS and PKC-βI-PS were from BIOSOURCE International (Camarillo, CA). Other chemicals and organic solvents of highest grade were obtained from Sigma.

Methods

Cell Culture and Adenoviral Infection—To generate the adenoviral vector expressing a constitutively active mutant of AMPK α1 (AMPK-CA), a rat cDNA encoding residues 1–312 of AMPKα1 and bearing a mutation of Thr172 into aspartic acid (T172D) was subcloned into a shuttle vector (p-shuttle CMV). The c-Myc epitope tag was fused in frame to the 5’-terminus of the coding sequence. The resulting plasmid was linearized by digesting with Pmel and co-transfected into Escherichia coli BJ5183 with the adenoviral backbone plasmid, pAdEasy-1. Homogenous recombinants were selected with kanamycin. The linearized recombinant plasmid was infected into transformed human embryonic kidney 293 cells. Recombinant adenoviruses were amplified on 293 cells and purified by two ultracentrifugation steps on cesium chloride gradients. The number of viral particles was assessed by measurement of the optical density at 260 nm.

BAEC were infected with adenovirus expressing a constitutively active PKCζ mutant (PKCζ-CA), a dominant negative mutant PKCζ (PKCζ-DN) (39, 40), or adenovirus coding constitutively active AMPK (AMPK-CA) (33, 34). A replication-defective adenoviral vector expressing green fluorescence protein (Ad-GFP) was used as control. BAEC were infected with the adenoviruses with a multiplicity of infection of at least 10 in medium deprived of serum overnight. The cells were then washed and incubated in fresh endothelial base medium without serum for an additional 18–24 h prior to experimentation. Under these conditions, infection efficiency was typically >80% as determined by GFP expression.

Hypoxia-Reoxygenation of BAEC—H/R was performed as described previously (34). Briefly, BAEC were cultured in 6-well plates. The cells were first infected with indicated adenoviruses for 2 days if required. The cells were placed in a water bath (37 °C, total volume of 2 liters) filled with 1 liter of prewarmed Kreb-Ringer’s buffer, gassed with 95% O₂, 5% CO₂. After 30 min of incubation, the oxygen tension was reduced abruptly from 95% O₂, 5% CO₂ to 95% N₂, 5% CO₂ and was maintained for 15 min as indicated. After this phase of hypoxia, 95% O₂, 5% CO₂ was resumed (reoxygenation) for 15 min. After that the cells were washed and incubated with phosphate-buffered saline buffer twice and collected for Western blot and immunoprecipitation assays. The control BAEC was gassed only with 95% O₂, 5% CO₂ for equivalent periods.

Assay of AMPK Activity—AMPK activity was assayed by using the SAMS peptide, as previously described (19, 33, 34). The difference between the presence and absence of AMP (200 μM) was calculated as AMPK activity.

LKB1 Activity Assay—LKB1 was immunoprecipitated from untreated (control) or treated cells with an antibody against LKB1 (Santa Cruz, catalogue number Sc-5638, D-19) overnight at 4 °C in the presence of protein A/G-agarose. LKB1 activity present in the immunoprecipitates was determined by its ability to activate recombinant AMPK as described previously (20).

In Vitro Kinase Assays—To determine the effects of PKCζ or PKCβII on AMPK on LKB1 or both, recombinant LKB1 or recombinant AMPK α1β1r1 (41) were incubated with PKCζ at concentrations indicated for 15 min at 37 °C in the presence of [32P]ATP (1 μCi) with or without AMP (200 μM). The SAMS peptides (final concentration, 200 μM) were added if needed. AMPK activity was calculated by counting the phosphorylated SAMS peptides in the supernatants (25 μl) as described previously (33, 34). The beads were added 20 μl of 3X sample buffer and boiled for 5 min at 95 °C. The proteins were separated with 12% SDS-PAGE, and the dried gels were subjected to radioautography.

Immunoprecipitation and Western Blotting—The proteins were immunoprecipitated with specific antibodies and Western blotted into
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FIGURE 1. Inhibition of protein kinase C attenuates ONOO−-enhanced AMPK-Thr172 phosphorylation and AMPK activity. Confluent BAEC were preincubated with vehicles (Me2SO) or with protein kinase inhibitors for 15 min before being exposed to ONOO− (50 μM). After treatment, the cells were lysed and extracted. Both phosphorylated AMPK-Thr172 and eNOS-Ser1179 were detected in Western blots by using the specific antibodies, and AMPK activity was assayed by [32P]ATP incorporation into the SAMS peptide, as described under “Experimental Procedures.” a, effects of protein kinase inhibitors on ONOO−-enhanced AMPK-Thr172 phosphorylation. The blot is representative of five blots from five individual experiments. b, inhibition of PKC attenuated ONOO−-enhanced AMPK activity (n = 6; *, p < 0.05, control versus ONOO− treated; †, p < 0.05, ONOO− versus ONOO− plus inhibitors). c and d, effects of protein kinase inhibitors on ONOO−-enhanced eNOS-Ser1179 phosphorylation. Of note is that PKC inhibitors, but not PKA nor PKG inhibitors, attenuated ONOO−-enhanced AMPK-Thr172 phosphorylation and eNOS phosphorylation. The blot is representative of five blots from five individual experiments (n = 6; *, p < 0.05, control versus ONOO− treated; †, p < 0.05, ONOO− versus ONOO− plus inhibitors).

Site-directed Mutagenesis of Ser428 and Asp194 of Human LKB1 and Plasmid Transfection—Human cDNA clone was purchased from Invitrogen (clone 3689780). Wild type LKB1 gene coding region was amplified by PCR. The PCR product was ligated into TA cloning vector pGEM-T easy (Promega). LKB1 gene was released with enzymes of EcoRI/NotI from TA cloning vector and cloned into pCI-neo mamalian expression vector (Promega; catalogue number E184). Ser428 of LKB1 was mutated into either alanine or aspartic acid. Aspartic acid 194 of LKB1, which is essential for maintaining LKB1 activity, was mutated into alanine. All of the site-directed mutagenesis was done by using the QuikChange kits from Stratagene according to the manufacturer’s instructions. All of the mutation vectors were confirmed by DNA sequencing. Plasmid DNA was extracted in large scale by using Qiagen EndoFree plasmid maxi kit (catalogue number 12362) and were transfected to HeLa-S3 by using Lipofectamine 2000 kit from Invitrogen (catalogue number 11668-019), according to the instruction provided by the supplier. Twenty four hours after transfection, the cells were treated as indicated. In this experiment, both LacZ expression vector and untreated cells are used as control.

Quantification of Western Blots—The intensity (area × density) of the individual bands on Western blots was measured by densitometry (model GS-700, Imaging Densitometer; Bio-Rad). The background was subtracted from the calculated area.

Statistical Analysis—The results were analyzed by using two-way analysis of variance. The values are expressed as the means ± S.E. of the mean for n assays. A p value of < 0.05 is considered statistically significant.

RESULTS

Inhibition of Protein Kinase C Attenuates ONOO−-enhanced AMPK Activity and eNOS Phosphorylation—We had previously shown that ONOO−, either given exogenously or generated endogenously during H/R, activated AMPK in cultured endothelial cells (19, 33, 34). In addition, inhibition of PI 3-kinase with pharmacological inhibitors or overexpression of dominant negative PDK1 abolished AMPK activation caused by either ONOO− or metformin (31–34).

PDK1 serves as an important link between PI 3-kinase and several other kinases in the so-called AGC family, consisting of protein kinase A (PKA), protein kinase G (PKG), and atypical PKC (43). To identify which of these kinases leads to AMPK activation, various selective protein kinase inhibitors were preincubated with BAEC before exposure to ONOO−. After treatment, AMPK activation was determined by monitoring both Thr172 phosphorylation of AMPK using a specific anti-phospho-antibody and AMPK activity by using [32P]ATP phosphorylation of the SAMS peptide (19, 34). As expected, ONOO− (50 μM) significantly increased AMPK-Thr172 phosphorylation and AMPK activity (Fig. 1, a and b). Interestingly, inhibition of PKC with either GO6983 (1 μM) or arcyriarubin A (1 μM) significantly attenuated ONOO−-enhanced AMPK-Thr172 phosphorylation and AMPK activity (Fig. 1b). In contrast, inhibition of either PKA with H89 (10 μM) or PKG with KT5823 (10 μM) had no effect (Fig. 1, a and b). These results suggest that ONOO− might activate AMPK via selective PKC activation.

eNOS residue Ser1179 (homologous to 1177 in the human sequence) is a substrate for several kinases including both AMPK and protein kinase B/Akt. We had previously reported that ONOO− induced AMPK-dependent phosphorylation of eNOS-Ser1179 in BAEC (33, 34). Thus, we examined the effects of protein kinase inhibitors on eNOS-Ser1179 in BAEC. As expected, ONOO− (50 μM) significantly increased the phosphorylation of eNOS-Ser1179. Neither H89 nor KT5823 altered ONOO−-enhanced eNOS-Ser1179 phosphorylation, whereas GO6983 and arcyriarubin A, both of which were shown to inhibit AMPK (Fig. 1, a and b), significantly attenuated ONOO−-enhanced eNOS-Ser1179 phosphorylation. These results again suggest that ONOO− might up-regulate AMPK and its downstream enzyme, eNOS, by activating PKC.

Activation of Protein Kinase Cγ by ONOO−—Because the employed PKC inhibitors cannot distinguish between these isoforms, we next determined which PKC isoforms are activated by ONOO−. Specific antibodies revealed that BAEC expressed several major isoforms of PKC such as PKCa, β, and atypical PKCγ (data not shown). Atypical PKCγ...
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FIGURE 2. ONOO\(^-\) increases PKC\( \zeta \) phosphorylation and the translocation of PKC from cytosolic to membranes. Confluent BAEC were exposed to ONOO\(^-\) or ONOO\(^+\) donor, SIN-1 (1 μm, 2 h). The translocation of PKC\( \zeta \) and PKC\( \zeta \) phosphorylation were assayed as described under “Experimental Procedures.” a, ONOO\(^-\) increased the phosphorylation of PKC\( \zeta \) in BAEC. The blot is representative of three blots obtained from three independent experiments. b, ONOO\(^-\) inhibits the phosphorylation of PKC\( \zeta \). The blot is representative of three blots from three independent assays. c, both Go6983 and arcyriarubin A inhibit ONOO\(^-\)-enhanced PKC\( \zeta \) phosphorylation. The blot is representative of three blots of three individual experiments. d, ONOO\(^-\) and SIN-1 increase the translocation of PKC\( \zeta \). The lower panel is the statistical analysis of ONOO\(^-\)-induced PKC\( \zeta \) phosphorylation (n = 5, \( p < 0.05 \), control versus ONOO\(^-\)-treated; \( t \), \( p < 0.05 \); ONOO\(^-\) versus ONOO\(^+\) plus inhibitors).

belongs to the PI 3-kinase/PDK1 family, and its activation is linked to phosphorylation at Thr\(^{638/641}\) that can be monitored by antibodies (43–45). Exposure of BAEC to either ONOO\(^-\) or ONOO\(^+\) donor SIN-1 significantly increased Thr\(^{638/641}\) phosphorylation without altering PKC\( \zeta \) expression (Fig. 2a). In contrast, ONOO\(^-\) decreased phosphorylated PKCa/β (Thr\(^{638/641}\)), an activated form of PKCa/β (Fig. 2b), suggesting that ONOO\(^-\) selectively activated PKC\( \zeta \) while inhibiting PKCa/β. In addition, PKC inhibitors Go6983 and arcyriarubin A, but not PKA/PKG inhibitors KT5823 and H89, significantly inhibited ONOO\(^-\)-induced phosphorylation of PKC\( \zeta \) (Fig. 2c), AMPK (Fig. 1, a and b), and eNOS (Fig. 1, c and d). These results again suggested that PKC\( \zeta \) might be involved in ONOO\(^-\)-triggered activation of AMPK.

Translocation of cytosolic PKC\( \zeta \) into the membrane is considered an important step for PKC\( \zeta \) activation (42). We therefore investigated whether ONOO\(^-\) altered the distribution of PKC\( \zeta \) within a cell. As shown in Fig. 2d, exposure of BAEC to either chemically synthesized ONOO\(^-\) or SIN-1, a donor of ONOO\(^-\), significantly increased the translocation of PKC\( \zeta \) from the cytosol into membrane, confirming the activation of PKC\( \zeta \) by ONOO\(^-\).

Pharmacological Inhibition of Protein Kinase \( \zeta \) Attenuates ONOO\(^-\)-enhanced Phosphorylation of Both AMPK-Th\(^{72}\) and eNOS-Ser\(^{179}\)—In an independent approach to establish PKC\( \zeta \) as a mediator of ONOO\(^-\) effects on AMPK, we used PKC\( \zeta \)-PS, a synthetic peptide that selectively inhibits PKC\( \zeta \) without affecting other PKC isoforms (46, 47). As shown in Fig. 3 (a and b), PKC\( \zeta \)-PS concentration-dependently attenuated ONOO\(^-\)-enhanced AMPK-Th\(^{72}\) phosphorylation as well as AMPK activity. In contrast, inhibition of PKC\( \zeta \) with the corresponding pseudosubstrate peptides did not alter AMPK-Th\(^{72}\) phosphorylation or AMPK activity (Fig. 3, a and b). In parallel, PKC\( \zeta \)-PS but not PKC\( \zeta \)-PS dose-dependently inhibited ONOO\(^-\)-enhanced eNOS-Ser\(^{179}\) phosphorylation, a downstream target of AMPK in BAEC (Fig. 3c). Taken together, these results imply that selective inhibition of PKC\( \zeta \) attenuated the effect of ONOO\(^-\) on AMPK in BAEC.

Genetic Inhibition or Activation of Protein Kinase \( \zeta \) Alters the Phosphorylation of Both AMPK-Th\(^{72}\) and eNOS-Ser\(^{179}\)—Further direct evidence for PKC\( \zeta \)-dependent AMPK activation was obtained from genetic inhibition and overexpression of constitutively active PKC\( \zeta \) mutants. First, we tested whether expression of a dominant negative PKC\( \zeta \) mutant (PKC\( \zeta \)-DN) alters ONOO\(^-\)-induced AMPK activation. As expected, overexpression of PKC\( \zeta \)-DN but not GFP prevented ONOO\(^-\)-induced translocation of PKC\( \zeta \) from cytosolic fractions into the membrane (Fig. 4a) and its Thr\(^{638/641}\) phosphorylation (data not shown). In addition, overexpression of PKC\( \zeta \)-DN but not GFP blunted ONOO\(^-\)-enhanced phosphorylation of both AMPK-Th\(^{72}\) and eNOS-Ser\(^{179}\) (Fig. 4, b and c). Taken together, these results provide strong evidence that PKC\( \zeta \) is required for ONOO\(^-\)-induced AMPK activation.

Activation of AMPK by Protein Kinase \( \zeta \) Is LKB1-dependent—Recent studies (25–27) suggested that LKB1 (also known as STK11) acts as AMPK kinase in vitro and in cultured cells. Recombinant LKB1 purified from mammalian cells phosphorylates and activates AMPK \( \alpha 1 \beta 1 \gamma 1 \) (25–27). LKB1 phosphorylates catalytically inactive mutants of AMPK on Thr\(^{172}\) within the \( \alpha \) subunit, and phosphorylation of AMPK requires LKB1. To study the dependence on LKB1 of AMPK activation by ONOO\(^-\), phosphorylation of AMPK was examined in HeLa S3 cells that lack LKB1. HeLa S3 cells, which are deficient of LKB1 but express normal amounts of both PKC\( \zeta \) and AMPK (Fig. 5a) (25–27), were exposed to ONOO\(^-\) (100 μM). ONOO\(^-\), which significantly increased the phosphorylation of both PKC\( \zeta \) and AMPK-Th\(^{172}\) in BAEC, did not alter the phosphorylation of AMPK-Th\(^{172}\), whereas phospho-PKC\( \zeta \) was increased in HeLa S3 (Fig. 5a). In addition, ONOO\(^-\) failed to alter AMPK activity, as assayed by AMPK activity assessed by formation of \([\beta ^3 P]SAMS \) peptide (Fig. 5b). Taken together, these results suggest that LKB1 is required for PKC\( \zeta \)-enhanced AMPK activation in BAEC.

We next determined whether ONOO\(^-\)-activated AMPK by increasing LKB1 activity. LKB1 was first immunoprecipitated from BAEC and then exposed to ONOO\(^-\) (50 μM). LKB1 activity was assayed by incubating with recombinant AMPKα1β1r1 (20 μg) as its substrate. As shown in Fig. 5c, exposure of LKB1 directly to ONOO\(^-\) (up to 50 μM) did not alter its activity (Fig. 5c). In addition, exposure of recombinant AMPK α1β1r1 to ONOO\(^-\) (up to 50 μM) instead significantly inhibited AMPK activity. These data exclude the possibility that ONOO\(^-\) directly up-regulates AMPK activity in BAEC.

 Activation of Protein Kinase \( \zeta \) Promotes the Association of LKB1 and AMPK—We have shown previously that metformin activates AMPK by increased production of ONOO\(^-\), which increased the association of

\(^3\) Z. Xie and M. H. Zou, unpublished data.
LKB1 with AMPK (19). Thus, we investigated whether ONOO\(^-\) may activate AMPK by increasing the interaction of AMPK and LKB1. Immunoprecipitated LKB1 was analyzed for AMPK and vice versa. As shown in Fig. 6a, immunoprecipitates from BAEC treated with ONOO\(^-\) showed significantly increased co-immunoprecipitation of LKB1 and AMPK-Thr\(^{172}\). We next determined whether PKC\(\varepsilon\) contributed to the increased association of LKB1 with AMPK. Although ONOO\(^-\) induced association of AMPK with LKB1 as above, this response was blunted by PKC\(\varepsilon\)-PS (Fig. 6b), suggesting that PKC\(\varepsilon\) activity enhances association of LKB1 with AMPK.

We next measured the amount of AMPK that was associated with LKB1. Because the SAMS peptide is a substrate for AMPK but not LKB1, the phosphorylation of \(^{32}\)P-SAMS peptides can be used as an index of the amount of AMPK that was co-immunoprecipitated with LKB1. As shown in Fig. 6c, ONOO\(^-\) significantly increased the amount of \(^{32}\)P-ATP incorporation into the SAMS peptides, suggesting increased AMPK activity. In contrast, PKC\(\varepsilon\)-PS reduced AMPK activity enhanced by ONOO\(^-\) (Fig. 6c). These results further indicate that ONOO\(^-\) activated PKC\(\varepsilon\), resulting in increased interactions of LKB1 with AMPK.

PKC\(\varepsilon\) is a member of a family consisting of 12 protein kinases called the AMPK family (30). Previous studies showed that stimuli such as AICAR, phenformin, or physical exercise activated AMPK without affecting the activities of LKB1 or other kinases from the AMPK family (31, 32). Because PKC\(\varepsilon\) activation affected LKB1 in BAEC, we analyzed whether, in addition to AMPK, other kinases from this family were activated. We analyzed MARK-3 as one family member, because commercial antibodies against this kinase are available. Co-immunoprecipitation of LKB1 with MARK-3 was slightly increased in ONOO\(^-\) treated BAEC as compared with controls (Fig. 6d). However, the association of LKB1 and MARK-3 was unchanged when PKC\(\varepsilon\) was inhibited with...
PKCζ-PS (Fig. 6d). Thus, unlike AMPK, PKCζ may not be involved in ONOO⁻-enhanced association of LKB1 with MARK-3.

**Protein Kinase Cζ Promotes LKB1 Phosphorylation at Ser⁴²⁸**—PKCζ increased the association of AMPK and LKB1 (Fig. 6). In addition, ONOO⁻ activated PKCζ in LKB1-deficient HeLa-S3 cells but without activating AMPK (Fig. 5a). These data suggested that PKCζ is an upstream AMPK kinase and prompt speculation that PKCζ might phosphorylate LKB1, resulting in increased association of LKB1 with AMPK. We next determined whether ONOO⁻ enhanced LKB1 phosphorylation in BAEC. Immunoprecipitated LKB1 was analyzed with an antibody recognizing all phosphorylated serine or threonine residues. As shown in Fig. 7a, ONOO⁻ significantly increased the Ser/Thr phosphorylation of LKB1. In addition, inhibition of PKCζ with PKCζ-PS significantly attenuated the effects of ONOO⁻ on LKB1 phosphorylation, suggesting a PKCζ-dependent process. To further identify the site(s) of LKB1 phosphorylation, we employed several commercially available antibodies against LKB1-Ser⁴²⁸, -Ser³³⁴, and -Thr¹⁸⁹. As depicted in Fig. 7b, ONOO⁻ increased phosphorylation of Ser⁴²⁸ in LKB1, which was significantly attenuated by PKCζ-PS. In contrast, ONOO⁻ did not alter the signal of phospho-Thr¹⁸⁹ and might have decreased phospho-Ser³³⁴ (Fig. 7c). Most importantly, incubation of purified recombinant LKB1 with recombinant PKCζ dose-dependently increased the LKB1 Ser⁴²⁸

**FIGURE 5.** LKB1 is required for ONOO⁻-dependent AMPK activation. a, ONOO⁻ activates AMPK in BAEC, but not LKB1-deficient HeLa S3 cells. The blot is representative of at least three independent assays. b, ONOO⁻ did not activate AMPK in LKB1-deficient HeLa-S3. Confluent HeLa-S3 cells were exposed to ONOO⁻, and AMPK activity was assayed as described under “Experimental Procedures” (n = 3). c, ONOO⁻ does not alter LKB1 activity. LKB1 was first immunoprecipitated from BAEC and then exposed to ONOO⁻. LKB1 activity was assayed by including recombinant AMPK as described under “Experimental Procedures” (n = 5).

**FIGURE 6.** ONOO⁻ via PKCζ enhances the co-immunoprecipitation of LKB1 and AMPK. a, ONOO⁻ increases the association of LKB1 and AMPK. LKB1 or AMPK were immunoprecipitated (IP) from BAEC and AMPK or LKB1 detected in Western blots (WB). The blot is representative of five blots from five independent experiments. b, inhibition of PKCζ attenuates ONOO⁻-enhanced association of LKB1 with AMPK. LKB1 was immunoprecipitated and stained for AMPK in Western blots. Of note is that ONOO⁻ increased LKB1 association with AMPK in BAEC, which was sensitive to PKCζ-PS. The blot is representative of five blots from five independent experiments. c, ONOO⁻ increases LKB1-associated AMPK activity. LKB1 were immunoprecipitated as described above. AMPK activity was assayed by [³²P]ATP phosphorylation of the SAMS peptide, as described under “Experimental Procedures” (n = 5; p < 0.05 control versus ONOO⁻; +, p < 0.05 ONOO⁻ versus ONOO⁻ plus PKCζ-PS. d, ONOO⁻ increased the co-immunoprecipitation of LKB1 with MARK-3. The lower panel is the results of MARK-3 staining in LKB1 immunoprecipitates (n = 3; *p < 0.05). Of note is that ONOO⁻ slightly increased the co-immunoprecipitation of LKB1 with MARK-3. However, PKCζ does not alter ONOO⁻-enhanced co-immunoprecipitation of LKB1 with MARK-3. The blot is representative of three blots from three individual experiments.
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Inhibition of Protein Kinase Cζ Blunts ONOO\(^{-}\)-induced Phosphorylation of AMPK-Thr\(^{172}\) and LKB1-Ser\(^{428}\) in Nonendothelial Cells Derived from Human, Rat, and Mouse—We next investigated whether ONOO\(^{-}\)-activated AMPK via LKB1-dependent mechanism in cells types other than BAEC. To this end, human retinal pericytes, cultured rat vascular smooth muscle cells, and mouse 3T3-L1 pre-adipocytes were exposed to ONOO\(^{-}\) in the presence or absence of PKCζ-PS (10 μM). As shown in Fig. 8a, ONOO\(^{-}\) significantly increased the phosphorylation of both AMPK-Thr\(^{172}\) and LKB1-Ser\(^{428}\) in all of the cell types tested. Mostly important, inhibition of PKCζ with PKCζ-PS attenuated the effects of ONOO\(^{-}\) on both AMPK and LKB1 in these cells (Fig. 8a), suggesting that ONOO\(^{-}\)-activated PKCζ might represent a common pathway for LKB1-AMPK activation in tissues other than endothelium.

Protein Kinase Cζ Is Implicated in AMPK Activation Caused by Hypoxia-Reoxygenation and Metformin—AMPK is activated by various stimuli including hypoxia and the antidiabetic drug metformin (12–14). We next investigated whether PKCζ is implicated in the AMPK activation caused by stimuli other than ONOO\(^{-}\). As shown in Fig. 8b, metformin (1 mM) significantly increased AMPK activity in BAEC. Interestingly, inhibition of PKCζ with PKCζ-PS concentration-dependently suppressed metformin-enhanced AMPK activation. Further, overexpression of PKCζ-DN but not GFP significantly blunted hypoxia-enhanced AMPK activity, whereas overexpression of PKCζ-CA enhanced hypoxia-induced AMPK activation (Fig. 8c). These data strongly suggest that PKCζ is involved in hypoxia-induced AMPK activation. Taken together, inhibition of PKCζ attenuates AMPK activation caused by known AMPK stimuli such as metformin and H/R. These data imply that PKCζ might play an important role in both AMP-dependent and AMP-independent AMPK activation.

Treatment of Phorbol 12-Myristate 13-aceyrate Activates AMPK via Protein Kinase Cζ—We next determined whether activation of PKCζ with pharmacological reagents such as phorbol 12-myristate 13-aceate (PMA) alters AMPK activation. Similar to ONOO\(^{-}\), PMA (5–10 mM) for 5 min significantly increased the phosphorylation of LKB1-Ser\(^{428}\) (Fig. 8d). In parallel, PMA significantly increased the phosphorylation of both AMPK-Thr\(^{172}\) and ACC-Ser\(^{79}\), which were abolished by PKCζ-PS (Fig. 8e). Taken together, these data strongly support the possibility that PKCζ-LKB1-AMPK is not unique to ONOO\(^{-}\) and might be a common pathway shared by other stimuli.

Mutation of Ser\(^{428}\) of LKB1 with Alanine Abolishes ONOO\(^{-}\)-induced AMPK Activation in HeLa-S3 Cells—We next investigated whether mutation of Ser\(^{428}\) of LKB1 altered ONOO\(^{-}\)-induced AMPK activation. Using direct mutagenesis techniques, Ser\(^{428}\) of LKB1 was mutated into alanine (loss of function). In addition, aspartic acid 194 of LKB1, which is essential for LKB1 activity, was also mutated with alanine. These plasmids were transfected into LKB1-deficient HeLa-S3 cells. As shown in Fig. 9, ONOO\(^{-}\) (100 μM) significantly increased the phosphorylation of both AMPK-Thr\(^{172}\) and ACC-Ser\(^{79}\), a downstream enzyme of AMPK. In HeLa-S3 cells transfected with LKB1 wild type but not in nontransfected HeLa-S3, implying the essential role of LKB1 in the activation of AMPK by ONOO\(^{-}\). In addition, cells transfected with kinase-inactive mutants (LKB1 aspartic acid 194 was replaced with alanine, LKB1-D194A) also blocked the effects of ONOO\(^{-}\) on AMPK, supporting the essential role of LKB1. Interestingly, mutation of LKB1 Ser\(^{428}\) with alanine (LKB1-S428A), like the kinase-inactive mutant D194A, abolished ONOO\(^{-}\)-enhanced phosphorylation of both AMPK-Thr\(^{172}\) and ACC-Ser\(^{79}\), implying the essential role of LKB1-Ser\(^{428}\) in the activation of AMPK by LKB1. Similar results were also obtained from A549 and HeLa cells (data not shown).

DISCUSSION

AMPK is an obligatory heterotrimeric enzyme consisting of three subunits: α, β, and γ. The α subunit contains the catalytic site, and phosphorylation of Thr\(^{172}\) in its activation loop by one or more upstream kinase (AMPKK) is absolutely required for activation (1–3). Recent works from several laboratories have demonstrated that LKB1 is one of the major upstream kinases for AMPK in both cell-free systems and mammalian cells (25–27). LKB1 is a tumor suppressor kinase and can phosphorylate the T-loop of all the members of the human AMPK family, which consists of 12 protein kinases (30). Paradoxically, neither the activity of LKB1 itself nor that of AMPK-related kinases is influenced directly by stimuli known to activate AMPK (H/R, metformin, etc.) (31–34). Thus, how these stimuli lead to LKB1-dependent AMPK activation has remained unclear so far. With the evidence presented here, we propose that PKCζ might act as a key factor in controlling phosphorylation (Fig. 7d), indicating a direct interaction of LKB1 with PKCζ. Collectively, these results suggest that PKCζ activated LKB1, likely via promoting LKB1-Ser\(^{428}\) phosphorylation.
LKB1-dependent AMPK activation. The key evidence may be summarized as follows. First, ONOO⁻/H₂O₂ increased PKCζ/δ activation in cultured human retinal pericytes, rat vascular smooth muscle cells, and mouse 3T3-L1 adipocytes. Of note is that ONOO⁻ increased the phosphorylation of both AMPK-Thr¹⁷² and LKB1-Ser⁴²⁸, whereas PKCζ-PS blocked the effects of ONOO⁻. Second, ONOO⁻/H₂O₂ activated AMPK in a variety of cells including human retinal pericytes, BAEC, vascular smooth muscle cell, 3T3-L1 preadipocytes. Importantly, inhibition of PKCζ attenuated ONOO⁻-enhanced AMPK activation in these cell types tested. Third, either pharmacological or genetic inhibition of PKCζ inhibits AMPK activation caused by known AMPK stimuli including metformin, ONOO⁻, and H/R. Fourth, activation of AMPK by ONOO⁻ was dependent on LKB1. Fifth, ONOO⁻ increased the co-immunoprecipitation of LKB1 with AMPK, which was in parallel with increased phosphorylation of LKB1-Ser⁴²⁸ and which was sensitive to PKCζ inhibition. Sixth, ONOO⁻ increased the co-immunoprecipitation of LKB1 with AMPK, which was in parallel with increased phosphorylation of LKB1-Ser⁴²⁸ and which was sensitive to PKCζ inhibition. Seventh, activation of PKCζ by ONOO⁻ was dependent on LKB1. Lastly, ONOO⁻ increased the co-immunoprecipitation of LKB1 with AMPK, which was in parallel with increased phosphorylation of LKB1-Ser⁴²⁸ and which was sensitive to PKCζ inhibition.
functions attributed to LKB1. The C-terminal region of LKB1 consists of 124 residues and contains several post-translational modifications. Five phosphorylation sites have been identified; two residues are auto-phosphorylation sites (Thr336 and Thr402), and three others (Ser325, Thr363, and Ser428) are phosphorylated by upstream kinases (28). In addition, LKB1 has been shown to undergo farnesylation at a cysteine residue located in the C-terminal region (Cys430 in human LKB1). Possibly, the LKB1 C-terminal region serves as a regulatory domain mediating dynamic interactions with several classes of proteins and promotes subcellular targeting (44). In the present study, we have provided evidence that PKCζ regulates AMPK activation by phosphorylating LKB1, which results in increased association of LKB1 with AMPK. The key findings can be summarized as follows. First, AMPK is not activated by ONOO− in cells lacking LKB1 (HeLa-S3 cells) but expressing normal levels of calcium calmodulin-dependent kinase kinase (CaMKK) (48–50). Second, ONOO− increased the co-immunoprecipitation of LKB1 with AMPK, which was sensitive to inhibition of PKCζ. Third, inhibition of PKCζ attenuated ONOO−-enhanced LKB1-Ser428 phosphorylation as well as the association of LKB1 with AMPK. Fourth, recombiant PKCζ in a concentration-dependent manner caused the phosphorylation of LKB1-Ser428 in vitro. Fifth, stimulation of BAEC with PMA activated AMPK in parallel with increased LKB1 phosphorylation at Ser428. Finally, mutation of Ser428 into alanine abolished ONOO−-enhanced AMPK activation. These results suggest that Ser428 located in the C terminus of LKB1 might play a crucial role in regulating AMPK activation. Indeed, a recent study carried out by Forcet et al. (51) suggests that naturally occurring C-terminal mutations, which neither disrupt LKB1 kinase activity nor interfere with LKB1-induced growth arrest, reduce LKB1-mediated activation of AMPK and impair downstream signaling. Inhibition of PKCζ abolished ONOO−-enhanced association of LKB1 with AMPK but not with MARK-3, another member of the AMPK family, suggesting that PKCζ-LKB1 signaling is exclusively active toward the AMPK pathway. These results are in line with the previous findings (31–33) that AMPK stimuli such as AICAR or exercise activate AMPK without altering the activities of LKB1 and other members of the AMPK family. This selective effect of PKCζ on AMPK may involve the PKCζ-dependent phosphorylation of LKB1-Ser428 within the C terminus of LKB1. Because phosphorylation of none of the other known regulatory sites on LKB1 was affected by PKCζ and a low level of LKB1/MARK3 interaction was independent of PKCζ inhibition, LKB1-Ser428 phosphorylation by PKCζ may be the specific step leading to LKB1-dependent AMPK activation. However, we cannot exclude the possibility that PKCζ might regulate the interaction of LKB1-AMPK by post-translational modifications on other sites. In addition, several other kinases such as PKA and p90Rsk are reported to phosphorylate LKB1-Ser428 (52, 53). If this site turns out to be indeed essential for LKB1-AMPK activation, one may speculate that these kinases could similarly regulate LKB1-dependent AMPK activation. Further studies are warranted to improve our understanding of these signaling events.

We have used endothelial cells to establish a novel PKCζ-LKB1-AMPK signaling axis. In addition, we have also replicated these findings in a variety of tissues including human retinal pericytes, rat smooth muscle cells, and mouse 3T3-L1 pre-adipocytes. Further, PKCζ inhibition attenuates AMPK activation by a variety of known AMPK stimuli including oxidant (ONOO−), pharmacological reagent (metformin), and H/R, implying that the PKCζ-LKB1-AMPK pathway is not unique to endothelium and might be a common pathway shared by other physiological stimuli in a variety of tissues. Thus, we can expect broader implications of this signaling pathway in cell biology. A number of reports have shown that PKCζ plays critical roles in signaling pathways that control cell growth, differentiation, and survival (28, 43). Thus, the interactions of PKCζ with LKB1 and AMPK that we have described in this study might affect our understanding of not only vascular biology but also cancer development. LKB1 is an upstream activator of AMPK, and regulation of the AMPK pathway is believed to be directly involved in LKB1 tumor suppressor function (54, 55). Consistent with this model, LKB1 has recently been identified as a negative regulator of mammalian targets of rapamycin (mTOR) signaling through the sequential stimulation of AMPK and of the TSC1/TSC2 tumor suppressor complex (56, 57). Furthermore, ACC, a substrate of AMPK, controls fatty acid synthetic metabolism, which is frequently dysregulated in tumors (54). We found that phosphorylation of LKB1-Ser428 in its C-terminal part increased association of LKB1 with AMPK as well as activation of its downstream pathways, thus reinforcing the idea that AMPK plays a role in the control of cell transformation. Indeed, C-terminal mutations in LKB1, which decrease LKB1-mediated AMPK activation, compromise the ability of LKB1 to establish and maintain polarity of both intestinal epithelial cells and migrating astrocytes. Our findings further support the notion that LKB1 tumor suppressor activity depends, at least in part, on the regulation of AMPK signaling and downstream effects on cell polarization.

To summarize, the major finding of the present study is that PKCζ promotes AMPK activation by increasing phosphorylation of and association with the AMPK kinase LKB1. This is an important finding and might explain earlier paradoxical results where several AMPK-activating stimuli like hypoxia, muscle contraction, phenformin, or AICAR failed to activate LKB1 or a group of AMPK-related kinases that are downstream of LKB1. Our results might help explain why contraction, phenformin, and AICAR are not directly stimulating LKB1 activity, although they activate AMPK. We have also provided evidence that PKCζ-dependent regulation via LKB1/AMPK association depends on the phosphorylation of LKB1-Ser428 by PKCζ. We conclude that PKCζ-dependent LKB1-mediated AMPK activation might play important roles in regulating not only cell energy metabolism but also signaling pathways that control cell growth, differentiation, and survival.

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