Protein Kinase Cζ-dependent LKB1 Serine 428 Phosphorylation Increases LKB1 Nucleus Export and Apoptosis in Endothelial Cells*

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LKB1 is a serine-threonine protein kinase that, when inhibited, may result in unregulated cell growth and tumor formation. However, how LKB1 is regulated remains poorly understood. The aim of the present study was to define the upstream signaling events responsible for peroxynitrite (ONOO⁻)-induced LKB1 nuclear export. We conclude that PKCζ signaling events responsible for peroxynitrite (ONOO⁻) phosphorylation in LKB1-deficient HeLa S3 cells or in HeLa S3 cells dependent Akt inhibition in response to ONOO⁻ are confirmed by the results of complementary experiments presented in accordance with 18 U.S.C. Section 1734 solely to indicate this fact. This article has been withdrawn by the authors. The Journal raised questions that the Akt immunoblot in Fig. 2B was reused in Fig. 6E, lanes 2 and 5 of the Akt immunoblot in Fig. 2C were duplicated, lanes 4 and 5 of the Akt immunoblot in Fig. 2C were reused in lanes 5 and 6 of the Akt immunoblot in Fig. 5C, lanes 2 and 5 of the Akt immunoblot were duplicated in Fig. 5C, the first lane of the PKCζ immunoblot in Fig. 4B was reused in Fig. 7A as Akt, and lanes 1 and 6 of the Akt immunoblot in Fig. 4C were duplicated. The authors were able to locate some of the original data and repeated experiments performed at the time of the original work, which the authors state support the conclusions of the paper. The authors state that the results of this paper are confirmed by the results of complementary experiments presented in the article, and some of the principal observations of this paper were further confirmed in publications from other laboratories (Elshaer, S. L. et al. (2018) Antioxidants 7, 47; Heo, K. S. et al. (2011) J. Cell Biol. 193, 867-884; Tommasini, I. et al. (2008) J. Immunol. 181, 5637-5645). The authors stand by the conclusions of the paper.

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2 The abbreviations used are: PKC, protein kinase C; GSK, glycogen synthase kinase; HUVEC, human umbilical vein endothelial cell; ONOO⁻, peroxynitrite; PKCζ-PS, PKCζ pseudosubstrate peptides; Sin-1, 1-amino-3-(4-morpholino)-1,2-oxiazadiazolium chloride; siRNA, small interference RNA; WT, wild type.
that PKCζ-dependent LKB1 phosphorylation at Ser428 is essential for ONOO \(^{-}\)-induced Akt inhibition and apoptosis.

**EXPERIMENTAL PROCEDURES**

**Materials**—Human umbilical vein endothelial cells (HUVECs) and cell culture medium were purchased from Cascade Biologicals (Portland, OR). HUVECs were maintained in Medium 200 supplemented with low serum growth supplement. HeLa S3 and A549 cells obtained from ATCC (Manassas, VA) were grown in F-12K media (ATCC) containing 10% heat-inactivated fetal bovine serum. Human breast cancer MDA-MB-468 cells were a generous gift from Dr. Bolin Liu (University of Colorado Health Science Center, Denver, CO) and were grown in Dulbecco’s modified Eagle’s medium with 10% heat-inactivated fetal bovine serum. Human breast cancer MDA-MB-468 cells were grown in F-12K media (ATCC) containing 10% heat-inactivated fetal bovine serum. Human breast cancer MDA-MB-468 cells were grown in F-12K media (ATCC) containing 10% heat-inactivated fetal bovine serum.

**Western Blot Analysis**—HUVECs were washed once with phosphate-buffered saline and lysed with ice-cold buffer from Cell Signalining Technology (Beverly, MA) containing 20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM NaEDTA, 1 mM EGTA, 1% Triton, 2.5 mM sodium pyrophosphate, 1 mM \(\beta\)-glycerophosphate, 1 mM phenylmethylsulfonyl fluoride, 10 \(\mu\)g/ml leupeptine, 10 \(\mu\)g/ml aprotinin, 1 mM NaNo, and 10 mM NaF. Lysates were separated by SDS-PAGE at 4 °C for 18 min. Protein concentrations were determined using the BCA protein assay (Pierce). Proteins were separated by SDS-PAGE and then transferred to nitrocellulose membranes. These membranes were blocked in 5% non-fat dry milk in Tris-buffered saline-Tween 20 for 1 h and incubated with the indicated primary antibody. The membranes were washed and incubated with a horseradish-peroxidase-linked secondary antibody. The reactive bands were detected by ECL Western blotting detection reagents (Amersham Biosciences).

**Akt Activity Assay**—Akt kinase activity was measured using an in vitro Akt kinase assay kit. Briefly, HUVECs were collected in lysis buffer according to the manufacturer’s instructions. Lysate (200 \(\mu\)g protein) was incubated overnight with the anti-Akt antibody provided in the kit. Captured Akt was incubated with 1 \(\mu\)g of recombinant glycogen synthase kinase-3\(\beta\) in the reaction buffer for 30 min at 37 °C. The reaction was terminated by the addition of SDS sample buffer, and reaction mixtures were electrophoresed on SDS-polyacrylamide gels. Phosphorylation of GSK-3\(\beta\) was used as an index of Akt activity and measured by Western blot using a phospho-GSK-3\(\beta\) (Ser21/9) antibody.

**PTEN Activity Assay**—Anti-PTEN antibody (10 \(\mu\)l) was incubated with 450 \(\mu\)g of cell lysates for 2 h, and the mixture was then incubated with protein A-Sepharose CL-4B beads overnight at 4 °C. Immunoprecipitates were washed with lysis buffer, and PTEN phosphatase activity was measured with a Malachite Green-based assay (Upstate) using phosphatidylinositol 3,4,5-trisphosphate as a substrate. An affinity-purified, constitutively active form of PTEN supplied with the kit was used as a positive control.

**LKB1 Immunocytochemical Staining**—HUVECs or A549 cells transfected with LKB1 wild type and mutated plasmids were cultured on cover glasses and then fixed with 4% paraformaldehyde. After blocking, HUVECs were incubated with a goat anti-LKB1 antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) overnight. Several commercially available
anti-LKB1 antibodies failed to recognize recombinant LKB1 in A549 cells. Because LKB1 plasmids encoded a His tag in the N terminus of the protein, mouse anti-His tag antibody (Upstate Cell Signaling Solutions, Temecula, CA) was used as an alternative. After three washes, the slides were incubated with a fluorescein isothiocyanate-conjugated donkey anti-goat or a fluorescein isothiocyanate-conjugated donkey anti-mouse IgG (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA), at a dilution of 1:150 for 1 h. The slides were then rinsed, counter-stained with 4’,6-diamidino-2-phenylindole, mounted in Vectashield medium (Vector Laboratories, Burlingame, CA), and viewed on a SLM 510 laser scanning confocal microscope (Carl Zeiss Meditec, Inc., Jena, Germany).

Preparation of Subcellular Fractions—Cellular cytosolic and nuclear fractions were prepared as described previously (20). Briefly, HUVECs were harvested in homogenization buffer (10 mM MOPS, pH 7.0, 10 mM KCl) containing protease inhibitors (Complete; Roche Applied Science) and processed in a Dounce homogenizer. The nuclei were pelleted by centrifugation at 800 \( \times g \) at 4 °C for 10 min, the supernatant was then centrifuged (40,000 \( \times g \), 4 °C) for 40 min, and the resulting supernatant (cytosolic fraction) was collected. Pelleted nuclei were resuspended in lysis buffer (50 mM Tris-HCl, pH 6.8, 6.5 mM urea, 2% SDS, 2 mM dithiothreitol, 1% Triton X-100, protease inhibitor mixture), and centrifuged at 14,000 \( \times g \) at 4 °C for 30 min, the resulting supernatants (nuclear fraction) were collected.

Cell Viability Assay—Cell viability was assessed using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay (ATCC). After being pretreated with uric acid for 30 min, HUVECs were incubated in 1 mM Sin-1 for 18 h. After treatment, the cells were lysed and nuclei-free supernatant was incubated with anti-histone antibodies. Fragmented nucleosomal DNA was detected with anti-DNA peroxidase-antibody conjugated antibody and the peroxidase substrate 2,2’-azino-bis(3-ethylbenzthiazoline-sulfonate). The absorbance was measured at 405 and 490 nm. DNA fragmentation was expressed as the number of terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling staining (TMR red) using a kit from Roche Applied Science and following the provided instruction manual. The percentage of apoptosis was calculated from the number of terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling-positive cells divided by the total number of cells counted.

In Vitro Kinase Assays—For determination of the effects of PKC\(\zeta\) on Akt activity, GSK-3β fusion protein was incubated with recombinant Akt1 with or without recombinant PKC\(\zeta\) for 30 min at 37 °C in kinase buffer (25 mM Tris, pH 7.5, 5 mM \( \beta \)-glycerophosphate, 2 mM dithiothreitol, 0.1 mM sodium orthovanadate, 10 mM MgCl\(\text{2}\)) with 0.2 mM ATP. To determine the effect of LKB1 Ser\(428\) phosphorylation on PTEN phosphorylation, PTEN was incubated with mutant (LKB1-S428A) or wild type LKB1 under the same reaction conditions. The reactions were terminated by adding SDS sample buffer and were heated for 5 min at 95 °C. The samples were then subjected to SDS-polyacrylamide gel electrophoresis and Western blotting using the indicated phospho-specific antibodies.

RESULTS

Protein Kinase C\(\zeta\) Mediates Akt Inhibition by ONOO\(^-\)—Protein kinase B/Akt plays a significant role in cell survival and insulin actions such as glycolysis, gluconeogenesis, protein synthesis, and adipogenesis. We first investigated the effects of ONOO\(^-\) on both Akt-Ser\(473\) phosphorylation and Akt activity in cultured HUVECs. As shown in Fig. 1 (A and B), exposure of HUVECs to a pathologically relevant concentration of ONOO\(^-\) (5 \( \mu \)M) (21, 22) for 15 min attenuated the Akt-Ser\(473\) phosphorylation (70%, \( p < 0.01 \)) and Akt activity (75% reduction, \( p < 0.01 \)). This concentration of ONOO\(^-\) did not alter Ser\(307\) phosphorylation of insulin receptor substrate 1 (data not shown).

ONOO\(^-\) is known to yield numerous reactive free radicals and oxidants including \( \text{NO}_{2}^- \), \( \text{NO}_3^- \), \( \text{CO}_2^- \), and HO\(^*\) (23, 24). However, exposure of HUVECs to decomposed ONOO\(^-\) did not alter Akt phosphorylation, excluding the possibility that the effects of ONOO\(^-\) were from \( \text{NO}_2^- \) or \( \text{NO}_3^- \), two major end products of ONOO\(^-\). Further, exposure of HUVECs to \( \text{H}_2\text{O}_2 \) (100 \( \mu \)M) or spermine \( \text{N}-[4-1-(3-aminopropyl)-2-hydroxy-2-nitrosodihydroazino]-butyl]-1,3-propanediamine (100 \( \mu \)M) increased Akt phosphorylation at Ser\(473\) (data not shown). Taken together, these results implied that ONOO\(^-\) plays a key role in the inhibition of Akt, although we could
PKCζ-dependent LKB1 Nuclear Export

ONOO⁻ is reported to activate PKCζ by increasing caspase-dependent cleavage (26). We next determined whether ONOO⁻ increased caspase-dependent PKCζ cleavage. As shown in Fig. 1E, ONOO⁻ increased the cleavage of both caspase 3 and PKCζ in HUVECs.

LKB1 Is Required for ONOO⁻-induced Akt Inhibition—We next determined whether PKCζ directly inhibited Akt. To that end, recombinant Akt1 were incubated with recombinant PKCζ. Interestingly, PKCζ increased Akt activity, as measured by GSK-3β phosphorylation. Consistently, PKCζ also increased Akt phosphorylation (Fig. 2, lane 4 versus lane 2). Consistent with our earlier report (25), PKCζ increased phosphorylated GSK-3β (Fig. 2, lane 3 versus lane 1).

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Not rule out the possible involvement of unstable products from inactivation of LKB1.

We next determined whether LKB1 and other kinases were involved in ONOO⁻-induced Akt inhibition. Exposure of HUVECs to KT5823, a potent protein kinase G inhibitor, or H-89 (1 μM), a selective protein kinase A inhibitor, did not alter the basal levels of Akt Ser473 phosphorylation or Akt activity in HUVECs (data not shown). Neither KT5823 nor H-89 altered ONOO⁻-induced Akt inhibition (Fig. 1, A and B). In contrast, bisindolylmaleimide I (5 μM), a nonselective PKC inhibitor (18), which had no effect on Akt phosphorylation or Akt activity in unstimulated cells, significantly attenuated ONOO⁻-induced suppression of both Akt Ser473 phosphorylation and Akt activity (Fig. 1, A and B). These results suggest that Akt inhibition caused by ONOO⁻ might be PKC-dependent.

ONOO⁻ caused a 2-fold increase of PKCζ phosphorylation at Thr410 (p < 0.01). Earlier studies from us suggested that ONOO⁻ activates PKCζ in bovine aortic endothelial cells (17).

To determine whether PKCζ was responsible for ONOO⁻-induced Akt inhibition, HUVECs were pretreated with PKCζ pseudosubstrate peptide (PKCζ-PS), a selective pharmacological inhibitor for PKCζ (25). As expected, treatment with PKCζ-PS prior to ONOO⁻ addition suppressed ONOO⁻-induced inhibition on both Akt-Ser473 phosphorylation and Akt activity in a dose-dependent manner (Fig. 1, C and D).

FIGURE 1. Inhibition of protein kinase Cζ attenuates ONOO⁻-induced Akt inhibition and Akt activity. A and B, HUVECs were treated with protein kinase inhibitors (protein kinase G inhibitor, KT5823; protein kinase A inhibitor, H-89; bisindolylmaleimide I (5 μM)), pan-PKC inhibitor) for 30 min before ONOO⁻ exposure (5 μM, 15 min). Akt phosphorylation at Ser473 (⁎) and Akt activity was assessed by phosphorylation of GSK-3 fusion protein. The blots are representative of three independent experiments (⁎⁎, p < 0.01 compared with control). C, phosphorylated PKCζ-Thr410 was detected by immunoprecipitation. D194A, ONOO⁻-induced Akt inhibition. Exposure of HUVECs to KT5823, a potent protein kinase G inhibitor, or H-89 (1 μM), a selective protein kinase A inhibitor, did not alter the basal levels of Akt Ser473 phosphorylation or Akt activity in HUVECs (data not shown). Neither KT5823 nor H-89 altered ONOO⁻-induced Akt inhibition (Fig. 1, A and B). In contrast, bisindolylmaleimide I (5 μM), a nonselective PKC inhibitor (18), which had no effect on Akt phosphorylation or Akt activity in unstimulated cells, significantly attenuated ONOO⁻-induced suppression of both Akt Ser473 phosphorylation and Akt activity (Fig. 1, A and B). These results suggest that Akt inhibition caused by ONOO⁻ might be PKC-dependent.

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PKCζ-dependent LKB1 Nuclear Export

FIGURE 2. LKB1 is required for ONOO−-induced Akt inhibition and recombinant Akt1 in the presence or absence of recombinant PKCζ. Representative Western blots are shown (n = 4, t, p < 0.001 compared with GSK-3β). LKB1-null HeLa S3 cells were exposed to ONOO− (25 μM, 15 min). The lysates were analyzed for PKCζ, cleaved PKCζ, and Akt (total and Ser473-phosphorylated) by Western blot. Representative blots are shown (n = 3, p < 0.01 compared with controls). C, cells transiently transfected with wild type LKB1 (WT), kinase-dead LKB1 (D194A), or LacZ prior to ONOO− treatment induced translocation of PKCζ from the cytoplasm into the nucleus. Intriguingly, ONOO− decreased the amount of LKB1 in the nucleus but increased LKB1 in the cytosol (Fig. 3, C and D). Consistent with previous reports (27), LKB1 was found to be localized primarily within the nucleus in cells overexpressing LKB1 mutants (LKB1-S428A). Further, ONOO− did not alter the subcellular distribution of LKB1-S428A mutants (Fig. 3D). These results suggest that LKB1 phosphorylation at Ser428 was required for LKB1 nuclear export, and ONOO− might increase LKB1 nucleus export by increasing PKCζ-dependent LKB1 phosphorylation at Ser428.

Inhibition of Akt by ONOO− Is PTEN-dependent—PTEN is a phosphatidylinositol 3,4,5-trisphosphate D3-phosphatase that inhibits Akt signaling by dephosphorylating phosphatidylinositol 3,4,5-trisphosphate (28, 29), and LKB1 is known to phosphorylate PTEN (10, 16). Therefore, we tested the effect of ONOO− on PTEN lipid phosphatase activity. Low concentrations (5 μM) of ONOO− enhanced PTEN activity by 60% in HUVECs (Fig. 4A).

To address whether PTEN is responsible for ONOO−-induced Akt inhibition, MDA-MB-468 cells, PTEN null breast cancer cells (30), were exposed to ONOO−. ONOO− increased the detection of LKB1 phosphorylation at Ser428 (Fig. 4B). In contrast to a strong inhibition seen in HUVECs, ONOO− markedly stimulated Akt Ser473 phosphorylation in a dose-dependent manner (Fig. 4B).

To further determine whether PTEN was required for LKB1-dependent Akt inhibition, MDA-MB-468 cells were transfected with wild type PTEN or phosphatase-defective PTEN mutants, C124S, before exposure of ONOO−. Compared with cells overexpressing either wild type PTEN or C124S PTEN mutants, Akt phosphorylation was significantly attenuated in the cells expressing wild type PTEN (Fig. 4C), implying that active PTEN was required for ONOO−-induced Akt inhibition.

LKB1 Ser428 Phosphorylation Is Required for LKB1-mediated PTEN Stabilization—We next determined the phosphorylation site of LKB1, which was important for LKB1-dependent PTEN stabilization and Akt inhibition. As shown in Fig. 5A, ONOO− (5 μM) significantly increased LKB1 Ser428 phosphorylation in both the nucleus and cytosol. Interestingly, more LKB1 phosphorylation remained in the nucleus with lower LKB1 levels in response to ONOO−. These data imply that LKB1 phosphorylation mainly occurs in the nucleus. Mutation of LKB1 serine 428 with alanine (LKB1 S428A) attenuated PTEN phosphorylation at Ser380/Thr382/383 in vitro (Fig. 5B, lane 4 versus lane 3), suggesting that LKB1 Ser428 phosphorylation was required for PTEN phosphorylation at Ser380/Thr382/383 in vitro. Because the phosphorylation of PTEN within the C-terminal tail is reported to increase its stability (31, 32), these data suggest that LKB1 may stabilize PTEN via increased phosphorylation at its C terminus.

To further examine the role of LKB1 phosphorylation in ONOO−-induced Akt inhibition, LKB1-deficient HeLa S3 cells were transiently transfected with WT LKB1 or phosphorylation-defect LKB1 mutants (LKB1-S428A). Overexpression of wild type LKB1 but not LKB1-S428A increased PTEN phosphorylation at Ser380/Thr382/383 but inhibited Akt Ser473 phosphorylation in response to ONOO− (Fig. 5C). In contrast, overexpression of LacZ or LKB1-S428A exhibited increased Akt phosphorylation when exposed to ONOO−.
PKCζ-dependent LKB1 Nuclear Export

PKCζ Participates in LKB1-PTEN Signaling—We next determined whether PKCζ was required for ONOO−-enhanced LKB1 phosphorylation at Ser428 and consequent Akt inhibition by PTEN. Because Ser428 phosphorylation was required for LKB1 nuclear export (Fig. 3D), we first determined whether PKCζ could mediate LKB1 nuclear export. As shown in Fig. 6 (A and B), ONOO− markedly increased the amount of LKB1 in the cytosol, whereas it lowered LKB1 in the nucleus. Interestingly, inhibition of PKCζ with PKCζ-PS abolished ONOO−-enhanced nuclear export of LKB1 (Fig. 6, A and B), suggesting that PKCζ was required for ONOO−-enhanced LKB1 nuclear export. LKB1 increased PTEN phosphorylation at Ser380/382 (Fig. 6C, lane 3 versus lane 1). Further, PKCζ significantly lowered LKB1-mediated PTEN phosphorylation (Fig. 6C, lane 4 versus lane 3). These results further implied that PKCζ might be required for LKB1-mediated but PTEN-dependent Akt inhibition in HUVECs.

PKCζ-LKB1-PTEN-Akt Axis Is Operated in ONOO−-induced Endothelial Cell Apoptosis—The fate of cell survival and cell death is determined by the balance of cell death/survival signals. Both LKB1 and PTEN function as tumor suppressors, whereas Akt is considered a major kinase for survival. Because PKCζ activation increased both LKB1 and PTEN but suppressed Akt, we reasoned that the PKCζ-LKB1-PTEN-Akt axis triggered by ONOO− might be involved in ONOO−-induced endothelial cell apoptosis (33). Exposure of HUVECs to Sin-1 (1 mM, 18 h), a ONOO− donor, markedly induced the phosphorylation of PKCζ, LKB1, and PTEN, whereas it markedly attenuated Akt phosphorylation (Fig. 7A). In addition, Sin-1 lowered cell viability by 2-fold (Fig. 7B) and increased apoptosis, as measured by histone-associated DNA fragmentation (Fig. 7C). Interestingly, the addition of PKCζ-PS or uric acid, a potent ONOO− scavenger, abolished Sin-1-enhanced apoptosis and the reduction of cell viability in HUVECs (Fig. 7, B and C).
LKB1 is thought to function as a tumor suppressor through its ability to negatively regulate the Akt signaling pathway. Thus, we determined whether LKB1 was required for endothelial apoptosis caused by Sin-1. Overexpression of LKB1 in fibrosarcoma cells induces cell death (3). Accordingly, introduction of WT LKB1 into LKB1-null A549 cells sensitized these cells to the effects of Sin-1 (Fig. 7D). In contrast, no significant difference existed between the apoptosis of A549 cells transfected with LacZ, WT, or mutants alone (data not shown). Active LKB1 appeared to be required for the induction of apoptosis by Sin-1, because neither of the two LKB1 inactive mutants (S428A or LKB1-D194A) accentuated Sin-1-induced apoptosis in HUVECs (Fig. 7D).

**DISCUSSION**

In the present study, we have provided evidence in support of the hypothesis that ONOO−/H11002 activates the PKCζ/H19256/LKB1 signal-ling axis, which induces apoptosis through suppression of Akt phosphorylation and Akt activity. We have shown that ONOO− significantly elevates the levels of phosphorylated PKCζ and induces PKCζ nuclear import, which then phosphorylates LKB1 at Ser428. LKB1, in turn, phosphorylates PTEN at Ser380/Thr382/383. Phosphorylation of PTEN at these sites increases its stability and leads to an accumulation of phosphatase activity, which appears to inhibit Akt signaling and induce apoptosis in response to ONOO−. Indeed, ONOO− increased Akt phosphorylation in LKB1-deficient HeLa S3 cells (Fig. 2B) because PTEN phosphorylation requires LKB1. This is further confirmed in PTEN-deficient MDA-MB-468 cells because ONOO− also increased Akt phosphorylation in these cells. Because ONOO− increased Akt phosphorylation in cells lacking LKB1 or PTEN and LKB1 activates Akt in vitro assays, these results can only be explained by LKB1-dependent
PTEN activation. Enhanced PKCζ in macrophages and in cardiac myocytes have also been shown to promote endothelial apoptosis (26). Although PKCζ can be activated via cytochrome c release-mediated caspase-dependent processing (41–43), we could not exclude that ONOO− may activate PKCζ via co-factor-dependent redox processing (44). Aside from apoptosis, PKCζ exerts an important role in insulin action. Multiple lines of evidence have suggested that insulin-activated PKCζ enhances GLUT4 translocation and glucose uptake in muscle cells (45) and adipocytes (46). Furthermore, insulin-enhanced PKCζ activity is reduced in skeletal muscles of patients with obesity and type 2 diabetes (47). Insulin-stimulated PKCζ activation may impair insulin signaling via receptor substrate-1 serine phosphorylation (48, 49), which is associated with insulin resistance. Our results suggest that ONOO− activates PKCζ, which activates LKB1 nuclear export and inhibits Akt. This pathway may represent an important mechanism underlying the development of insulin resistance. Our results, in combination with previous results suggesting a crucial role for PKCζ in regulation of endothelial cell dysfunction (50), suggest that insulin sensitization may be manipulated by targeting this novel signaling pathway.

In the present study, we have also found that ONOO− activates caspase that leads to PKCζ cleavage and activation in endothelial cells. ONOO− may release cytochrome c to activate caspase (43); then activated caspase processes PKCζ at 3 aspartate residues (Asp210, Asp222, and Asp239) and promotes relief of the autoinhibitory state by separating the kinase domain from the pseudosubstrate autoinhibitory sequence (amino acids 116–122) (26, 41, 42, 51). However, we cannot exclude other possibilities. Low concentrations of ONOO− warrant further investigation.

Another important finding in the present study is the regulation of LKB1, a tumor suppressor, by PKCζ. Human LKB1 is a serine-threonine kinase of 433 amino acids that contains both a kinase domain and a nuclear localization signal in its N-terminal region (55). Germline mutations of the LKB1 gene underlie the cancer-prone disorder Peutz-Jeghers syndrome. The
PKCζ-dependent LKB1 Nuclear Export

The majority of Peutz-Jeghers syndrome missense mutations are located in the region encoding the kinase domain and abolish enzymatic activity, disrupting all functions attributed to LKB1. The C-terminal region of LKB1 consists of 124 residues and contains several post-translational modifications. Several phosphorylation sites have been identified: two residues are auto-phosphorylation sites (Thr336 and Thr402) and three others (Ser325, Thr363, Ser428) are phosphorylated by upstream kinases (55). In addition, LKB1 has been shown to undergo farnesylation at a cysteine residue located in the C-terminal region (Cys429 in human LKB1). The C-terminal region may also possibly serve as a regulatory domain mediating dynamic interactions with several classes of proteins and promoting subcellular targeting. In the present study, we have provided evidence that PKCζ phosphorylates LKB1 at Ser428, resulting in increased LKB1-PTEN interaction and subsequently, Akt inhibition. PTEN-LKB1 interaction plays an essential role in Akt inhibition. This is best demonstrated by the finding that Akt is not inhibited by ONOO− in PTEN-deficient cells (MDA-MB-468 cell line). Similarly, ONOO− activated Akt in LKB1-deficient cells (HeLa S3 or A549 cells), implying that LKB1 is required for the inhibitory effects of ONOO− on Akt. Transfection of wild type, but not mutant, LKB1 restored the effects of ONOO− on Akt. Indeed, recombinant PKCζ significantly phosphorylated LKB1 at Ser428, and substitution of Ser428 with alanine, like kinase-dead LKB1 mutants, abolished ONOO−-induced Akt inhibition. Finally, pharmacological or genetic inhibition of LKB1 abolished the effects of ONOO−, suggesting that LKB1 is upstream of LKB1 and that Ser428 in LKB1 might play a crucial role in Akt regulation. However, we cannot exclude that PKCζ might regulate LKB1-Akt interactions through post-translational modifications in LKB1.

The first order rate constant for ONOO− decomposition measured over a pH range from 7.0 to 8.0 indicates that the pK_a is 7.49 ± 0.06 at 37 °C, and the half-life of ONOO− is 1.9 s at pH 7.4 (23). Therefore, most exogenously added ONOO− will be quenched before reaching its cellular targets. Although there is no way to quantify how much ONOO− added was taken up by the cells, we believe this concentration of ONOO− is likely pathologically relevant and can be generated within tissues. Although NO− is normally present at 1–20 nM levels in biological tissues and up to 100–150 nM levels in stimulated vessels, NO− concentrations of 0.1 μM up to several μM may occur in pathological states of ischemia or inflammation (56–58). In post-ischemic tissues, such as the heart, it has been shown that the levels of O2− production measured by spin trapping of the vascular effluent are 0.2–1.0 μM; and in the presence of PMNs, this O2− generation is further increased (56–58). Therefore, it is very likely that submicromolar to micromolar levels of NO−, O2−, and ONOO− are formed in stimulated or post-ischemic tissues. In cultured cells, the rate of ONOO− production in macrophages is estimated to be as high as 50–100 μM per min (59). Thus, the PKCζ-LKB1-PTEN-Akt axis we have described here might be implicated in many pathological conditions including hypoxia-reoxygenation, diabetes, and atherosclerosis.

In summary, PKCζ potently phosphorylates LKB1 at Ser428, and LKB1 Ser428 phosphorylation is required for ONOO−-induced Akt inhibition, which might play a crucial role in cell survival and insulin signaling. We conclude that PKCζ acts upstream of LKB1-dependent, PTEN-mediated Akt inhibition.

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