Thromboxane $A_2$ Receptor Activates a Rho-associated Kinase/LKB1/PTEN Pathway to Attenuate Endothelium Insulin Signaling*

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This study was conducted to elucidate the molecular mechanisms of thromboxane A2 receptor (TP)-induced insulin resistance in endothelial cells. TP inhibits either insulin-stimulated Akt phosphorylation and eNOS at Ser1177 or Akt at Ser473. These effects were abolished by pharmacological activation of phosphoinositide 3-kinase (PI3K) or dephosphorylation of both Akt (at Ser473) and eNOS (at Ser1177). This study was further confirmed in publications from other laboratories (Soliman, H. et al. (2015) Am. J. Physiol. Heart Circ. Physiol. 309, H70--H81; Lee, S-H. et al. (2014) Am. J. Physiol. Endocrinol. Metab. 306, E332--E343). The authors stand by the conclusions of the paper.

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This article has been withdrawn by the authors. The Journal raised questions that the Akt immunoblot in Fig. 2A was reused in Fig. 5B; lanes 1, 2, and 4 of the actin immunoblot in Fig. 4A are the same; the LKB1 immunoblot in Fig. 7B was reused in Fig. 7D; and lane 2 of the tubulin immunoblot in Fig. 9A was reused in lane 1 for Fig. 9C. The authors were able to locate some of the original data and were able to locate some repeated experiments performed at the time of the original work, which the authors state support the conclusions of the paper. The authors offered to publish amended figures based upon that data and, alternatively, offered to repeat the experiments. However, the Journal declined both offers, a decision with which the authors disagree. Further, the authors state that the results of this paper are confirmed by the results of complementary experiments presented in the article and that some of the principal observations of this article were further confirmed in publications from other laboratories (Soliman, H. et al. (2015) Am. J. Physiol. Heart Circ. Physiol. 309, H70--H81; Lee, S-H. et al. (2014) Am. J. Physiol. Endocrinol. Metab. 306, E332--E343). The authors stand by the conclusions of the paper.

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[2] The abbreviations used are: eNOS, endothelial nitric-oxide synthase; EC, endothelial cell; HFD, high fat diet; HUVECs, human umbilical vein endothelial cells; IBOP, [15-(1α,2β)(5Z,3α(1E,3R,4α)-7-[3-(3-hydroxy-4-(4'-iodophenoxy)-1-butenyl)-7-oxabicyclo[2.2.1]heptan-2-yl] 5'-heptenoic acid; IR, insulin resistance; MEACs, mouse aortic endothelial cells; PTx, pertussis toxin; ROCK, Rho-associated kinase; siRNA, small interference RNA; SQ29548, [[15]α,2β(5Z,3S,4α)-7-[3-[2-[(phenylamino)carbonyl]hydrazino]methyl]-7-oxabicyclo[2.2.1]hept-2-yl]; TP, thromboxane A2 receptor; U46619, 15S-hydroxy-12α,9α(epoxymethano)prosta-5Z,13E-dienoic acid; TXA2, thromboxane A2; FBS, fetal bovine serum.

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bition by TP stimulation remains undefined. Moreover, whether TP activation impairs basal insulin signaling is also unclear.

Here, we investigated whether TP ligands interfere with insulin signaling. Our results reveal that activation of TP using a potent and stable ligand (IBOP) abrogates insulin signaling in ECs. We also show that Rho/ROCK/LKB1-mediated PTEN (phosphatase and tensin homolog deleted on chromosome ten) up-regulation is required for TP-induced inhibition of insulin signaling in ECs.

EXPERIMENTAL PROCEDURES

Materials—Human umbilical vein endothelial cells (HUVECs) were purchased from Invitrogen. HUVECs were maintained in endothelial cell basal medium (EBM) supplemented with EGM™ SingleQuots from LONZA (Walkersville, MD). Culture media were supplemented with penicillin (100 units/ml) and streptomycin (100 μg/ml). Mouse aortic endothelial cells (MAECs) were isolated and maintained in cultured, as described previously (21). Recombinant human ROCK1 and ROCK2 proteins and antibodies against PTEN, phospho-PTEN-Ser1177, Akt, phospho-Akt-Ser473, eNOS, phosho-eNOS-Ser1177, phospho-LKB1-Ser428, and β-tubulin were obtained from Cell Signaling Technology (Beverly, MA). Recombinant human active PTEN was from R & D Systems, Inc. (Minneapolis, MN). Anti-β-actin was from Abcam, Inc. (Cambridge, MA). Recombinant human Akt1 was from BIOSOURCE International, Inc. (Camarillo, CA). Recombinant LKB1 and the PTEN malachite green assay kit were obtained from Upstate Group LLC (Lake Placid, NY). LKB1 siRNA and antibodies against LKB1 were from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). IBOP, U46619, SQ29548, and rabbit polyclonal antibodies against ROCK1 and ROCK2 were purchased from Cayman Chemical. All other chemicals and organic solvents were of the highest grade and were obtained from Sigma-Aldrich.

Cell Treatment—HUVECs cultured in 0.5% serum medium were preincubated for 30 min with the indicated concentration of SQ29548 (TP blocker) or Y27632 (ROCK inhibitor). They were then treated with IBOP or U46619 overnight.

siRNA-mediated Gene Silencing—A series of 21-nucleotide siRNAs were chemically synthesized, desalted, deprotected, and PAGE-purified by Applied Biosystems (Austin, TX). TP synthase was inhibited using the following oligoribonucleotides (TP siRNA): 5'-GGAGCUGUCUAUCACUUGUU-3' and 5'-CAAGUAGAUGAGCAGCUCCUU-3'. PTEN siRNA as well as a nonsilencing control siRNA were obtained from Cell Signaling Technology. The siRNA duplexes were applied at a concentration of 100 nM. HUVECs were transfected with gene target-specific siRNA or nonspecific control siRNA for 48 h using Lipofectamine™ 2000 (Invitrogen) according to the manufacturer’s instructions. Transfected cells were cultured in 0.5% serum medium and exposed to the indicated agents overnight.

In Vitro Kinase Assays—To determine the effect of ROCK on Akt activation, we incubated glycogen synthase kinase-3β fusion protein with recombinant Akt1 in the presence or absence of recombinant ROCK1 or ROCK2 for 20 min at 37 °C. The reaction mixture consisted of kinase buffer (25 mM Tris, pH 7.5, 5 mM β-glycerophosphate, 2 mM dithiothreitol, 0.1 mM Na3VO4, 10 mM MgCl2) supplemented with 0.2 mM ATP. The
effects of the ROCK proteins on LKB1-mediated PTEN phosphorylation were investigated by incubating recombinant LKB1 and PTEN with or without recombinant ROCK1 or ROCK2. To examine the involvement of the ROCK proteins on LKB1-mediated PTEN phosphorylation, we incubated recombinant LKB1 and PTEN in the presence or absence of recombinant ROCKs. In both cases, the reaction conditions were the same as described above. 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-PAGE and Western blotting using phospho-specific antibodies.

**Western Blot Analysis**—HUVECs were washed once with cold phosphate-buffered saline and lysed with ice-cold buffer from Cell Signaling Technology (Beverly, MA) containing 20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM Na3EDTA, 1 mM EGTA, 1% Triton, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM phenethylsulfonfluoride, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 1 mM Na3VO4, and 10 mM NaF. The lysates were clarified by centrifugation at 4°C for 18 min. Protein concentrations were measured using the BCA protein assay (Pierce). Samples containing 20–50 μg of protein were separated on a polyacrylamide gel with Tris-glycine-SDS running buffer (Bio-Rad) and transferred onto nitrocellulose membranes (Bio-Rad) for 2 h. The membranes were blocked in 5% milk in Tris-buffered saline with Tween 20 for 20 min and incubated with primary antibodies. The membranes were washed and incubated with a peroxidase-linked secondary antibody. The reactive bands were detected using ECL™ Western blotting detection reagents (Amersham Biosciences).

**Rhodekin Pulldown Assay for Rho Activation**—HUVECs were plated in 0.5% serum medium in 10-cm culture dishes and treated with 0.4 μmol/liter IBOP, with or without pretreatment of SQ29548. After 1 h of culture, the cells were rapidly lysed on ice and processed for assay of the levels of GTP-bound Rho. The assays were performed according to the manufacturer’s instructions (Thermo Fisher Scientific, Inc.).

**PTEN Activity Assay**—Anti-PTEN antibody (10 μl) was incubated with 500 μg of cell lysate for 1 h. The mixture was then incubated with protein A-Sepharose 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 substrate. An affinity-purified, constitutively active form of PTEN supplied with the kit served as a positive control.

**Animals and Feeding Protocol**—Male C57BL/6 mice were obtained from Jackson Laboratory (Bar Harbor, ME) and maintained on a regular diet. The mice were housed in temperature-controlled cages (20–22°C), fed *ad libitum*, and maintained on a 12-h/12-h light/dark cycle. All of the animal experiments were approved by the Institutional Animal Care and Use Committee at University of Oklahoma Health Sciences Center. Age-matched groups (6–8 weeks of age, n = 10/group) were fed equicaloric diets containing a normal or high amount of fat (60% saturated fat) (Research Diets, New Brunswick, NJ). The animals received a normal or high fat diet (HFD) for 13 weeks. One-half of the animals in each group received either vehicle or SQ29548 (5 mg/kg/day) in drinking water for 5 weeks, with the water being changed twice daily. At the end of this time (18 weeks), the mice were subjected to an intraperitoneal glucose tolerance test and then sacrificed. For the glucose tolerance test, the mice were fasted overnight, and blood was collected from the tail vein at 0, 5, 15, 30, 60, 120, and 240 min after intraperitoneal injection of glucose (2 g/kg of body weight). Blood glucose levels were assayed using a blood glucose meter (LifeScan, Inc.). The trapezoidal rule was used to determine the area under the curve for glucose concentrations in each animal. After the mice were sacrificed, the thoracic aortas were immediately collected and snap frozen in liquid nitrogen.
mouse aortas were subsequently homogenized for analysis of eNOS, Akt, and PTEN.

**Western Blot Quantification**—The integrated intensity (area × density) of individual bands was quantified by densitometry (AlphaEaseFC, Alpha Innotech). The background was subtracted from the calculated area.

**Statistical Analysis**—All of the quantitative variables are presented as the means ± S.D. Differences between individual groups were analyzed using a one-way, repeated measures analysis of variance with Student’s *t* tests. *p* < 0.05 was considered significant.

**RESULTS**

**TP Activation Abrogates Basal and Insulin-stimulated eNOS-Ser1177 Phosphorylation**—Treatment of HUVECs with the TP agonist IBOP (400 nmol/liter) (13, 22) overnight significantly inhibited the phosphorylation of eNOS at Ser1177 (an eNOS activation site) (23, 24) (Fig. 1A). Short term (less than 8 h) treatment with TP agonists did not significantly block eNOS phosphorylation (data not shown). Similar results were obtained with another TP agonist, U46619 (25) (Fig. 1A). To determine whether IBOP- or U46619-induced inhibition of eNOS phosphorylation is mediated by TP receptors, we pretreated HUVECs with SQ29548 (4 μmol/liter), a TXA2 receptor antagonist that has higher affinity to TPs than IBOP (26). As shown in Fig. 1A, SQ29548 pretreatment selectively blocked the dephosphorylation of eNOS elicited by either IBOP or U46619. We also investigated the effect of TP activation on insulin-stimulated phosphorylation of eNOS. HUVECs preincubated with IBOP or U46619 were cultured in serum (0.5% serum) overnight before being challenged with insulin or U46619. Insulin stimulated eNOS phosphorylation, and this effect was blocked by either IBOP or U46619 (Fig. 1B). These results demonstrate that TP stimulation effectively antagonizes insulin-induced eNOS phosphorylation. Interestingly, the inhibitory effects of either IBOP or U46619 were completely reversed by SQ29548 pretreatment (Fig. 1B). The ability of this TP antagonist to block either IBOP- or U46619-induced inhibition of both basal and insulin-stimulated eNOS phosphorylation suggests that these TXA2 mimetics attenuate eNOS activation through TP.

To confirm that TP participates in IBOP-induced inhibition of insulin signaling, we transfected HUVECs with TP-specific siRNA to knockdown TP. As shown in Fig. 1C, TP siRNA clearly blocked IBOP-induced inhibition of eNOS-Ser1177 phosphorylation, whereas control siRNA did not. Interestingly, TP siRNA also abrogated IBOP-mediated inhibition of insulin-stimulated eNOS phosphorylation. However, TP siRNA alone did not further enhanced insulin-stimulated eNOS phosphorylation compared with control siRNA with insulin (data not shown). These data strongly suggest that TP activation suppresses insulin signaling in ECs.

**TP Activation Blocks Both Basal and Insulin-stimulated Akt Phosphorylation**—Because insulin activates eNOS through Akt (27), we investigated whether TP activation affects both basal and insulin-enhanced Akt activation, as assessed by Ser473 phosphorylation. We found that TP activation blocked both
basal and insulin-stimulated Akt phosphorylation at Ser^473 (Fig. 2, A and B). Pretreatment with the TP antagonist SQ29548 abolished the inhibitory effects of either IBOP or U46619 on both basal and insulin-stimulated Akt phosphorylation (Fig. 2, A and B). TP-specific siRNA blocked IBOP suppression of both basal and insulin-stimulated Akt phosphorylation (Fig. 2, C and D), whereas TP siRNA alone did not further stimulate insulin-induced Akt phosphorylation (data not shown). These results confirm that TP activation impairs endothelial insulin signaling.

TP Activation Inhibits the Phosphorylation of Akt and eNOS in Freshly Isolated MAECs—To test whether TP activation contributes to the development of atherosclerosis, freshly isolated MAECs were treated with either IBOP or U46619 in the presence or absence of insulin. As shown in Fig. 3 (A and B), both IBOP and U46619 significantly attenuated the phosphorylation of both eNOS and Akt. SQ29548 also blocked the inhibition of TP agonists (IBOP or U46619) on both basal and insulin-stimulated Akt phosphorylation in MAECs (Fig. 3, B and D). Similar results were also obtained with IBOP and U46619 in cultured human aortic endothelial cells (Fig. 3, E and F).

TP-induced Inhibition of Insulin Signaling Requires PTEN—To begin to understand what molecules TP may target to block insulin signaling, we investigated IBOP-induced changes in PTEN, a lipid phosphatase that participates in insulin resistance (28, 29). As shown in Fig. 4A, IBOP induced PTEN phosphorylation at Ser^380/Thr^382/383, a modification that is essential for PTEN stability (30–32). IBOP also up-regulated PTEN protein levels (Fig. 4A), exposure of HUVECs to IBOP increased the specific lipid phos-
ROCK/LKB1-mediated PTEN Up-regulation

FIGURE 6. ROCK/ROCK participates in TP-induced inhibition of insulin signaling.

A

B

C

D

E

ROCK Functions as an Upstream Kinase for LKB1, Which Phosphorylates PTEN—Next, we determined whether ROCK directly inhibits Akt. Incubation of recombinant Akt1 with recombinant ROCK1 or ROCK2 did not inhibit Akt-Ser473 or Akt-Thr308 phosphorylation. Indeed, phosphorylation of Akt-Ser473 was increased to some extent. Both ROCK1 and ROCK2

Ser428 phosphorylation by IBOP, suggesting that LKB1 is required for TP-induced inhibition of Akt.

To determine whether LKB1 is required for the inhibitory effects of TP activation, HUVECs were transiently transfected with LKB1 siRNA. Transfection of LKB1-specific siRNA, but not control siRNA, reduced LKB1 protein levels by 80% (Fig. 5B). LKB1 siRNA blocked TP-induced inhibition of insulin-stimulated eNOS and Akt phosphorylation, whereas control siRNA had no effect (Fig. 5B). LKB1 siRNA also blocked TP-induced increases in PTEN levels and PTEN phosphophorylation at Ser380/Thr382/383 (Fig. 5C). As before, control siRNA had no effect on these TP-induced changes (Fig. 5C).

ROCK Participates in the Inhibition of Insulin Signaling in Human ECs—ROCK negatively regulates eNOS phosphorylation by inhibiting Akt phosphorylation. However, whether the Rho kinase pathway participates in TP-induced inhibition of insulin signaling is unknown. To investigate whether ROCK participates in TXA2 signaling, we determined whether stimulation of TP activates Rho. Rhotekin pulldown assays revealed that IBOP did stimulate Rho activation (Fig. 6A), consistent with the ability of U46619 to activate Rho in prostate carcinoma PC-3 cells (36). This activation was attenuated by pretreatment of cells with SQ29548 (Fig. 6A). These data suggest that IBOP activates Rho through TXA2 receptor stimulation.

To investigate the requirement for Rho/Rho-associated kinase (ROCK) in TP-induced inhibition of insulin signaling, we evaluated the effects of Y27632, a specific inhibitor of ROCK (37). As shown in Fig. 6B, Y27632 (10 μmol/liter) pretreatment blocked TP-induced inhibition of basal Akt and eNOS phosphorylation in HUVECs. Y27632 also considerably disrupted TP-induced increases in PTEN, PTEN-Ser380/Thr382/383 phosphorylation, and PTEN lipid phosphatase activity (Fig. 6, C and D). Y27632 pretreatment dramatically ablated the inhibitory effect of IBOP on insulin-stimulated both Akt and eNOS phosphorylation (Fig. 6E).

Taken together, these data show that Rho activation is required for TP-induced inhibition of insulin signaling and that Rho/ROCK may serve as intermediate component of the TXA2-TP signaling pathway regulating the insulin response.

**Phosphatase activity of PTEN**

To determine whether TP-induced inhibition of Akt phosphorylation is mediated by PTEN, HUVECs were transiently transfected with PTEN-specific siRNA. Transfection of PTEN-specific siRNA, but not control siRNA, markedly reduced endogenous PTEN in HUVECs (Fig. 4C). However, PTEN-specific siRNA did not alter either Akt-Ser473 or eNOS-Ser1177 phosphorylation (Fig. 4). Moreover, PTEN-specific siRNA, but not control siRNA, blocked IBOP-induced dephosphorylation of Akt and eNOS (Fig. 4E). Y27632 (10 μmol/liter) significantly increased Akt-Ser473 phosphorylation, whereas SQ29548 pretreatment clearly blocked the induction of LKB1-Thr308 phosphorylation. Indeed, phosphorylation of Akt-Thr308 was increased to some extent. Both ROCK1 and ROCK2

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FIGURE 7. ROCK is an upstream kinase of LKB1, which up-regulates PTEN phosphorylation (at Ser473 and Thr308) of recombinant Akt1 in the presence or absence of recombinant ROCK1 or ROCK2. Akt1 activity was assessed by phosphorylation of glycogen synthase kinase-3 fusion protein. Representative Western blots from five separate experiments are shown. A, in vitro kinase assay showing LKB1-mediated PTEN-Ser380/Thr382/383 phosphorylation in the presence or absence of recombinant ROCK1 or ROCK2. LKB1 in the presence or absence of recombinant ROCK1 or ROCK2 dramatically increased LKB1-Ser428 phosphorylation in the presence or absence of recombinant ROCK1 or ROCK2. Similar results were obtained in three independent assays. C, in vitro kinase assay showing LKB1-mediated PTEN phosphorylation in the presence or absence of recombinant ROCK1 or ROCK2. Representative Western blots from four independent assays. D, Y27632 inhibits IBOP-induced LKB1-Ser428 phosphorylation. The cells were pretreated with Y27632 (10 μmol/liter, 20 min) and then exposed to IBOP overnight (10 μmol/liter, 20 min) and then exposed to IBOP overnight (10 μmol/liter, 20 min) and then exposed to IBOP overnight (10 μmol/liter, 20 min). Pretreatment of HUVECs with a ROCK inhibitor abolished IBOP-induced LKB1-Ser428 phosphorylation and eNOS phosphorylation and an increase in PTEN levels. SQ29548 normalized PTEN phosphorylation and PTEN levels in HFD-fed animals (Fig. 9C).

DISCUSSION

In the present study, we provide evidence that TP activation, through the G, G protein family, stimulates the Rho/ROCK → LKB1 → PTEN signaling axis, which suppresses both basal and insulin-stimulated Akt and eNOS phosphorylation, effects that cause insulin resistance (Fig. 9D). These data may have important implications in clinical settings. TP activation is thought to contribute to the development of diabetic (types 1 and 2) complications (14, 45, 46), and diabetic mice have elevated PTEN levels (32, 47). In a diabetic state, increased PTEN levels impair insulin signaling, acting to hamper EC responses to insulin, inhibit NO

G, Contributions to TP-induced Inhibition of Insulin Signaling—The major signaling pathways initiated by TXA2-receptor binding may be mediated by G proteins (40–42). RhoA is activated by G protein-coupled receptors including the Gα11 heterotrimeric G proteins, which are activated by IBOP. We determined whether a pertussis toxin (PTx)-sensitive G protein (43) mediates IBOP-induced Akt dephosphorylation. Preincubation of HUVECs with 50 ng/ml PTx for 30 min completely inhibited TP-induced dephosphorylation of both Akt and eNOS under basal conditions (Fig. 8A) and in the presence of IBOP (Fig. 8B). These results are consistent with the concept that TP activation in HUVECs was mediated by a PTx-sensitive G protein (15).

To determine how LKB1 participates in ROCK-mediated Akt inhibition following TP activation, we performed in vitro kinase assays and measured LKB1-Ser428 phosphorylation in the presence or absence of ROCK. In line with earlier reports (38), recombinant LKB1 underwent autophosphorylation at Ser428 when incubated alone (Fig. 7B, first lane). The addition of recombinant ROCK1 or ROCK2 dramatically increased LKB1 Ser428 phosphorylation (Fig. 7B, second and third lanes versus first lane), suggesting that ROCK may be an upstream kinase for LKB1. Consistent with earlier reports (32, 38), recombinant LKB1 phosphorylated PTEN at Ser380/Thr382/383 (Fig. 7C, lane 2 versus lane 1). Recombinant ROCK1 did not phosphorylate PTEN (Fig. 7C, lane 3). On the other hand, ROCK2 slightly phosphorylated PTEN (Fig. 7C, lane 4), in agreement with the ability of overexpressed ROCK to phosphorylate PTEN (39). Both ROCK1 and ROCK2 markedly enhanced LKB1-mediated PTEN phosphorylation at Ser380/Thr382/383 (Fig. 7C, lanes 5 and 6 versus lane 2). Pretreatment of HUVECs with a ROCK inhibitor abrogated IBOP-enhanced LKB1-Ser428 phosphorylation (Fig. 7D), further supporting the idea that ROCK acts as an LKB1 kinase.

dramatically inhibited the phosphorylation of a direct substrate, glycogen synthase kinase-3, and these results indicate that ROCK needs a partner to inhibit Akt activation in response to TP stimulation.

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normal diet (data not shown). The HFD also significantly decreased eNOS-Ser1177 phosphorylation and eNOS levels in thoracic aorta (Fig. 8A). A 5-week administration of SQ29548 in the drinking water reversed this repression of both eNOS-Ser1177 phosphorylation and eNOS expression (Fig. 8A), although it did not block glucose intolerance and body weight gain (data not shown). The HFD also notably decreased Akt-Ser473 phosphorylation in the thoracic aorta, with SQ29548 treatment abolishing this effect (Fig. 8B). HFD-induced dephosphorylation of Akt was accompanied by enhanced PTEN-Ser380/Thr382/383 phosphorylation and a 2-fold increase in PTEN phosphorylation. Preincubation of HUVECs with 50 ng/ml PTx for 30 min completely inhibited TP-induced dephosphorylation of both Akt and eNOS under basal conditions (Fig. 8A) and in the presence of IBOP (Fig. 8B). These results are consistent with the concept that TP activation in HUVECs was mediated by a PTx-sensitive G protein (15).

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release, and increase the risk of smooth muscle cell hypertrophy and neointima formation. Our results have uncovered a novel mechanism whereby TP activation induces impairments in endothelial insulin signaling, which may cause vascular endothelium dysfunction contributing to cardiovascular disease.

We show here, for the first time, that TP activation up-regulates PTEN. Several lines of evidence are consistent with the hypothesis that TP activation enhances PTEN stability and leads to an accumulation of lipid phosphatase activity, which represses basal and insulin-stimulated phosphorylation of both Akt and eNOS. First, the TXA₂ mimetic IBOP markedly increased PTEN Ser³⁸⁰/Thr³⁸²/³⁸³ phosphorylation, which increases PTEN stability (30, 31), and a TP antagonist attenuated this effect. Second, a TP antagonist as well as TP-specific siRNA blocked IBOP-induced suppression of insulin signaling. Third, siRNA-mediated knock down of PTEN restored insulin responsiveness in the presence of TP activation. Fourth, a HFD dramatically increased aortic PTEN phosphorylation and PTEN levels in a TP antagonist-reversible manner. Together, these findings suggest that TP stimulation up-regulates PTEN, which potently modulates insulin signaling and function. This suggests that PTEN is a viable target for diabetes therapies aimed at improving EC function, including antiatherogenic responses to insulin and angiogenesis.

The Rho GTPase family, which includes RhoA, Cdc42, and Rac, is critical for dynamic changes in cell shape and adhesion that govern polarity and drive migration (48–50). Recent studies reveal that Rho/ROCK pathways play a critical role in diabetic retinal microvascularopathy (51). Here, we show that the Rho/ROCK pathway participates in TP-induced impairment of endothelial insulin signaling. TP stimulation appreciably activated Rho in HUVECs. In addition, the ROCK-specific inhibitor, Y27632, removed TP-induced suppression of insulin signaling and blocked TP-induced elevation of PTEN and its lipid phosphatase activity. Our results revealed that ROCK functioned as an upstream kinase for LKB1 and in this way up-regulated phosphorylation of PTEN at sites known to regulate PTEN stability. These findings suggest that Rho kinase inhibition could ameliorate endothelial insulin signaling, which contributes to endothelial function (52) in human subjects with TP activation.

In conclusion, Rho/ROCK-mediated, LKB1-dependent up-regulation of PTEN in response to TP activation attenuates
insulin signaling in ECs. This results in EC dysfunction, which contributes to atherosclerosis and impaired angiogenesis. These findings suggest that inhibition of TP stimulation could enhance EC insulin sensitivity in patients with types 1 and 2 diabetes.

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