Reactive Nitrogen Species Induced by Hyperglycemia Suppresses Akt Signaling and Triggers Apoptosis by Upregulating Phosphatase PTEN (Phosphatase and Tensin Homologue Deleted on Chromosome 10) in an LKB1-Dependent Manner

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**Background**—Oxidative stress plays a causal role in vascular injury in diabetes mellitus, but the mechanisms and targets remain poorly understood.

**Methods and Results**—Exposure of cultured human umbilical vein endothelial cells to either peroxynitrite (ONOO−) or high glucose significantly inhibited both basal and insulin-stimulated Akt phosphorylation at Ser473 and Akt activity in parallel with increased apoptosis, phosphorylation, and activity of phosphatase and tensin homologue deleted on chromosome 10 (PTEN). Furthermore, protein kinase B/Akt inhibition induced by ONOO− or high glucose and apoptosis triggered by high glucose could be abolished by transfection of PTEN-specific small interfering RNA, suggesting that PTEN mediated the Akt inhibition by ONOO−. In addition, exposure of human umbilical vein endothelial cells to ONOO− or high glucose remarkably increased Ser428 phosphorylation of LKB1, a tumor suppressor. Interestingly, the ONOO−-enhanced PTEN phosphorylation and Akt inhibition can be blocked by LKB1-specific small interfering RNA. Consistently, LKB1 phosphorylated PTEN at Ser380/Thr382/383 in vitro, suggesting that LKB1 might act as an upstream kinase for PTEN. Compared with nondiabetic mice, the levels of PTEN, LKB1-Ser428 phosphorylation, and 3-nitrotyrosine (a biomarker of ONOO−) were significantly increased in the aortas of streptozotocin-induced diabetic mice, which was in parallel with a reduction in Akt-Ser473 phosphorylation and an increase in apoptosis. Furthermore, administration of PTEN-specific small interfering RNA suppressed diabetes-enhanced apoptosis and Akt inhibition. Finally, treatment with Tempol, a superoxide dismutase mimetic, and insulin, both of which reduced the ONOO− formation, markedly reduced diabetes-enhanced LKB1-Ser428 phosphorylation, PTEN, and apoptosis in the endothelium of mouse aortas.

**Conclusion**—We conclude that hyperglycemia triggers apoptosis by inhibiting Akt signaling via ONOO−-mediated LKB1-dependent PTEN activation. (*Circulation. 2007;116:1585-1595.*)

**Key Words:** apoptosis ■ endothelium ■ endothelium-derived factors ■ hyperglycemia ■ peroxynitrite
activation of specific PKC isoforms, and accelerated nonenzymatic formation of advanced glycation end products. Recently, Du and coworkers\(^4\) proposed overproduction of mitochondrial superoxide anions ($O_2^{•−}$) as the unifying mechanism for hyperglycemia-induced vascular injury. However, the molecular mechanisms tying hyperglycemia to vascular injury remain poorly understood.

Activation of phosphoinositide-3'-kinase (PI3K) generates lipid products, including PI (3,4,5)P\(_3\), initiating a cascade of serine kinase activation whereby phosphoinositide-dependent kinase-1 (PDK-1) is phosphorylated, leading to the phosphorylation and activation of Akt, other serine kinases, and their downstream substrates. The PI3K–PDK-1–Akt axis plays essential roles in cell biology, including cell growth and cell death. In addition, this pathway ultimately culminates in the pleiotropic biological actions of insulin in vascular function. In addition to its known effects on metabolism, insulin, via the PI3K–Akt axis, increases nitric oxide (NO) release by phosphorylating serine (Ser) 1177 of endothelial NO synthase.\(^5\)

The phosphatase and tensin homolog deleted on chromosome 10 (PTEN), originally identified as a tumor suppressor gene mutated in a large percentage of human cancers,\(^6\) is considered to be a key negative regulator of the PI3K/Akt pathway.\(^7\)\(^8\) Previous studies have demonstrated that deletion of PTEN in muscle protects mice from insulin resistance and diabetes mellitus caused by high-fat feeding.\(^9\) Other studies provide evidence that increased PTEN might contribute to impaired NO release in endothelial cells exposed to either free fatty acids\(^10\) or resistin,\(^11\) although the mechanism by which diabetes mellitus activates PTEN remains unknown.

Peroxynitrite (ONOO\(^−\)), a highly reactive oxidant formed by the diffusion-controlled reaction of $O_2^{•−}$ and NO, is formed during sepsis, inflammation, diabetes mellitus, ischemia-reperfusion, and atherosclerosis and contributes to all of these pathophysiological processes.\(^12\)\(^−\)\(^14\) Hyperglycemia induces $O_2^{•−}$ and ONOO\(^−\) overproduction,\(^4\)\(^5\)\(^16\) and our laboratory has recently demonstrated that ONOO\(^−\) dose dependently inhibits Akt activity.\(^17\) However, the underlying mechanism is not understood. Because PTEN has a critical role in antagonizing PI3K pathways, we reasoned that ONOO\(^−\) generated by hyperglycemia blocks PI3K/Akt activation by upregulating PTEN. In the present study, we demonstrate that ONOO\(^−\) significantly increased the phosphorylation of LKB1, a tumor suppressor,\(^18\) resulting in enhanced association and phosphorylation of PTEN by LKB1, which increased the phosphorylation, stability, and activation of PTEN in vitro and in vivo. Thus, our results suggest that diabetes mellitus, via hyperglycemia-driven ONOO\(^−\), resulted in accelerated apoptosis by LKB1-mediated but PTEN-dependent Akt inhibition.

**Methods**

A full description of materials and methods used, including Akt activity assay, small interfering RNA (siRNA) gene silencing, measurement of endogenous ONOO\(^−\), PTEN activity assay, in vitro kinase assays, quantitative detection of DNA fragmentation by ELISA, and immunohistochemistry, can be found in the online-only Data Supplement.

**Cell Culture and Treatment With ONOO\(^−\) and Insulin**

Human umbilical vein endothelial cells (HUVECs) and endothelial cell culture medium were purchased from Cascade Biologics (Portland, Ore). HUVECs were maintained in Medium 200 supplemented with a low-serum-growth supplement kit, penicillin (100 U/mL), and streptomycin (100 \(μg/mL\)). The concentrations of ONOO\(^−\) were determined spectrophotometrically in 0.1 mol/L NaOH ($\varepsilon_{250}\approx1670$ mol $\cdot$ L$^{-1} \cdot$ s$^{-1}$). To avoid a pH shift, ONOO\(^−\) was diluted in 0.1 mol/L NaOH. After serum starvation for 6 hours, HUVECs were treated with 5 $\mu$mol/L ONOO\(^−\) as described previously.\(^19\) Thirty-five minutes after ONOO\(^−\) treatment, HUVECs were stimulated with insulin (100 $\mu$mol/L) for 10 minutes.

**High-Glucose Treatment of HUVECs**

After reaching confluence, HUVECs were exposed to normal glucose (NG; 5 mmol/L D-glucose), high glucose (HG; 30 mmol/L D-glucose), or hyperosmotic control (5 mmol/L D-glucose plus 25 mmol/L L-glucose) for up to 72 hours with a daily change of culture media. When needed, pharmacological inhibitors, including uric acid (0.5 mmol/L), Tempol (0.05 mmol/L), or $N^\text{N}$-nitro-$L$-arginine methyl ester (L-NAME; 0.5 mmol/L), were preincubated 1 hour before HG. For siRNA experiments, control or specific siRNA was added to the HUVECs and kept for the time indicated.

**Streptozotocin-Induced Diabetes Mellitus**

After 4 hours of fasting, 10-week-old male mice were made diabetic by injection of streptozotocin (STZ; 50 mg/kg of body weight daily) for 5 consecutive days.\(^20\) Diabetes mellitus is defined as random blood glucose levels of $>300$ mg/dL. Control and STZ-injected mice were given Tempol (44 mg/kg body weight daily) in their drinking water for 28 days\(^5\) or insulin injections (2.5 U/kg body weight twice daily). STZ animals also were injected retro-orbitally with either mouse-specific PTEN siRNA or control siRNA (200 $\mu$L; final dose, 1 mg/kg) every 3 days for 6 days using in vivo jetPEI (Polyplus-Transfection, Illkirch, France) according to the manufacturer’s recommendations. At the time indicated, mice were euthanized by isoflurane inhalation, and aortas were removed and immediately frozen in liquid nitrogen or fixed in 4% paraformaldehyde. For further details, see the online-only Data Supplement.

**Statistical Analysis**

Values are presented as mean±SD. Differences between experimental groups were determined by 1-way or 2-way ANOVA, followed by Student t test as appropriate. An unpaired Student t test was performed for single comparisons between groups. Values of $P<0.05$ were 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**

**ONOO\(^−\) Inhibits Akt-Ser473 Phosphorylation and Activity**

To determine the effects of ONOO\(^−\) on the insulin signaling cascade, we first investigated the effects of ONOO\(^−\) on Akt-Ser473 phosphorylation and Akt activity in cultured HUVECs. Confluent HUVECs were treated with insulin (100 $\mu$mol/L) with or without ONOO\(^−\). As expected, insulin significantly increased the phosphorylation of Akt-Ser473 and Akt activity (Figure 1A) as measured by Akt-dependent GSK-3β fusion protein phosphorylation in in vitro assays. Exposure of HUVECs to pathologically relevant concentrations of ONOO\(^−\) (5 $\mu$mol/L) significantly attenuated the basal and insulin-stimulated Akt-Ser473 phosphorylation (70% and...
76% reduction; \( P < 0.01 \) and Akt activity (80% and 88% reduction; \( P < 0.01 \)) (Figure 1A).

We next determined whether ONOO\(^-\) altered the phosphorylation of PDK1, PI3K, and Akt. The phosphorylation of PDK1 at Ser241 is reported to correlate with its activity.\(^{22}\) As expected, insulin dramatically increased the Ser241 phosphorylation of PDK1 in HUVECs (Figure 1B). However, exposure of HUVECs to ONOO\(^-\) (5 \( \mu \)mol/L) markedly diminished insulin-enhanced Ser241 phosphorylation of PDK1.

Because a decrease in PDK-1 and Akt might be due to inhibition of insulin receptor substrate 1 (IRS)-1–dependent PI3K activation, we next determined whether ONOO\(^-\) altered the Ser307 phosphorylation of IRS-1, which is reported to negatively modulate IRS-1–associated PI3K activity.\(^{23}\) Unexpectedly, ONOO\(^-\) inhibited the Ser307 phosphorylation of IRS-1 in HUVECs (data not shown), suggesting that the effects of ONOO\(^-\) on both PDK-1 and Akt were not from decreased activation of PI3K by IRS-1.

We next determined whether ONOO\(^-\) altered the phosphorylation of both PDK-1 and Akt enhanced by growth factors such as vascular endothelial growth factor (VEGF) and insulin-like growth factor (IGF). As shown in Figure 1C, both VEGF and IGF significantly increased the Ser473 phosphorylation of Akt and Akt activity. The addition of ONOO\(^-\) significantly ablated VEGF- or IGF-enhanced Akt-Ser473 phosphorylation or Akt activity (Figure 1C). Concomitantly, VEGF or IGF significantly increased the phosphorylation of PDK1, whereas ONOO\(^-\) (5 \( \mu \)mol/L) abolished VEGF- and IGF-stimulated PDK1 phosphorylation (Figure 1D). Taken together, our results suggest that ONOO\(^-\) inhibited both PDK-1 and Akt, likely via the suppression of the PI3K pathway.

**Inhibition of Akt by ONOO\(^-\) Is PTEN Dependent**

The phosphorylation of both PDK1 and Akt is concomitantly controlled by levels of phosphatidylinositol-3,4,5-P3, which is regulated by PI3K and PTEN.\(^{25,26}\) PTEN is a PtdIns-3,4,5-P3 D3-phosphatase that inhibits both PDK and Akt signaling by dephosphorylating phosphatidylinositol-3,4,5-P3.\(^{24}\) Phosphorylation of PTEN at Ser380/Thr382/383 is essential for its stability because it prevents degradation.\(^{25,26}\) Therefore, we examined the effects of ONOO\(^-\) on the levels of p-PTEN-S380/T382/383 and total PTEN. As shown in Figure 2A, ONOO\(^-\) (5 \( \mu \)mol/L) significantly increased the levels of phosphorylated PTEN (S380/T382/383) by 1.5-fold. Similarly, compared with HUVECs treated with vehicles, ONOO\(^-\) increased the levels of PTEN by 40% (\( P < 0.05 \); Figure 2B). Concomitantly, ONOO\(^-\) increased PTEN activity by 2-fold under basal and insulin-stimulated conditions (Figure 2C).

To determine whether PTEN was responsible for ONOO\(^-\)-induced Akt inhibition, HUVECs were transfected with PTEN-specific siRNA or control siRNA. As shown in Figure 2D, transfection of siRNA but not control siRNA significantly suppressed both endogenous PTEN and PTEN phosphorylation in HUVECs. However, neither PTEN-specific
siRNA nor control siRNA altered the levels of Akt-Ser473 phosphorylation or Akt activity (Figure 2D). PTEN-specific siRNA but not control siRNA reversed ONOO⁻-induced inhibition on both Akt phosphorylation and Akt activity in HUVECs. In parallel, PTEN siRNA but not control siRNA abolished ONOO⁻-enhanced PTEN phosphorylation in HUVECs (Figure 2D). Concomitantly, transfection of PTEN siRNA but not control siRNA abolished ONOO⁻-suppressed PDK1 phosphorylation in HUVECs (Figure 2D). Taken together, these results imply that ONOO⁻-induced Akt inhibition is PTEN-mediated.

**PTEN-Dependent Akt Inhibition by ONOO⁻ Is LKB1 Mediated**

We previously demonstrated that ONOO⁻ significantly increases Ser428 phosphorylation of the serine/threonine kinase LKB1 in bovine aortic endothelial cells. Moreover, evidence exists that LKB1 phosphorylates PTEN in cancer cells; however, LKB1 phosphorylation site(s) had not been identified. We further assayed whether increased PTEN phosphorylation was mediated by LKB1 in HUVECs and, if so, the phosphorylation site(s) of PTEN by LKB1. As depicted in Figure 3A, ONOO⁻ significantly increased both basal and insulin-treated LKB1-Ser428 phosphorylation by 60% (P<0.01). Although LKB1 is located primarily in the nucleus, ONOO⁻ significantly increased the translocation of LKB1 from the nucleus to the cytoplasm and plasma membrane (Figure 3B). In contrast, PTEN was located mainly in cytosol in unstimulated HUVECs. Exposure of HUVECs to ONOO⁻ increased membrane-associated PTEN (Figure 3C).

To further examine the role of LKB1 in ONOO⁻-induced, PTEN-mediated Akt inhibition, HUVECs were transfected with LKB1 siRNA or control siRNA. Transfection of LKB1-specific siRNA but not control siRNA reduced LKB1 by 80% (Figure 3D). Interestingly, LKB1 siRNA but not control siRNA abolished ONOO⁻-enhanced PTEN phosphorylation at Ser380/Thr382/383 and AMPK phosphorylation in HUVECs (Figure 3D). Furthermore, LKB1 siRNA abolished ONOO⁻-enhanced inhibition of Akt phosphorylation, whereas control siRNA had no effect (Figure 3D).

We next investigated whether ONOO⁻ increased the interaction of LKB1-PTEN. LKB1 was immunoprecipitated and Western blotted with the antibody against PTEN or vice versa. Compared with a weak association of LKB1 with PTEN in control cells, ONOO⁻ significantly increased the association of LKB1 with PTEN (Figure 4A and 4B).

**LKB1 Phosphorylates PTEN at Ser380/Thr382/383**

Phosphorylation of PTEN at Ser380/Thr382/383 within its C-terminal tail is reported to increase its stability. Because ONOO⁻ increased the association of PTEN with LKB1, we first determined whether LKB1 phosphorylated PTEN in vitro. Incubation of recombinant LKB1 with recombinant PTEN in vitro, the phosphorylation site(s) of PTEN by LKB1. In addition, exogenous PTEN had no effect on Akt activity (Figure 4C, lane 5 versus lane 4), implying that PTEN did not act as a protein phosphatase to inhibit Akt. Furthermore, LKB1 significantly increased the phosphorylation of PTEN at Ser380/Thr382/383 (Figure 4D, lane 2). Because neither LKB1 nor PTEN inhibited the phosphorylation of Akt or GSK-3β by LKB1. In addition, exogenous PTEN had no effect on Akt activity (Figure 4C, lane 5 versus lane 4), implying that PTEN did not act as a protein phosphatase to inhibit Akt. Furthermore, LKB1 significantly increased the phosphorylation of PTEN at Ser380/Thr382/383 (Figure 4D, lane 2). Because neither LKB1 nor PTEN inhibited the phosphorylation of Akt or GSK-3β in vitro, the inhibitory effects of ONOO⁻ on Akt/PDK1 in HUVECs are likely via a PTEN-dependent degradation of lipid molecules [PtdIns(3,4,5)P3] of PI3K. Because ONOO⁻ increased PTEN phosphorylation at Ser380/Thr382/383, resulting in an increased PTEN half-life, increased association of LKB1 with PTEN might be responsible for ONOO⁻-enhanced PTEN phosphorylation and membrane association.
HG Induces PTEN-Dependent Endothelial Cell Apoptosis in HUVECs

Both diabetes mellitus and hyperglycemia are known to increase ONOO\(^{-}\), as assayed by 3-nitrotyrosine (3-NY), a footprint of ONOO\(^{-}\) in cultured cells.\(^{15,16}\) We next determined whether HG, via ONOO\(^{-}\), altered the LKB1-PTEN-Akt axis in HUVECs. Consistent with earlier reports,\(^{31,32}\) exposure of HUVECs to HG (30 mmol/L) for 72 hours inhibited Akt Ser473 phosphorylation by 80% (\(P<0.01\)) (Figure 5A); increased the levels of both PTEN and phosphorylated PTEN (Figure 5B); and decreased the phosphorylation of Akt (Figure 5C).

Figure 3. LKB1 is required for ONOO\(^{-}\)-induced Akt inhibition. A, ONOO\(^{-}\) enhances phosphorylation of LKB1 at Ser428 (n=6; 2-way ANOVA: \(\Delta P<0.01\), ONOO\(^{-}\) vs control; \(\Delta P<0.05\) vs insulin). No significance existed between ONOO\(^{-}\) and ONOO\(^{-}\) plus insulin-induced LKB1 phosphorylation. B, ONOO\(^{-}\) increases the translocation of LKB1 from the nucleus to the cytoplasm and membrane (n=4; unpaired t test: \(\Delta P<0.01\), ONOO\(^{-}\) vs control; \(\Delta P<0.05\), ONOO\(^{-}\) vs control). Nuclear, cytosolic, and membrane fractions were isolated as described in the online-only Data Supplement. LKB1 or PTEN was detected in Western blots using the specific antibody. C, ONOO\(^{-}\) enhances membrane-bound LKB1 and PTEN. The photo is a representative of 4 individual experiments by confocal microscopy. D, HUVECs were transfected with control siRNA or LKB1 siRNA (100 nmol/L) for 48 hours and treated with ONOO\(^{-}\) (5 \(\mu\)mol/L) for 45 minutes. The blot is a representative blot from 4 independent experiments. Notably, LKB1 siRNA but not control siRNA attenuates ONOO\(^{-}\)-induced PTEN phosphorylation and Akt inhibition (n=4; 1-way ANOVA: \(\Delta P<0.01\), LKB1 siRNA vs control; \#\(P<0.01\), ONOO\(^{-}\) vs control; \(\Delta P<0.01\), LKB1 siRNA/ONOO\(^{-}\) vs control siRNA/ONOO\(^{-}\)).

Figure 4. ONOO\(^{-}\) enhances LKB1 and PTEN interactions. A and B, ONOO\(^{-}\) increases the association of LKB1 and PTEN. LKB1 or PTEN was immunoprecipitated, and PTEN or LKB1 was detected by Western blotting (n=4; 2-way ANOVA: \(\Delta P<0.01\), ONOO\(^{-}\) vs control; \(\Delta P<0.01\), ONOO\(^{-}\)/insulin vs insulin). C, Recombinant LKB1 enhances Akt-Ser-473 phosphorylation and activity in vitro. GSK-3 fusion protein was incubated with kinase buffer alone (lane 1), recombinant Akt1 (lane 2), recombinant LKB1 (lane 3), or recombinant Akt1, LKB1, and PTEN (lane 4) in the presence of ATP. Reactions were allowed to proceed for 30 minutes at 37°C (n=3; 1-way ANOVA: \(\Delta P<0.01\), Akt1+LKB1+GSK3\(^{\beta}\) or -PTEN vs Akt1+GSK3\(^{\beta}\); \(\Delta P<0.01\), LKB1 phosphorylates recombinant human PTEN at Ser380/Thr382/383. Recombinant PTEN was incubated with either kinase buffer alone (lane 1) or recombinant LKB1 (lane 2) in the presence of ATP for 30 minutes at 37°C. Representative Western blots for phosphorylated PTEN-S380/T382/383 (top) and total PTEN (bottom) are shown (n=6; unpaired t test: \(\Delta P<0.01\) vs PTEN alone).
phorylated PTEN (S380/T382/383) by 50% and 70%, respectively (P < 0.01) (Figure 5A and 5D); and increased the Ser428 phosphorylation of LKB1 (Figure 5D). Importantly, transfection of PTEN siRNA but not control siRNA, which did not alter LKB1 phosphorylation, abolished HG-induced inhibition of Akt phosphorylation (Figure 5A). These data suggest that LKB1 might be an upstream kinase for PTEN in HUVECs.

Both LKB1 and PTEN are tumor suppressors, whereas Akt functions as a survival factor. Thus, an increase in the first 2 and/or suppression of the last by ONOO• are expected to cause apoptosis. Indeed, HG significantly reduced the cell viability, as assayed by 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide, and increased endothelial cell apoptosis by 130%, as measured by DNA fragmentation (Figure 5B). As expected, PTEN siRNA abrogated the HG-induced apoptosis in HUVECs, whereas control siRNA had no effect (Figure 5B).

**HG Via ONOO• Causes the Upregulation of LKB1 and PTEN in HUVECs**

To assay the formation of ONOO•, we measured HG-induced dihydrorhodamine 123 oxidation in HUVECs. Earlier studies suggest that dihydrorhodamine 123 reacts with ONOO• but not with its precursor, O2•− or NO.33 Compared with normal cells, HG but not hyperosmotic control glucose significantly increased the oxidation of dihydrorhodamine 123 (Figure 5C), implying that HG increased ONOO•. Concomitantly, HG but not hyperosmotic control glucose significantly increased 3-NY, a footprint in cultured endothelial cells (P.S., J.X., and M.-H.Z., unpublished data, 2007). Furthermore, administration of either an O2•− scavenger, Tempol (0.05 mmol/L), or an NO synthase inhibitor, L-NAME, both of which prevent ONOO• formation, like ONOO• scavenger uric acid, abolished HG-induced dihydrorhodamine oxidation. Because uric acid, Tempol, and L-NAME alone had no effect on basal dihydrorhodamine oxidation (data not shown), these data suggest that HG significantly increased the formation of ONOO• in HUVECs.

We next determined whether ONOO• generated by HG was involved in HG-induced PTEN-mediated Akt inhibition. Because ONOO• has a half-life of <1 second at physiological pH 7.4,34 and ONOO• can initiate both nitrosative and oxidative reactions in both in vitro and in vivo,12,13 scavenging of O2•− or inhibition of NO with L-NAME was used to obtain indirect evidence for ONOO• in HUVECs. As shown in Figure 5C and 5D, either scavenging of O2•− or inhibition of NO with L-NAME was used to obtain indirect evidence for ONOO• in HUVECs. As shown in Figure 5C and 5D, either scavenging or inhibition of NO with L-NAME was used to obtain indirect evidence for ONOO• in HUVECs. As shown in Figure 5C and 5D, either scavenging of O2•− or inhibition of NO with L-NAME was used to obtain indirect evidence for ONOO• in HUVECs. As shown in Figure 5C and 5D, either scavenging or inhibition of NO with L-NAME was used to obtain indirect evidence for ONOO• in HUVECs. As shown in Figure 5C and 5D, either scavenging or inhibition of NO with L-NAME was used to obtain indirect evidence for ONOO• in HUVECs. As shown in Figure 5C and 5D, either scavenging or inhibition of NO with L-NAME was used to obtain indirect evidence for ONOO• in HUVECs. As shown in Figure 5C and 5D, either scavenging or inhibition of NO with L-NAME was used to obtain indirect evidence for ONOO• in HUVECs. As shown in Figure 5C and 5D, either scavenging or inhibition of NO with L-NAME was used to obtain indirect evidence for ONOO• in HUVECs. As shown in Figure 5C and 5D, either scavenging or inhibition of NO with L-NAME was used to obtain indirect evidence for ONOO• in HUVECs. As shown in Figure 5C and 5D, either scavenging or inhibition of NO with L-NAME was used to obtain indirect evidence for ONOO• in HUVECs. As shown in Figure 5C and 5D, either scavenging or inhibition of NO with L-NAME was used to obtain indirect evidence for ONOO• in HUVECs. As shown in Figure 5C and 5D, either scavenging or inhibition of NO with L-NAME was used to obtain indirect evidence for ONOO• in HUVECs. As shown in Figure 5C and 5D, either scavenging or inhibition of NO with L-NAME was used to obtain indirect evidence for ONOO• in HUVECs. As shown in Figure 5C and 5D, either scavenging or inhibition of NO with L-NAME was used to obtain indirect evidence for ONOO• in HUVECs. As shown in Figure 5C and 5D, either scavenging or inhibition of NO with L-NAME was used to obtain indirect evidence for ONOO• in HUVECs. As shown in Figure 5C and 5D, either scavenging or inhibition of NO with L-NAME was used to obtain indirect evidence for ONOO• in HUVECs. As shown in Figure 5C and 5D, either scavenging or inhibition of NO with L-NAME was used to obtain indirect evidence for ONOO• in HUVECs. As shown in Figure 5C and 5D, either scavenging or inhibition of NO with L-NAME was used to obtain indirect evidence for ONOO• in HUVECs. As shown in Figure 5C and 5D, either scavenging or inhibition of NO with L-NAME was used to obtain indirect evidence for ONOO• in HUVECs. As shown in Figure 5C and 5D, either scavenging or inhibition of NO with L-NAME was used to obtain indirect evidence for ONOO• in HUVECs. As shown in Figure 5C and 5D, either scavenging or inhibition of NO with L-NAME was used to obtain indirect evidence for ONOO• in HUVECs. As shown in Figure 5C and 5D, either scavenging or inhibition of NO with L-NAME was used to obtain indirect evidence for ONOO• in HUVECs. As shown in Figure 5C and 5D, either scavenging or inhibition of NO with L-NAME was used to obtain indirect evidence for ONOO• in HUVECs. As shown in Figure 5C and 5D, either scavenging or inhibition of NO with L-NAME was used to obtain indirect evidence for ONOO• in HUVECs. As shown in Figure 5C and 5D, either scavenging or inhibition of NO with L-NAME was used to obtain indirect evidence for ONOO• in HUVECs. As shown in Figure 5C and 5D, either scavenging or inhibition of NO with L-NAME was used to obtain indirect evidence for ONOO• in HUVECs. As shown in Figure 5C and 5D, either scavenging or inhibition of NO with L-NAME was used to obtain indirect evidence for ONOO• in HUVECs. As shown in Figure 5C and 5D, either scavenging or inhibition of NO with L-NAME was used to obtain indirect evidence for ONOO• in HUVECs.
had the same effects. Because none of these reagents altered the basal levels of LKB1 and PTEN in HUVECs (data not shown), these results implied that reactive nitrogen species, likely ONOO⁻, might play a causal role in the upregulation of LKB1 and PTEN caused by HG exposure in HUVECs.

Inhibitory Effects of Hyperglycemia on Akt Are PTEN Dependent in Diabetic Mice

We further investigated the upregulation of LKB1 and PTEN and accelerated apoptosis in diabetes mellitus in vivo. Diabetes mellitus was induced in mice by STZ injection, and mice were concomitantly treated with an SOD mimetic, Tempol, or insulin. Injection of STZ significantly increased serum glucose (460/11006 20 mg/dL) compared with the mice with vehicle (180/11006 15 mg/dL; n=5; P<0.01). Administration of insulin markedly reduced the glucose to levels (185/11006 19 mg/dL) similar to those seen in vehicle. Treatment with Tempol for 4 weeks did not alter the blood glucose levels of the control mice (188/11006 16 mg/dL; n=5; P>0.05) or the STZ-induced diabetic mice (465/11006 23 mg/dL; n=5; P>0.5). No significant difference in body weight was observed among these groups (data not shown).

We first determined whether diabetes mellitus increased ONOO⁻ in vivo. 3-NY, a stable marker for ONOO⁻, was measured in aortic homogenates in Western blots. As shown in Figure 6A, diabetes mellitus significantly increased the levels of 3-NY–positive proteins. Both insulin and Tempol treatments alone significantly attenuated the diabetes-enhanced 3-NY levels (Figure 6A) whereas neither PTEN-siRNA nor control siRNA altered the levels of 3-NY (Figure 6A). In parallel with 3-NY reduction, administration of insulin or Tempol or PTEN-specific siRNA significantly attenuated diabetes-enhanced DNA fragmentation in mouse aortic homogenates, suggesting that diabetes mellitus via hyperglycemia-driven reactive oxygen and nitrogen species caused accelerated apoptosis.

We next determined whether LKB1-dependent PTEN-mediated Akt inhibition was operated in vivo. As shown in Figure 6B, STZ injection significantly increased LKB1 phosphorylation at Ser428. Administration of insulin or Tempol abolished STZ-induced LKB1 phosphorylation (Figure 6B). In parallel, STZ injection significantly increased the PTEN phosphorylation and PTEN levels, which was sensitive to insulin or Tempol (Figure 6C). Interestingly, administration of PTEN siRNA suppressed diabetes-enhanced PTEN phosphorylation and PTEN levels (Figure 6C) but did not alter the phosphorylation of LKB1 (Figure 6B), suggesting that LKB1 might be an upstream kinase of PTEN. Moreover, administration of either PTEN siRNA or insulin or Tempol abolished diabetes-induced Akt inhibition (Figure 6D), suggesting that diabetes mellitus via reactive oxygen or nitrogen species suppressed Akt via an LKB1-dependent PTEN-mediated manner in vivo.

We next investigated the changes in endothelium caused by diabetes mellitus. As shown in Figure 7, diabetes mellitus significantly increased 3-NY, PTEN, and apoptosis, localized mainly in endothelium (Figure 7B, 7H, and 7N). Interest-
ingly, insulin, which lowered STZ-induced hyperglycemia, reduced STZ-enhanced 3-NY, PTEN, and apoptosis (Figure 7C, 7I, and 7O). Similarly, administration of Tempol in STZ-injected mice also reversed the effects of diabetes mellitus on 3-NY, PTEN, and apoptosis (Figure 7F, 7L, and 7R). Administration of PTEN-specific siRNA but not control siRNA lowered the levels of PTEN in the endothelium of aortic rings by 70% (Figure 7K and 7J) and significantly reduced diabetes-enhanced apoptosis (Figure 7Q) without altering diabetes-enhanced 3-NY (Figure 7E). Taken together, these data imply that diabetes mellitus, via hyperglycemia-driven ONOO⁻/H₂O₂, upregulated PTEN, resulting in accelerated apoptosis in endothelium.

Discussion

In the present study, we have presented evidence that hyperglycemia via ONOO⁻ can significantly elevate levels of PTEN, which appears to mediate the inhibitory effects of HG on PI3K/Akt signaling in vascular endothelium. We also have demonstrated that LKB1 was required for ONOO⁻-induced Akt inhibition. Our data support the hypothesis that ONOO⁻ production associated with HG triggers LKB1-dependent activation of PTEN, which suppresses Akt signaling and enhances apoptosis in both cultured HUVECs and vascular endothelium of the aortas from diabetic mice (Figure 8). Thus, our results unveil a novel mechanism by which diabetes mellitus may cause vascular endothelial dysfunction and cell death.

Several lines of evidence are consistent with the hypothesis that hyperglycemia, via ONOO⁻, enhances PTEN stability, which suppresses PI3K-dependent phosphorylation of Akt. First, HG significantly increased ONOO⁻ in HUVECs, as assayed by dihydrorhodamine 123 oxidation and 3-NY-positive proteins. Second, ONOO⁻ or HG treatment of HUVECs significantly inhibited both basal and insulin-stimulated phosphorylation of Akt-Ser473 and Akt activity. Third, inhibition of PTEN with siRNA but not with control siRNA prevented ONOO⁻ or HG-induced Akt inhibition and apoptosis. Consistent with these findings, diabetic mice exhibited a parallel reduction in Ser473 phosphorylation of Akt in the aortas, along with a marked increase in PTEN. Fourth, administration of insulin, which lowered blood serum glucose to levels comparable to those in nondiabetic controls, attenuated diabetes-enhanced PTEN and Ser428 phosphorylation of LKB1. These results suggest that hyperglycemia is responsible for diabetes-enhanced PTEN upregulation. Importantly, insulin treatment also ablated 3-NY staining in

Figure 7. ONOO⁻ generation, PTEN upregulation, and single-strand DNA breakage in diabetic blood vessels. A through F. Immunohistochemical staining for 3-NY (brown) in vehicle rings (A), in rings from STZ mice (B), in STZ mice treated with insulin (C), in STZ mice injected with control siRNA (D), in mice injected with PTEN siRNA (E), or in mice fed Tempol (F). G through L. Immunohistochemical staining for PTEN (red). M through R. Terminal deoxynucleotidyl transferase–mediated dUTP nick-end labeling (TUNEL) (red fluorescent), an indicator of DNA-strand breakage. Magnification ×200. Similar immunohistochemical profiles were seen in 5 to 6 aorta rings per group. S-Ctrl indicates control siRNA; S-PTEN, PTEN siRNA.

Figure 8. Proposed mechanism for diabetes-enhanced apoptosis in endothelial cells. STZ-induced hyperglycemia enhances LKB1 and PTEN phosphorylation, PTEN levels, and consequent inhibition of Akt with resultant apoptosis. Inhibition of ONOO⁻ formation, by inhibition of either its constituent O₂⁻ via the SOD mimetic Tempol or nitric oxide by L-NAME or its scavenger uric acid, blocks hyperglycemia-augmented PTEN and concomitant Akt inhibition. Overall, we consider in diabetes mellitus that hyperglycemia via ONOO⁻ increases apoptosis via an LKB1-mediated PTEN-dependent Akt inhibition.
diabetes mellitus, suggesting that hyperglycemia might be a driving factor for ONOO\textsuperscript{−}. Fifth, elevated LKB1 and PTEN phosphorylation and PTEN levels induced by HG were significantly ablated by either the inhibition of ONOO\textsuperscript{−} formation (Tempol and L-NAME) or the ONOO\textsuperscript{−} scavenger, uric acid. Finally, administration of Tempol, an SOD mimetic, which suppressed ONOO\textsuperscript{−} formation as evidenced by decreased 3-NY staining, increased Akt phosphorylation but suppressed apoptosis in parallel with decreased levels of PTEN and LKB1 phosphorylation in diabetic mice, supporting a potential role of ONOO\textsuperscript{−} in diabetes mellitus. Taken together, our results indicate that STZ-induced hyperglycemia inhibited PI3K/Akt by upregulating PTEN via increased stability.

We provided further evidence that the tumor suppressor LKB1 might play an important role in the development of vascular injury and insulin resistance in the vasculature. Previous studies have demonstrated that a skeletal muscle-selective knockout of LKB1 can increase insulin sensitivity,\textsuperscript{35} and several studies have suggested that LKB1 may interact with PTEN to suppress tumor growth.\textsuperscript{28,36} Our data reveal that LKB1 significantly increased the phosphorylation of PTEN in vitro at S380/T382/T383 sites known for prolonged stability of PTEN. Our findings are consistent with the idea that LKB1 increases the stability of PTEN by phosphorylating PTEN at Ser380/Thr382/383. First, Ser428 LKB1 phosphorylation in response to ONOO\textsuperscript{−} or hyperglycemia was accompanied by increased PTEN phosphorylation. Second, ONOO\textsuperscript{−} notably augmented the translocation of LKB1 from the nucleus to the cytoplasm and cytoplasmic membranes. Third, knockdown of LKB1 with LKB1-specific siRNA clearly normalized phosphorylated PTEN and restored Akt phosphorylation inhibited by ONOO\textsuperscript{−}. Fourth, LKB1 and PTEN interactions, which were weak under control or insulin-treated conditions, were significantly enhanced after ONOO\textsuperscript{−} treatment. Finally, in STZ-induced diabetic mice, increases in aortic levels of PTEN, Ser428-phosphorylated LKB1, and 3-NY occurred in parallel with a reduction in Ser473 Akt phosphorylation. To the best of our knowledge, this is the first direct evidence that ONOO\textsuperscript{−} augments the functional link between LKB1 and PTEN.

Recent evidence indicates that diabetes mellitus and hyperglycemia cause oxidant stress.\textsuperscript{37} Endothelial cells are capable of generating ONOO\textsuperscript{−} because of their capacity to simultaneously produce O\textsubscript{2}\textsuperscript{−} and NO.\textsuperscript{38,39} Previous studies have demonstrated that HG augments O\textsubscript{2}\textsuperscript{−} release,\textsuperscript{40,41} which traps the vasorelaxant NO, leading to increased ONOO\textsuperscript{−} levels in endothelial cells.\textsuperscript{15} Recent clinical data found that 3-NY staining was associated with increased apoptosis in diabetes mellitus, suggesting a correlation between apoptosis and ONOO\textsuperscript{−} generation. For example, Frustaci et al\textsuperscript{1} found that apoptosis increased 61- and 85- fold in endothelial cells and cardiomyocytes, respectively, in ventricular myocardial biopsies from diabetic humans. However, these studies did not establish a causal role of ONOO\textsuperscript{−} in diabetic apoptosis. In the present study, we have for the first time demonstrated that ONOO\textsuperscript{−}-dependent PTEN caused apoptosis in diabetes mellitus. However, our results might be applied only to cells or tissues when NO is present because ONOO\textsuperscript{−} formation requires NO. In the absence of NO, O\textsubscript{2}\textsuperscript{−} might become hydrogen peroxide, which, unlike ONOO\textsuperscript{−}, causes oxidative inactivation as a result of disulfide bond formation in PTEN.\textsuperscript{42–44}

Recently, it has been shown that upregulation of PTEN is involved in the inhibitory effects of resistent and free fatty acids on insulin signaling in endothelial cells.\textsuperscript{10,11} Indeed, Nakashima et al\textsuperscript{46} demonstrated that overexpression of PTEN in 3T3-L1 cells inhibits glucose uptake and GLUT4 translocation in vitro, whereas microinjection of a PTEN antibody increases basal and insulin-stimulated GLUT4 translocation in vitro. Tissue-specific deletion of PTEN in liver,\textsuperscript{46} muscle,\textsuperscript{9} and adipose tissue\textsuperscript{47} results in insulin hypersensitivity. More interestingly, systemic administration of PTEN antisense oligonucleotides once a week for 4 weeks can reverse hyperglycemia in db/db (mutations in the gene that encodes the receptor for leptin) and ob/ob diabetic mice.\textsuperscript{48} Thus, our observations may hold importance for insulin resistance in type 2 diabetes mellitus. Although we have shown that administration of PTEN siRNA for 6 days can completely restore Akt phosphorylation in mice aortas, this treatment did not reverse hyperglycemia. Nevertheless, our experimental paradigm in endothelial cells suggests that PTEN may be a promising target for therapeutic intervention for diabetic complications. In the insulin-resistant state, the metabolic IRS/PI3K/PDK/Akt branch becomes insensitive to insulin stimulation. More importantly, in the absence of defective insulin-stimulated glucose uptake, a defect in insulin-induced endothelium-dependent vasodilation also exists, suggesting a systemic deregulation of the PI3K/Akt pathway responsible for changes in insulin-regulated metabolism and cardiovascular functions. Our results show that hyperglycemia can increase PTEN levels and that PTEN-specific siRNA can reverse the inhibitory effects of hyperglycemia on Akt activation. These results suggest that the upregulated PTEN may mediate the inhibitory effects of hyperglycemia on insulin signaling and endothelial NO synthase activation in endothelial cells. Because PTEN plays an essential role in maintaining cell survival/death signals, upregulation of PTEN by ONOO\textsuperscript{−}-dependent LKB1 may provide a link between oxidative stress and endothelial injury, an early phenomenon observed in type 1 and 2 diabetes mellitus.

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Disclosures
None.

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**CLINICAL PERSPECTIVE**

The major clinical manifestations of diabetes mellitus that result in most of the morbidity and mortality are results of its vascular pathology. The inner layers of vessels and capillaries are formed by endothelial cells. High blood sugar (glucose) alters the features of these cells by increasing the number of dead cells (apoptosis), which is regarded as an important factor in the pathogenesis of diabetes mellitus. PI-3 kinase/Akt has been shown to be associated with antiapoptosis, angiogenesis, and carcinogenesis. Our laboratory and others have demonstrated that PI-3 kinase/Akt, which is attenuated by high blood sugar, might play a causal role in increased deaths of endothelial cells in diabetes mellitus. However, the underlying mechanism is not fully understood. Because the phosphatase and tensin homologue deleted on chromosome 10 (PTEN) has a critical role in antagonizing PI3K pathways, we reasoned that peroxynitrite (ONOO$^-$) generated by high blood sugar blocks PI3K/Akt activation by upregulating PTEN. In the present study, we demonstrate that ONOO$^-$ significantly increased the phosphorylation of LKB1, a tumor suppressor, resulting in enhanced association and phosphorylation of PTEN by LKB1, which increased the phosphorylation, stability, and activation of PTEN in both cultured endothelial cells and the aortas from diabetic mice. In addition, pharmacological or genetic inhibition of either PTEN or LKB1 prevents diabetes-enhanced apoptosis and Akt inhibition. Thus, our results suggest that diabetes mellitus, via hyperglycemia-driven ONOO$^-$, resulted in accelerated apoptosis by LKB1-mediated but PTEN-dependent Akt inhibition. Our studies may lead to the development of therapeutic agents that protect against diabetes-related cardiovascular disease.
Reactive Nitrogen Species Induced by Hyperglycemia Suppresses Akt Signaling and Triggers Apoptosis by Upregulating Phosphatase PTEN (Phosphatase and Tensin Homologue Deleted on Chromosome 10) in an LKB1-Dependent Manner

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