Thromboxane Receptor Activates the AMP-Activated Protein Kinase in Vascular Smooth Muscle Cells via Hydrogen Peroxide

Miao Zhang, Yun-Zhou Dong, Jian Xu, Zhong-Lin Xie, Yong Wu, Ping Song, Melissa Guzman, Jiliang Wu, Ming-Hui Zou

Abstract—Thromboxane A$_2$ receptor (TPr) stimulation induces cellular hypertrophy in vascular smooth muscle cells (VSMCs); however, regulation of VSMC hypertrophy remains poorly understood. Here we show that TPr stimulation activates AMP-activated kinase (AMPK), which in turn limits TPr-induced protein synthesis in VSMCs. Exposure of cultured VSMCs to either TPr agonists, IBOP and U46619, or exogenous hydrogen peroxide (H$_2$O$_2$) caused time- and dose-dependent AMPK activation, as evidenced by increased phosphorylation of both AMPK-Thr172 and acetyl-coenzyme A carboxylase–Ser79, a downstream enzyme of AMPK, whereas SQ29548, a selective TPr antagonist, significantly attenuated TPr-enhanced AMPK activation. In parallel, both IBOP and U46619 significantly increased the production of reactive oxygen species such as H$_2$O$_2$. Furthermore, adenoviral overexpression of catalase (an H$_2$O$_2$ scavenger) abolished, whereas superoxide dismutase (which catalyzes H$_2$O$_2$ formation) enhanced, IBOP-induced AMPK activation, suggesting that TPr-activated AMPK was mediated by H$_2$O$_2$. Consistently, exposure of VSMCs to either TPr agonists or exogenous H$_2$O$_2$ dose-dependently increased the phosphorylation of LKB1 (at serines 428 and 307), an AMPK kinase, as well as coimmunoprecipitation of AMPK with LKB1. In addition, direct mutagenesis of either Ser428 or Ser307 of LKB1 into alanine, like the kinase-dead LKB1 mutant, abolished both TPr-stimulated AMPK activation and coimmunoprecipitation. Finally, genetic inhibition of AMPK significantly accentuated IBOP-enhanced protein synthesis, whereas adenoviral overexpression of constitutively active AMPK abolished IBOP-enhance protein synthesis in VSMCs. We conclude that TPr stimulation triggers reactive oxygen species–mediated LKB1-dependent AMPK activation, which in return inhibits cellular protein synthesis in VSMCs. (Circ Res. 2008;102:0-0.)

Key Words: thromboxane receptor ■ AMPK ■ oxidative stress ■ vascular smooth muscle cells

AMP-activated protein kinase (AMPK) is a well-conserved eukaryotic protein kinase that is a sensor for changes in cellular energy state.$^1$-$^3$ AMPK activity is stimulated by an increase in the intracellular AMP-to-ATP ratios in response to stresses such as exercise,$^4$ hypoxia,$^5$ oxidative stress,$^6$ and glucose deprivation.$^7$ The activation of AMPK turns on catabolic pathways that produce ATP and turns off anabolic pathways that consume ATP.$^8$-$^10$ The activation of AMPK leads to the phosphorylation of a number of proteins, resulting in increased glucose uptake and metabolism and fatty acid oxidation, and simultaneously results in inhibition of hepatic lipogenesis, cholesterol synthesis, and glucose production.$^1$-$^3$,$^10$ Because AMPK activation could have beneficial metabolic consequences for diabetic patients, AMPK has emerged as a potential target for the treatment of obesity and type 2 diabetes.$^3$ Activation of AMPK requires the phosphorylation of Thr172 in the activation loop of the α subunit by at least 2 upstream kinases,$^{11}$ LKB1$^{12}$-$^{14}$ and Ca$^{2+}$/calmodulin-dependent kinase kinase (CaMKK)-β.$^{15}$-$^{17}$

There is overwhelming evidence that excessive production of reactive oxygen species (ROS) causes oxidative damage to macromolecules of a host cell, which play an important role in the etiology of many disease processes, including cancer, atherosclerosis, and diabetes.$^{18}$ Griendling et al have found that smooth muscle cells exposed to angiotensin II exhibit increased superoxide generation via NADH/NADPH oxidase-like enzymatic activity.$^{19}$ This enzymatic system now appears to be involved in a number of “maladaptive” characteristics of atherosclerosis, such as smooth muscle cell hypertrophy,$^{19}$-$^{20}$ diabetic retinopathy,$^{21}$ platelet-derived growth factor–induced cell proliferation,$^{21}$-$^{22}$ and impaired NO bioactivity.$^{21}$-$^{23}$ Other sources of ROS in the vasculature may include xanthine oxidase, mitochondrion, NO synthase, and P450 enzymes.$^{24}$ One emerging concept is that ROS-
mediated signaling is not restricted to pathologic events. Indeed, angiotensin II and platelet-derived growth factor are important mediators of vascular signals that, in part, depend on ROS as mediators of signal transduction. Recent evidence from our group suggests that both hypoxia-reoxygenation and metformin requires peroxynitrite (ONOO⁻) as a signaling molecule to activate AMPK in endothelial cells. In addition, AMPK is activated by exogenous hydrogen peroxide (H₂O₂). Thromboxane (Tx)A₂ is a product of arachidonic acid through the cyclooxygenase pathway and is synthesized after activation of a variety of cells, including platelets, vascular smooth muscle cells (VSMCs), endothelial cells, and macrophages. TxA₂ exerts potent biological activity, causing platelet aggregation and secretion, vasoconstriction, and mitogenesis and stimulating hypertrophy in VSMCs. These biological effects are the consequence of the interaction of TxA₂ with membrane receptors (TxA₂ receptors [TPrs]), which belong to the heptahedral superfamily of G protein-coupled receptors. Although not completely understood, there is evidence that TxA₂-induced hypertrophy and proliferation appear to involve the activation of mitogen-activated protein kinase and P70S6 kinase. Interestingly, TxA₂ has been found to promote the formation of superoxide anions in pulmonary artery and cultured cells. However, the physiological role of TPr-derived superoxide anions remains unknown.

Therefore, we hypothesize that AMPK may respond to TxA₂-induced ROS production. The aim of the present study was to elucidate the mechanisms by which TPr triggers AMPK activation and the physiological functions of AMPK in cultured rat VSMCs.

Methods and Materials
An expanded Materials and Methods section is available in the online data supplement at http://circres.ahajournals.org. Briefly, rat VSMCs were cultured from rat thoracic aortas. VSMCs were stimulated by the TxA₂ mimetic IBOP or U46619. Phosphorylations of LKB1, AMPK, and acetyl-coenzyme A carboxylase (ACC) after stimulation were detected using Western blotting, and intracellular ROS production was monitored by 2',7'-dichlorodihydrofluorescein (DCF). [³H]Leucine incorporation was performed to investigate the effects of AMPK on IBOP-induced protein synthesis. After infection by adenoviral vectors Ad-AMPK-CA, Ad-AMPK-DN, or Ad–green fluorescent protein (Ad-GFP), VSMCs were treated with IBOP for 5 or 48 hours, and then the protein synthesis was assessed by [³H]leucine (1 μCi/mL) incorporation.

Results
Thromboxane Mimetics Activate AMPK in Rat VSMCs
Because activation of AMPK requires the phosphorylation of Thr172 in the activation loop of α1 and α2 subunits, AMPK activity was determined in Western blots by using specific antibodies (a and c). Exposure of VSMCs to IBOP (b) and U46619 (d) increased both AMPK-Thr172 and ACC-Ser79 in a dose-dependent manner. Data are means ± SEM (b, n = 4; d, n = 3). P < 0.05; ‡P < 0.01, treated vs untreated control cells.

Figure 1. TxA₂ mimetics activate AMPK in cultured VSMCs. Confluent VSMCs were treated with the TxA₂ mimetics IBOP and U46619 at the concentrations indicated for 10 minutes. Both phosphorylated AMPK-Thr172 and ACC-Ser79 were detected in Western blots by using specific antibodies (a and c). Exposure of VSMCs to IBOP (b) and U46619 (d) increased both AMPK-Thr172 and ACC-Ser79 in a dose-dependent manner. Data are means ± SEM (b, n = 4; d, n = 3). P < 0.05; ‡P < 0.01, treated vs untreated control cells.
shown in Figure 1a and 1b, exposure of VSMCs to IBOP (0.001 to 1 μmol/L), a TxA2 mimetic, for 10 minutes dose-dependently increased the phosphorylation of both AMPK-Thr172 and ACC-Ser79, implying that IBOP activated AMPK in VSMCs. We also included 2 other structurally related TxA2 mimetics, U46619 and carboxy-TxA2. As depicted in Figure 1c and 1d, exposure of VSMCs to U46619 for 10 minutes, similar to IBOP, dose-dependently increased both AMPK-Thr172 and ACC-Ser79. Exposure of VSMCs to carboxy-TxA2 also increased the phosphorylation of both AMPK-Thr172 and ACC-Ser79 in a dose-dependent manner (Figure Ia in the online data supplement). Furthermore, concentrations as low as 0.1 μmol/L for both IBOP and U46619, which are pathologically relevant,38 were found to increase AMPK-Thr172 by at least 2-fold, thereby reaching levels similar to that caused by AICAR (1 mmol/L, 1 hour) (supplemental Figure Ib).

The activation of AMPK by a TxA2 mimetic was also time-dependent. Peak phosphorylation of AMPK was reached between 5 and 15 minutes after stimulation with either IBOP (1 μmol/L) or U46619 (1 μmol/L) (Figure 2a and 2b). The phosphorylation of both AMPK and ACC started to decline at 30 minutes (Figure 2a and 2b) and remained elevated at 5 hours (supplemental Figure Iib) after treatment with either IBOP or U46619. In contrast, no change in the expression of AMPK α subunits was observed in VSMCs exposed to the TxA2 mimetic IBOP up to 96 hours (supplemental Figure Iib), suggesting that altered AMPK-Thr172 phosphorylation by TxA2 mimetics was not because of increased expression of AMPK.

**TPr-Dependent AMPK Activation**

We first determined whether TPr agonists altered TPr in VSMCs. TPr expression was determined by Western blotting using a specific antibody. As shown in Figure 2c, exposure of VSMCs to IBOP or U46619 for 24 hours did not alter the expression of TPr. TPr was detected at 55 and 64 kDa (Cayman’s TPr polyclonal antibody detects the TPr receptor at 55 and 64 kDa according to the production information) via Western blotting (d). The TPr antagonist SQ29548 abolishes TPr-induced AMPK activation. Confluent VSMCs were preincubated with or without the TPr antagonist SQ29548 at 1 μmol/L or 10 μmol/L for 30 minutes before being exposed to IBOP (1 μmol/L) for either 5 or 15 minutes. The blot is representative of 3 blots from 3 individual experiments.

![Image](https://via.placeholder.com/150)

**Figure 2.** TxA2 mimetics activate AMPK via TPr in VSMCs. TPr activation causes a time-dependent AMPK activation in VSMCs (a and b). VSMCs were treated with either IBOP (1 μmol/L) or U46619 (1 μmol/L) for 5, 15, and 30 minutes. Data are means±SEM (n=4). †P<0.05, ‡P<0.01, treated vs control cells. VSMCs were treated with either IBOP (1 μmol/L) or U46619 (1 μmol/L) for 24 hours (c). TPr was detected at 55 and 64 kDa (Cayman’s TPr polyclonal antibody detects the TPr receptor at 55 and 64 kDa according to the production information) via Western blotting (d). The TPr antagonist SQ29548 abolishes TPr-induced AMPK activation. Confluent VSMCs were preincubated with or without the TPr antagonist SQ29548 at 1 μmol/L or 10 μmol/L for 30 minutes before being exposed to IBOP (1 μmol/L) for either 5 or 15 minutes. The blot is representative of 3 blots from 3 individual experiments.

**TPr Increases ROS Generation in VSMCs**

There is evidence that TPr activation promotes superoxide production in both pig pulmonary artery VSMCs36 and corpus
We next tested whether TPr increased ROS production in cultured aortic VSMCs. As shown in Figure 3a, treatment of VSMCs with either IBOP (1 μmol/L) or U46619 (1 μmol/L) markedly increased ROS release 10 minutes after incubation, as detected by H2O2-sensitive DCF fluorescence.

**Figure 3.** TxA2-induced AMPK activation is mediated by ROS production. TPr stimulation increases ROS production in VSMCs (a). ROS were determined by measuring DCF after incubation with either IBOP (1 μmol/L) or U46619 (1 μmol/L) for 10 minutes. Data are means±SEM [a, n=4 [▲P<0.05]; b, n=3 [▲P<0.05]]. Infection of VSMCs with adenoviruses encoding catalase or SOD1 increased the expression of both catalase and SOD1 in VSMCs, respectively (b). The blot is representative of at least 3 blots of 3 independent experiments. Adenoviral overexpression of catalase increased catalase activity in VSMCs (b). Catalase activity was examined after 48 hours of infection with adenoviral GFP or catalase. Catalase activity is shown in nanomoles per minute per milliliter (means±SEM, n=3). †P<0.01 catalase vs GFP controls. Adenoviral overexpression of catalase suppresses IBOP-induced (1 μmol/L, 10 minutes) AMPK activation, whereas overexpression of SOD1 (Cu/Zn SOD) increases AMPK activation (c). The blot is representative of 3 blots from 3 individual experiments. Exogenous H2O2 at concentrations indicated for 10 minutes increases AMPK activation in VSMCs (d). Data are means±SEM (n=3). †P<0.05; ‡P<0.01, treated vs control cells.

**Activation of AMPK by Exogenous H2O2 in VSMCs**

Earlier studies have demonstrated that exogenous H2O2 activates AMPK; therefore, we next investigated whether H2O2 could activate AMPK in VSMCs. As depicted in Figure 3d, exposure of VSMCs to H2O2 increased the phosphorylation of AMPK-Thr172 and ACC-Ser79 in a dose-dependent manner.

**TPR-Induced AMPK Activation Is H2O2-Dependent**

We next determined whether TPR-enhanced H2O2 production contributes to TPR-dependent AMPK activation in VSMCs. To this end, catalase, which can detoxify H2O2, was overexpressed by adenoviruses encoding catalase. Adenoviral infection of catalase in VSMCs greatly increased catalase expression and catalase activity (Figure 3b). As expected, adenoviral overexpression of catalase abolished IBOP-enhanced phosphorylation of both AMPK Thr172 and ACC Ser79 (Figure 3c). On the other hand, adenoviral overexpression of superoxide dismutase (SOD1) increased SOD1 expression (Figure 3b), which presumably results in enhanced H2O2 production, significantly increased IBOP-enhanced phosphorylation of both AMPK and ACC (Figure 3c). Because SOD enhanced, whereas catalase suppressed, the effects of IBOP on AMPK, these results imply that IBOP-activated AMPK is H2O2-dependent.
There is evidence that the tumor suppressor LKB1 acts as a major upstream kinase for AMPK. We next investigated whether LKB1 was required for TxA2-enhanced activation of AMPK. As shown in Figure 4a and 4b, exposure of VSMCs to either IBOP (1 μmol/L) or U46619 (1 μmol/L) time-dependently increased the phosphorylation of LKB1 at Ser428, a phosphorylation site that may play a crucial role in regulating AMPK activation.39 IBOP treatment significantly increased LKB1-Ser428 phosphorylation as early as 1 minute following exposure. IBOP also increased LKB1-Ser307 phosphorylation (Figure 4c). In parallel with AMPK-Thr172 phosphorylation, LKB1-Ser307 phosphorylation reached a peak at 10 minutes and then returned to basal levels within 30 minutes.

As seen in AMPK-Thr172 phosphorylation, overexpression of SOD significantly enhanced the phosphorylation of LKB1 at Ser428, whereas overexpression of catalase blunted IBOP-induced LKB1-Ser428 phosphorylation (Figure 5a). In addition, exposure of VSMCs to exogenous H2O2 for 10 minutes also increased LKB1 Ser428 phosphorylation (Figure 5b). Taken together, these results suggest that TPr, via H2O2, increases LKB1 phosphorylation at both Ser428 and Ser307.

### Phosphorylation of Both Ser428 and Ser307 of LKB1 Is Required for TPr-Stimulated AMPK Activation

Our previous studies have demonstrated that Ser428 of LKB1 is required for ONOO−-enhanced AMPK activation.39 Ser307 of LKB1 is also required for LKB1-dependent AMPK activation (Xie et al, unpublished data). We next determined whether the phosphorylation of LKB1 at Ser307 and Ser428 is required for IBOP-induced AMPK activation. As shown in Figure 6a, IBOP did not activate AMPK either in LKB1-
Figure 6. TPr-induced AMPK activation requires LKB1. Activation of AMPK by IBOP is LKB1-dependent (a). LKB1-deficient A549 cells were transfected for 24 hours with LKB1 wild-type (WT) or LKB1 mutants (S307A, S428A, and D194A). The plasmid encoding LacZ.

(Continued)
deficient A549 cells or A549 cells transfected with the LKB1 mutants of D194A, S307A, or S428A but increased AMPK-Thr172 in A549 transfected with wild-type LKB1, implying that LKB1 is required for IBOP-induced AMPK activation. Furthermore, direct mutagenesis of either S307A or S428A, like the kinase-dead mutants (D194A), also abolished the effect of IBOP on AMPK in VSMCs (Figure 6b), suggesting that the phosphorylation of LKB1 at Ser307 and Ser428 was required for IBOP-enhanced AMPK phosphorylation.

**TPr Increases the Association of AMPK With Its Upstream Kinase LKB1**

We have reported previously that ONOO− activates AMPK by increasing the association of LKB1 with AMPK.50 Therefore, we next investigated whether TPr increases the interactions between AMPK and LKB1. LKB1 was first immunoprecipitated and then Western blotted for AMPK or vice versa. As shown in Figure 6c, IBOP significantly increased the coimmunoprecipitation of LKB1 with AMPK-α in VSMCs. However, overexpression of LKB1-S307A, LKB1-S428A, or LKB1-D194A abolished IBOP-enhanced coimmunoprecipitation of LKB1 with AMPK.

We next determined whether TPr agonists alter LKB1 activity by measuring LKB1 activity after treatment with IBOP or U46619. Neither IBOP nor U46619 altered LKB1 activity in VSMCs (supplemental Figure IV). These results suggest that TPr stimulation activated AMPK by increasing the association of AMPK with LKB1, which is an upstream kinase of AMPK, in VSMCs.

**TPr-Induced AMPK Is Independent of CaMKK**

Recent studies reveal that CaMKKβ serve as AMPK kinases.15–17 We next determined whether CaMKKβ involved in the AMPK activation induced by TPr. Pharmacologic inhibition with the CaMKK inhibitor STO-609 (1 μmol/L) did not alter IBOP-induced AMPK phosphorylation in VSMCs (Figure 6d). Transfection of CaMKKβ-specific small interfering RNA but not scrambled small interfering RNA, which largely reduced the levels of CaMKKβ in bovine aortic endothelial cells, significantly reduced the basal levels of AMPK-Thr172. However, inhibition of CaMKKβ with CaMKKβ-specific small interfering RNA did not affect IBOP-induced AMPK-Thr172 phosphorylation in bovine aortic endothelial cells (Figure 6d). Taken together, these data suggest that activation of AMPK by TPr may be independent of CaMKKβ.

**AMPK Inhibition Accentuates IBOP-Induced Protein Synthesis in VSMCs**

Exposure of VSMCs to IBOP for 5 hours increased the phosphorylation of both AMPK at Thr172 and ACC at Ser79 (supplemental Figure IIA), implying that IBOP activated AMPK in VSMCs. Because earlier studies15,40 have shown that TPr stimulation induces hypertrophy in VSMCs, we first determined the role of AMPK activation in TPr-enhanced protein synthesis by assessing [3H]leucine incorporation in the VSMCs infected with adenoviruses encoding either dominant-negative AMPK mutants (AMPK-DN) or constitutively active AMPK mutants (AMPK-CA) or GFP (supplemental Figure V) for 48 hours following 5 hours of IBOP treatment. Because IBOP significantly increased VSMC apoptosis (data not shown), VSMC protein synthesis was assessed by increased [3H]leucine (cmp) incorporation per cell. As expected, exposure of VSMCs to IBOP (1 μmol/L) for 5 hours significantly increased [3H]leucine incorporation per cell in GFP-infected VSMCs (Figure 7a), confirming that IBOP significantly inhibits protein synthesis in VSMCs. Importantly, Inhibition of AMPK by overexpression of AMPK-DN (loss-of-function) further enhanced IBOP-induced protein synthesis (Figure 7a). On the other hand, overexpression of AMPK-CA (gain-of-function) abolished IBOP-induced protein synthesis (Figure 7a), suggesting that AMPK activation inhibited IBOP-induced protein synthesis in VSMCs.

We next assayed the long term effects of AMPK inhibition on IBOP-increased protein synthesis. As expected, IBOP significantly increased protein synthesis in VSMCs at 48 hours. Inhibition of AMPK by AMPK-DN overexpression significantly enhanced IBOP-induced protein synthesis in VSMCs (by 2-way ANOVA; Figure 7b). Taken together, these results suggested that AMPK inhibition enhanced IBOP-induced VSMC protein synthesis.

**Discussion**

AMPK is a serine/threonine protein kinase and a member of the Snf1/AMPK protein kinase family. It is known that AMPK activity is stimulated by an increase in the intracellular AMP-to-ATP ratios in response to stresses such as exercise, hypoxia, oxidant stress, and glucose deprivation. Here we demonstrate TPr-dependent AMPK activation. Exposure of VSMCs to TPr stimulation caused dose- and time-dependent activation of AMPK. In parallel, both IBOP and U46619 increased the production of ROS, as detected by DCF. Furthermore, exogenous H2O2 increased the phosphorylation of AMPK, as seen in NIH 3T3 cells and rat VSMCs,6,28 as well as LKB1 phosphorylation. Moreover, inhibition of H2O2 production by overexpression of catalase (promoting the conversion of H2O2 to water and molecular
oxygen) attenuated IBOP-induced AMPK activation, whereas the overexpression of SOD further enhanced AMPK activation. In parallel, IBOP-enhanced phosphorylation of LKB1 at Ser428 was largely diminished by overexpression of catalase. Therefore, it is suggested that H2O2 mainly contributes to enhanced activation of AMPK by LKB1. How ROS such as H2O2 increase LKB1 phosphorylation, however, is unknown and is under investigation in this laboratory.

Another important finding in the present study is that both Ser428 and Ser307 of LKB1 were required for both TPr- and H2O2-stimulated AMPK activation. The key evidence can be summarized as follows. First, both IBOP and U46619 increased the phosphorylation of LKB1 at Ser428 and Ser307, and according to a previous study in this laboratory,39 the phosphorylation site Ser428 of LKB1 may play a crucial role in regulating AMPK activation. Second, IBOP did not induce AMPK activation in LKB1-deficient A549 cells, whereas IBOP increased AMPK Thr172 phosphorylation in VSMCs. These results suggest that activation of AMPK by IBOP is dependent on LKB1. Third, mutation of Ser307 (S307A) or Ser428 (S428A) in VSMCs and A549 cells abolished IBOP-dependent AMPK activation, implying important roles of Ser428 and Ser307 of LKB1 in VSMCs and A549 cells.

In the present study, we provide evidence that TxA2 is a potent stimulator of ROS and that activation of AMPK by TPr-derived ROS inhibited VSMC protein synthesis. This finding is consistent with recent reports showing that the TXA2 analogue, U46619, promotes the formation of superoxide in intact pulmonary arteries and in pulmonary artery corpus cavernosal smooth muscle cells.36,37 TPr expression and serum levels of multiple TPr ligands are elevated, both locally and systemically, in patients with several vascular and thrombotic diseases, including ischemia, angioplasty, unstable angina, myocardial infarction, and reoclusion after coronary thrombolysis.8,30,38,42 TPr density is increased in atherosclerotic coronary arteries and in vessels with severe intimal hyperplasia.38 Because there is overwhelming evidence that ROS play a causal role in the development of cardiovascular diseases and diabetes, TPr-stimulated ROS production might contribute to the excessive oxidant stress observed in these diseases, and ROS might serve as the common pathway for TPr-induced vascular pathways. Indeed, we found that TPr stimulation increased both superoxide and ONOO⁻, decreased NO bioactivity, and increased protein tyrosine nitration in cultured endothelial cells.

Figure 7. Activation of AMPK by IBOP limits cellular protein synthesis in VSMCs. a, AMPK-dependent inhibition of protein synthesis in 5-hour IBOP-treated VSMCs. Forty-eight hours after having being infected with GFP, AMPK-DN, or AMPK-CA adenoviral vectors, VSMCs were treated for 5 hours with IBOP (1 μmol/L) or vehicle. Protein synthesis was assayed by [3H]leucine incorporation, as described in Materials and Methods. At same time, the cell number was counted using a hemocytometer. VSMC protein synthesis was calculated by dividing the total [3H]leucine (cmp) by the number of cells in each well and the vehicle treated GFP was used as 100%. Two-way ANOVA indicated significant effect of IBOP (P < 0.01) for leucine incorporation and effect of AMPK mutation (P < 0.01) for leucine incorporation. Interaction effect between IBOP and AMPK mutation approached significance (P < 0.05). Data are means ± SEM (n = 4). • P < 0.01, GFP vs GFP plus IBOP; † P < 0.01, AMPK-DN vs AMPK-DN plus IBOP; ‡ P < 0.05, AMPK-DN plus IBOP vs GFP plus IBOP; ¶ P < 0.05, AMPK-CA plus IBOP vs GFP plus IBOP. b, Inhibition of AMPK by AMPK-DN enhanced IBOP-induced protein synthesis in VSMCs. Confluent VSMCs infected with adenovirus of either GFP or AMPK-DN for 48 hours were treated for 48 hours with IBOP (1 μmol/L) or vehicle. Two-way ANOVA indicated significant effect of IBOP treatment (P < 0.01) and AMPK-DN transfection (P < 0.01) for leucine incorporation. Interaction effect between IBOP and AMPK-DN transfection approached significance (P < 0.01) for leucine incorporation. Data are means ± SEM (n = 3). • P < 0.01, GFP vs GFP plus IBOP; † P < 0.01, AMPK-DN vs AMPK-DN plus IBOP; ‡ P < 0.05, AMPK-DN plus IBOP vs GFP plus IBOP.
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(Original paper, unpublished data). Therefore, excessive production of ROS might be a common pathway for TPr-induced vasculopathy.

The function of AMPK activation is still not fully understood. Here we have preliminary evidence that inhibition of AMPK accentuates IBOP-induced protein synthesis. It is known that AMPK activation suppresses protein synthesis and prevents cardiac myocyte hypertrophy by regulation of the eEF2 kinase/eEF2 axis and/or TSC2-mTOR-P70S6 pathways. How AMPK suppresses TPr-induced protein synthesis in VSMCs remains unclear. Further study is warranted to elucidate the molecular mechanisms underlying AMPK-mediated inhibition of protein synthesis.

In conclusion, we have provided evidence that TPr stimulation increases ROS, which mediates AMPK activation in VSMCs. In addition, we demonstrate that TPr-activated AMPK is LKB1-dependent, which requires the phosphorylation of both Ser307 and Ser428 of LKB1 resulting in association of AMPK with LKB1. Finally, we have provided evidence that AMPK activation may serve as an endogenous inhibitor for IBOP-induced cellular protein synthesis in VSMCs. Thus, AMPK may be a therapeutic target in preventing cardiovascular diseases such as diabetes and atherosclerosis.

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Disclosures

None.

References


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Expanded Materials and Methods

Materials

Dulbecco's modified Eagle's medium/Ham's F12 medium (DMEM/F12) were purchased from Mediatech, Inc (Herndon, VA) and ATCC (Rockville, MD), respectively. Fetal calf FCS (FCS), 5-(and-6)-carboxy-2′,7′ -dichlorodihydrofluorescein diacetate (carboxy-H2DCF-DA), and phospho-LKB1 antibody (LKB1-Ser 307) were from Invitrogen Corporation (Carlsbad, CA). All culture media were supplemented with penicillin (100 Units/ml) and streptomycin (100 µg/ml). IBOP ([1S-(1 alpha,2 beta(5Z),3 alpha(1E,3R),4 alpha)]-7-[(3- (3-hydroxy-4- (4′-iodophenoxy) -1-butenyl)-7- oxabicyclo-[2.2.1] heptan-2-yl] -5-h eptenoic acid), U46619, SQ29548, the polyclonal antibody against TPr, and catalase assay kits were obtained from Cayman Chemical (Ann Arbor, MI). Antibodies against phospho- AMPK alpha (Thr172), AMPK alpha, phospho-Acetyl-CoA carboxylase (ACC) (Ser79) and phospho-LKB1 (Ser428) were from Cell Signaling (Danvers, MA). LKB1 monoclonal antibody was from Santa Cruz (Santa Cruz, CA). LKBtide peptide (SNLYHQGKFLQTFCGSPLYRRR corresponding to amino acids 196-215 of human NUAK2) was synthesized by Mimotopes, Australia. Protein A-Sepharose was purchased from GE Healthcare (Piscataway, NJ). STO-609 and other chemicals or organic solvents were obtained from Sigma (St. Louis, MO).

Cell Culture
Rat VSMC were cultured from rat thoracic aortas as described previously. Cultured cells were used between passages 5 and 12 and cultured in DMEM/F12 medium with 10% FCS, penicillin (100 units/ml), and streptomycin (100 µg/ml). When confluent, the cells were washed with FCS-free medium and then maintained in DMEM/F12 with 0.1% FCS for 24 h. LKB1-deficient lung cancer cell line A549 cells (ATCC) were grown in Ham’s F-12K medium supplemented with 10% FCS, penicillin, and streptomycin.

**Fluorescent Measurement of Intracellular Reactive Oxygen Species**

Cells were inoculated at a density of $6 \times 10^4$ cells per well in a 24-well plate overnight and were then incubated in DMEM/F12 with 0.1% fetal calf serum (FCS) for 24 h. Cells were loaded with 20 µM DCF-DA in phenol-free culture medium for 30 min at 37°C. The cells were then washed with culture medium and treated with either IBOP or ethanol in culture medium. The change in fluorescence was measured using a Synergy HT Microplate Fluorescence Reader from Bio-Tek Instruments (Winooski, VT) at excitation and emission wavelengths of 485 nm and 545 nm, respectively.

**Site-Directed Mutagenesis and Adenovirus Construction**

The AMPK-CA adenoviral vector was constructed from a rat cDNA encoding residues 1–312 of AMPK-α1 and bearing a mutation of Thr172 into aspartic acid (T172D); the AMPK-DN adenoviral vector was constructed from AMPK-α2 bearing a mutation altering lysine45 to arginine (K45R) as described previously. The wild-type (WT) and mutant (Ser428A/Ser307A/Asp194A) human LKB1 cDNAs in the mammalian expression vector pCI-neo
were established in our laboratory as previously described\(^3\). Briefly, the wild type LKB1 gene coding region was amplified by PCR, the PCR product was then ligated into a pGEM-T easy TA cloning vector, the LKB1 gene was released using EcoRI/NotI enzymes from the TA cloning vector, and was cloned into the pCI-neo mammalian expression vector (Promega; catalogue number E184). Ser428, Ser 307, or Asp194 of LKB1 was mutated into alanine by site-directed mutagenesis (Stratagene, Cat# 200518). All of the mutation vectors were confirmed by DNA sequencing.

For adenovirus construction, the LKB1 ORF for wild type (WT) point mutations were released from the plasmid DNA of the WT-LKB1 pCI-neo, S307A/pCI-neo, S428A/pCI-neo, and D194A/pCI-neo vectors, and then cloned into the EcoRI/NotI sites of transfer vector pCR259 in the Transpose-Ad adenoviral vector system (Q-Biogen, cat# AES3000; Canada). The resulting vectors, WT-LKB1/pCR259, S307A/pCR259, S428A/pCR259, and D194A/pCR259, were transformed to Transpose-Ad 294 E. coli cells for homolog-based recombination with the adenovirus genome. Positive clone selection, characterization, and large-scale adenovirus amplification were carried out according to the manufacturer’s instructions.

**siRNA-mediated knockdown of CaMKKβ**

Small interfering RNA (siRNA) duplex oligonucleotides used in this study are based on the human cDNAs encoding CaMKKβ. CaMKKβ siRNA and a nonsilencing control siRNA were obtained from Santa Cruz Biotechnology Inc (Santa Cruz, CA). Transfection was performed according to the manufacturer’s instructions.
**Adenoviral Infection**

Confluent VSMC were infected in medium with 0.1% FCS overnight. The cells were then washed and incubated in fresh DMEM/F12 medium with 0.1% FCS for an additional 24 h prior to experimentation. VSMC were infected with indicated adenoviruses, and an adenovirus expressing green fluorescence protein (GFP) was used as a control. All adenovirus titrations were measured using the BD Clontech Adeno-X Rapid Titer kit (Cat# K1653-1). Confluent VSMC were infected with a MOI of 100, which was required to achieve infection efficiency of over 80% as determined by GFP expression.

**Assay of Catalase Activity**

Catalase activity was determined using catalase assay kits (Cayman Chemical Co, Ann Arbor, MI) according to the manufacturer’s protocol.

**Assay of LKB1 activity**

Endogenous LKB1 was immunoprecipitated from 600 µg of VSMC lysate protein using 10 µl of LKB1 antibody, and LKB1 activity was measured using the LKBtide substrate assay, as described previously.

**Immunoprecipitation and Western Blotting**

Cell lysates (1 mg of total proteins) were incubated with specific antibodies overnight at 4°C with rotation. Immune complexes were then incubated with protein A-Sepharose beads for 3 h and washed three times with IP buffer (lysis buffer plus 1 M NaCl), and...
immunoprecipitates were boiled in 1x SDS sample buffer for 5 min. After separation by 8% SDS-PAGE, samples were analyzed using the Western blotting as previously described \(^3\). The density of each band was quantified using AlphaEaseFC Software (Alpha Innotech Co, San Leandro, CA).

**Measurement of Intracellular AMP, ADP, and ATP Levels**

After treatment with IBOP or U46619, VSMC were immediately covered with ice-cold 1% trichloroacetic acid and kept on ice for 5 min. The cells were then scraped and centrifuged, and the supernatants were neutralized by ether extraction. ATP, ADP, and AMP content was assayed by bioluminescent methods as described previously \(^5\).

**Protein Synthesis Measurement**

To measure synthesis of new protein, VSMC were cultured in 6-well plates and allowed to grow to confluence in DMEM/F12 containing 10% FCS (two parallel incubations with same passage and identical initial cell number were applied for \(^{3}\)H]leucine incorporation and cell number counting, respectively). VSMC were made quiescent by incubating in 0.1% FCS medium for 24 hours and then infected by Ad-AMPK CA, Ad-AMPK DN or Ad-GFP according to the method described above. The cells were then cultured in medium with 2.5% FCS and with or without IBOP (1 µM). L-[4,5-\(^{3}\)H]leucine (1 µCi/mL) was added to each well 5 hours before the end of the incubation period. Cells were washed twice with Dulbecco’s PBS and twice with ice-cold trichloroacetic acid (10%) and then incubated with 0.3N NaOH for 60 min at room temperature. Relative \(^{3}\)H]leucine incorporation was determined by liquid scintillation counting.
For cell counting, the cells were removed by trypsinization, and the number of viable cells was counted in a hemocytometer with the use of trypan blue staining.

**Statistical Analyses**

Values are expressed as mean ± SEM. Statistical comparisons were performed with Student’s t test or one-way ANOVA with Bonferroni post hoc analysis. To determine the effects of IBOP and AMPK on protein synthesis, two-way ANOVA followed by Bonferroni post hoc analyses was conducted. P<0.05 was considered statistically significant.
Supplemental Figure 1. TxA2 mimetics increase AMPK phosphorylation. (a).
Confluent VSMC were treated with the TxA₂ mimetic carbocyclic TxA2 (CTA2) at concentrations indicated for 10 min. Both phosphorylated AMPK-Thr172 and ACC-Ser79 were detected in western blots by using specific antibodies. (b). VSMC were treated with IBOP (0.1 µM, 10 min), U46619 (1 µM, 10 min), or AICAR (1mM, 1 h). Data shown as mean±SEM (n=3). ‡ p< 0.01, treated versus untreated control cells.

Supplemental Figure 2. (a) Activation of AMPK in VSMC by 5h IBOP treatment. Confluent VSMC were exposed to IBOP for 5 h and the phosphorylation of AMPK and ACC were detected in western blots. The blot is a representative of 3 blots from 3 independent experiments. (b) Expression of AMPK α in VSMC after 96 h of IBOP (1µM) treatment. (c). TPr antagonist S18886 abolishes TPr-induced AMPK activation. Confluent VSMC were preincubated with or without S18886 at 1 µM for 30 min before being exposed to IBOP (1 µM) or U46619 (1 µM) for 15min. The blot is a representative of three blots from three individual experiments.

Supplemental Figure 3. IBOP-induced AMPK activation is AMP/ATP independent.
Rat VSMC were treated with IBOP (1 µM, 10 min) or U46619 (1 µM, 10 min). Intracellular AMP, ADP, and ATP levels were assayed by a bioluminescent method as described in Materials and Methods. AMP, ADP, and ATP levels were expressed as µmol/mg protein. Values are expressed as mean±SEM.
**Supplemental Figure 4. LKB1 activity in VSMC.** VSMC were treated with IBOP (1 µM) or U46619 (1 µM) for 10 min. LKB1 activity was measured using the LKBtide substrate assay. Data shown as mean±SEM (n=3).

**Supplemental Figure 5.** Ovexpression of AMPK-DN or AMPK-CA in VSMC. VSMC were transfected with GFP, AMPK-DN or AMPK-CA for 48 h and then treated with IBOP (1 µM) for 5h. AMPK expression was detected in western blots. The blot is a representative of 3 blots from three individual experiments.

**References**


a. 

P-ACC
P-AMPK
AMPK

0 h 5 h
IBOP (1µM)

b. 

AMPK α
β-actin

0 h 96 h
IBOP (1µM)

c.

P-ACC
P-AMPK
AMPK

IBOP - + +
U46619 - + +
S18886 - - +

Zhang et al. Supplemental Figure 2
Zhang et al. Supplemental Figure 3

a. 

b. 

Zhang et al. Supplemental Figure 3
Zhang et al. Supplemental Figure 4
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