Inhibition of the AMP-Activated Protein Kinase-α2 Accentuates Agonist-Induced Vascular Smooth Muscle Contraction and High Blood Pressure in Mice

Shuangxi Wang, Bin Liang, Benoit Viollet, Ming-Hui Zou

Abstract—The aim of the present study was to determine the effects and molecular mechanisms by which AMP-activated protein kinase (AMPK) regulates smooth muscle contraction and blood pressure in mice. In cultured human vascular smooth muscle cells, we observed that activation of AMPK by 5-aminoimidazole-4-carboxamide 1-β-D-ribofuranoside inhibited agonist-induced phosphorylation of myosin light chain (MLC) and myosin phosphatase targeting subunit 1 (MYPT1). Conversely, AMPK inhibition with pharmacological or genetic means potentiated agonist-induced the phosphorylation of MLC and MYPT1, whereas it inhibited both Ras homolog gene family member A and Rho-associated kinase activity. In addition, AMPK activation or Rho-associated kinase inhibition with Y27632 abolished agonist-induced phosphorylation of MLC and MYPT1. Gene silencing of p190-guanosine triphosphatase-activating protein abolished the effects of AMPK activation on MLC, MYPT1, and Ras homolog gene family member A in human smooth muscle cells. Ex vivo analyses revealed that agonist-induced contractions of the mesenteric artery and aortas were stronger in both AMPKα1−/− and AMPKα2−/− knockout mice than in wild-type mice. Inhibition of Rho-associated kinase with Y27632 normalized agonist-induced contractions of AMPKα1−/− and AMPKα2−/− vessels. AMPKα2−/− mice had higher blood pressure along with decreased serine phosphorylation of p190-guanosine triphosphatase–activating protein. Finally, inhibition of the Ras homolog gene family member A/Rho-associated kinase pathway with Y27632, which suppressed MYPT1 and MLC phosphorylation, lowered blood pressure in AMPKα2−/− mice. In conclusion, AMPK decreases vascular smooth muscle cell contractility by inhibiting p190-GTP-activating protein–dependent Ras homolog gene family member A activation, indicating that AMPK may be a new therapeutic target in lowering high blood pressure.

Key Words: AMPK ■ Rho A ■ ROCK ■ p190-GAP ■ blood pressure

AMP-activated protein kinase (AMPK) is a key component of an energy-sensing/signaling system that allows cells to sense changes in their energy status.1,2 AMPK consists of 3 subunits, designated α, β, and γ. The α subunit contains the catalytic kinase domain, which transfers phosphate from ATP to the target protein. Phosphorylation of Thr172 on the α subunit by upstream signaling kinases activates AMPK,3 and AMP allosterically promotes phosphorylation of this residue.4 Activated AMPK phosphorylates a number of target proteins, resulting in increased glucose uptake, increased metabolism, and increased fatty acid oxidation. At the same time, AMPK activation inhibits hepatic lipogenesis, cholesterol synthesis, and glucose production.5 Because AMPK activation has beneficial metabolic consequences for patients with metabolic syndrome and diabetes mellitus, it is a new target for the treatment of obesity and type 2 diabetes mellitus.6

Recent studies found that AMPK-mediated cellular functions have protective effects that counteract many cardiovascular diseases.7–10 In addition, both 5-aminoimidazole-4-carboxamide 1-β-D-ribofuranoside [AICAR], an AMPK agonist and resveratrol (a botanical phytoalexin AMPK agonist) are reported to lower blood pressure (BP) in insulin-resistant and obese rats.11,12 However, these studies did not establish the direct effects of AMPK on BP, because AMPK activation is known to improve insulin resistance and metabolic anomalies in these models. Whether AMPK regulates vascular tone and/or BP remains unknown. Because vessel smooth muscle function is directly related to vascular tone,13 and abnormal vascular smooth muscle cell (VSMC) relaxation or contraction is known to induce hypertension in both humans and animal models,14 we hypothesized that activation of AMPK might lower BP by inhibiting vessel contraction. The aim of the present study was to elucidate the mechanisms by which AMPK activation attenuates agonist-induced contraction of smooth muscle and the physiological functions of AMPK in the regulation of BP in mice.

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A full description of materials, animals, and methods used, including cell culture, gene transfection of cells, transfection of small-interfering RNA (siRNA) into cells, Western blot analysis, rotokine pull-down assay for Ras homolog gene family, member A (RhoA) activation, Rho-associated kinase (ROCK) activity assay, measurement of vessel tension in mice, BP measurement, and statistical analysis can be found in the online Data Supplement. Please see http://hyper.ahajournals.org.

Methods and Materials

AICAR Inhibits Agonist-Induced Myosin Light Chain Phosphorylation in a Time- and Dose-Dependent Manner

Vessel tone is determined by the status of myosin light chain (MLC) (phosphorylation or dephosphorylation at ser19) in VSMCs. We first determined whether AMPK activation could affect phenylephrine (PE)-induced phosphorylation of MLC. To test this, we treated human smooth muscle cell (HSMC) with agonists and measured MLC phosphorylation by immunoblot analysis. Treatment of HSMC with PE (1 μmol/L, 30 minutes) significantly increased the phosphorylation of MLC (Figure 1A and 1B) and AMPK phosphorylation at Thr172 (Figure S1A and S1B, available in the online Data Supplement). As expected, pretreatment with AICAR (2 mM) increased PE-induced AMPK-Thr172 phosphorylation (Figure 1C). However, AMPK pretreatment abolished PE-induced MLC phosphorylation (Figure 1A and 1B).

Next, to investigate whether AICAR pretreatment altered MLC phosphorylation caused by other agonists, we assayed the effects of AICAR on PE-unrelated agonist–induced MLC phosphorylation in HSMCs. As depicted in Figure 1C, 9,11-dideoxy-11-,9-epoxy-methano-prostaglandin F2 (U46619) or [1S-(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 (IBOP; 1 nmol/L) for 30 minutes. n=3, *P<0.05 vs PE plus WT.

Figure 1. Activation of AMP-activated protein kinase (AMPK) inhibits agonist-induced myosin light chain (MLC) phosphorylation in vascular smooth muscle cells (VSMCs). A and B, Western blot analysis of Thr172-phosphorylated AMPK and Ser19-phosphorylated MLC in phenylephrine (PE)-stimulated human smooth muscle cell (HSMC) pretreated with or without 5-aminomimidazole-4-carboxamide 1-b-d-ribofuranoside (AICAR). Cells were pretreated with varying (A) concentrations or (B) times of AICAR and then stimulated with PE (1 μmol/L) for 30 minutes. n=3, *P<0.05 vs control, #P<0.05 vs PE alone. C, Western blot analysis of phosphorylated MLC in cells incubated with AICAR (2 mmol/L) for 1 hour and then challenged with 9,11-dideoxy-11-,9-epoxy-methano-prostaglandin F2 (U46619; 30 nmol/L) or [1S-(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 (IBOP; 1 nmol/L) for 30 minutes. n=3, *P<0.05 vs basal, #P<0.05 vs PE plus control siRNA. D-F, MLC phosphorylation in primary mouse VSMCs from wild-type (WT), AMPKα1/–, and AMPKα2/– mice aortas treated with PE (1 μmol/L) for 30 minutes. n=3, *P<0.05 vs WT alone, #P<0.05 vs PE plus WT.
Importantly, AICAR significantly lowered U46619- or IBOP-enhanced MLC phosphorylation.

PE-Induced MLC Phosphorylation Is Dependent on AMPK
To exclude potential off-target effects of AICAR, we next investigated whether genetic inhibition of AMPK by adenovirus-mediated expression of dominant-negative AMPK (AMPK-DN) affected PE-induced MLC activation. As expected, PE-induced MLC phosphorylation in green fluorescent protein–expressing control HSMCs (Figure 1D). In AMPK-DN–expressing HSMCs, PE-induced MLC phosphorylation was greater than those in green fluorescent protein–expressing cells; conversely, MLC phosphorylation was absent in AMPK-constitutively active (CA)-expressing HSMCs (Figure 1D). Taken together, these data suggest that AMPK negatively regulates PE-induced MLC activation.

To investigate the roles of endogenous AMPKα in MLC phosphorylation, HSMCs that had been transfected with AMPKα-specific siRNA or primary MSMSCs isolated from AMPKα1−/− or AMPKα2−/− mice were exposed to PE. Western blot analysis revealed that either AMPKα1- or α2-targeted siRNA reduced the levels of AMPKα1 or α2, respectively (Figure S2A). As shown in Figure 1E, PE-induced MLC phosphorylation was greater in cells receiving AMPKα1 or AMPKα2-targeted siRNA than in wild-type (WT) or nontargeted control siRNA-transfected cells. Consistently, PE-induced MLC phosphorylation was significantly higher in AMPKα1- or α2-null cells compared with those in WT cells (Figure 1F). These experiments further support that AMPK negatively regulates PE-induced MLC activation in HSMCs.

AMPK Regulates Thr696 Phosphorylation of Myosin Phosphatase Targeting Subunit 1, Which Lies Upstream of MLC
MLC phosphatase suppresses smooth muscle contractions by dephosphorylating its substrate, MLC, which is bound to myosin heavy chain. Myosin phosphatase targeting subunit 1 (MYPT1), as a subunit of MLC phosphatase complex, through the MYPT1 NH2 terminus, reduces the MLC phosphatase complex catalytic activity for myosin II. Western blot analysis revealed that PE-induced MYPT1 phosphorylation at Thr696 was significantly greater in HSMCs treated with compound C or expressing AMPK-DN than in control cells (Figure 2A and 2B). Conversely, either AICAR treatment or AMPK-CAXA expression attenuated PE-induced MYPT1 phosphorylation. The effects of AMPK inhibition on PE-induced MYPT1 phosphorylation were further confirmed by siRNA-mediated AMPKα knockdown and gene-specific knockout mice. Knockdown of AMPKα1 or α2 by siRNA significantly increased PE-induced MYPT1 phosphorylation at Thr696 (Figure 2C). Similarly, MYPT1 phosphorylation at Thr696 was significantly higher in VSMCs isolated from AMPKα1 or α2 when compared with those in WT cells (Figure 2D). Taken together, these data indicate that AMPK negatively regulates Thr696 phosphorylation of MYPT1, an upstream regulator of MLC.

ROCK Participates in the Regulation of MYPT1 by AMPK
The ROCK-dependent signal pathway is essential in mediating agonists-induced smooth muscle vessel contrac-
AMPK Negatively Regulates PE-Induced RhoA Activation

Smooth muscle MYPT1 is a G protein–regulated enzyme. RhoA belongs to the Ras low-molecular-weight G protein superfamily. RhoA binds GTP to activate ROCK, and this in turn leads to MYPT1 phosphorylation, MLC activation, and smooth muscle cell contraction. As shown in Figure 3D and 3E, both PE and U46619 activated RhoA by increasing the amount of GTP-bound RhoA. AICAR inhibited RhoA activation, but compound C increased its activation. These results were confirmed by transduction of cells with AMPK-CA or AMPK-DN and measurement of the RhoA activation status. PE-induced RhoA activation was lower in AMPK-CA–expressing cells that overexpressed AMPK-CA than in control cells, suggesting that AMPK activation is RhoA dependent.
Activation of AMPK Promotes the Binding of p190-GTP–Activating Protein to RhoA in Agonist-Stimulated VSMCs

RhoA cycles between an active GTP-bound state and an inactive GDP-bound state through nucleotide exchange and intrinsic guanosine triphosphatase activity. The RhoA activation status is controlled by guanine nucleotide exchange factors and p190-GTP-activating proteins (GAPs). We tested whether AMPK inactivates RhoA by promoting the association of RhoA with guanosine triphosphatase activity. The RhoA activation status is measured by rhotekin pull-down assays to measure RhoA activation (Figure 4A). Overexpression of AMPK-CA mimicked the effect of AICAR on p190-GAP/RhoA binding in PE-treated HSMCs (Figure 4B). Similarly, the effect of compound C was mimicked by overexpression of AMPK-DN or AMPKα knockdown using AMPKα-specific siRNA (Figure 4B and 4C). Taken together, these data suggest that activation of AMPK promotes the binding of p190-GAP to RhoA in agonist-stimulated HSMCs.

p190-GAP Silencing Abolishes AICAR Suppression of RhoA Activation, MYPT1 Phosphorylation, and MLC Phosphorylation in VSMCs

To establish whether p190-GAP mediated the effects of AMPKα on RhoA inactivation, VSMCs were transfected with p190-GAP–targeted siRNA or nontargeted control siRNA. Silencing of p190-GAP expression was confirmed by Western blot analysis (Figure S2B). As expected, in PE-stimulated HSMC cells that were transfected with nontargeted control siRNA, AICAR treatment significantly reduced the levels of active RhoA (Figure 4D), phosphorylated MYPT1 (Figure 4E), and phosphorylated MLC (Figure 4F). In contrast, gene silencing of p190-GAP abolished the effects of AICAR, implying that p190-GAP is required for AMPK-mediated RhoA inhibition.

Inhibition of AMPK Potentiates Agonist-Induced Contraction of Mesenteric and Aortic Vessels in Mice

Because peripheral resistance is determined by the contractile status of VSMCs in small arterioles, we then determined...
vascular smooth muscle function in resistance vessels from WT, AMPKα1−/−, and AMPKα2−/− mice. Both PE and U46619 were used to induce contraction of mouse mesenteric artery ring explants in an organ bath (Figure S3A and S3B). Both PE and U46619 significantly increased mesenteric artery contraction force than explants from WT mice, which is in line with an earlier report.21

ROCK Dependent

Next, we tested whether ROCK mediates the effect of AMPK on vessel contraction. Aortas and mesenteric arteries from AMPKα1−/− and AMPKα2−/− mice were pretreated with Y27632 (2 μmol/L) for 30 minutes, and this significantly inhibited agonist-induced increases in contraction (Figure S3A and S3B and Figure S4C). These data show that inhibition of AMPK potentiates agonist-induced contraction of murine conduit and resistance vessels, likely through regulation of ROCK.

Deletion of AMPK Suppresses p190-GAP Serine Phosphorylation and Binding to Rhoga In Vivo

p190-GAP activity is regulated in a serine or tyrosine phosphorylation-dependent manner.22 We hypothesized that AMPK, which is a serine/threonine kinase, directly phosphorylates p190-GAP. Because no site-specific phosphoserine p190-GAP antibodies are available, we relied on immunoprecipitation of total p190-GAP and then Western blot analysis using a general phosphoserine antibody (Figure 5). Immunoprecipitation isolated p190-GAP in cells from WT mice was detected by the phosphoserine antibody; however, deletion of either AMPKα1 or α2 dramatically suppressed the serine phosphorylation of p190-GAP (Figure 5A), suggesting that AMPKα deletion may suppress p190-GAP activity by preventing its phosphorylation. In addition, deletion of AMPKα1 or α2 also inhibited the binding of RhoA by p190-GAP (Figure 5B), and this, in turn, increased RhoA activation (Figure 5C).

Y27632-Inhibitable Hypertension in AMPKα2−/− Mice

We reported previously that AMPKα1−/− mice exhibit splenomegaly and moderate anemia23 and were, therefore, not suitable for BP studies. Thus, we determined the systolic BP, diastolic BP, and mean BP in WT and AMPKα2−/− mice. As depicted in Figure 6A, all of the BP measurements (systolic BP, diastolic BP, and mean BP) in AMPKα2−/− mice were higher than those in WT mice under basal conditions. Importantly, Y27632 (10 mg/kg, 4 hours, given IP) significantly reduced the BP values (Figure 6A, systolic BP, diastolic BP, and mean BP), ROCK activity (Figure 6B), MYPT1 phosphorylation (Figure 6C), and MLC phosphorylation (Figure 6D) in AMPKα2−/− mice. Collectively, these data indicate that upregulation of the p190-GAP–dependent RhoA/ROCK/MYPT1/MLC pathway contributes to hypertension in AMPKα2−/− mice.

Discussion

The major finding of this study is that AMPK activation lowers BP by suppressing VSMC contractility via the inhibition of MYPT1/MLC phosphorylation. Mechanistically, we found that AMPK decreases VSMC contractility by inhibiting p190-GAP-dependent RhoA activation.

One of the most important findings of this study is that AMPK activation suppresses VSMC contractility by inhibiting MYPT1/MLC phosphorylation. This appears to occur indirectly through MYPT1-Thr396 dephosphorylation, which, in turn, inhibits MLC phosphatase activity. This conclusion is supported by several findings. First, deletion of AMPKα1 or α2 enhanced agonist-induced contraction in explanted murine mesenteric arteries, and inhibition of AMPK with a pharmacological inhibitor (compound C) reproduced this effect.
Second, activation of AMPK by AICAR attenuated agonist-induced contraction. Third, systemic BP was higher in AMPKα2−/− mice than in WT mice under normal physiological conditions. Although AMPKα1−/− mice were excluded from this study because knockout of this gene results in splenomegaly and moderate anemia,23,24 we expect that the hypertensive phenotype observed in the AMPKα2−/− mice would also be observed in AMPKα1−/− mice. Finally, AMPK-CA or AICAR treatment abolished PE-induced phosphorylation of MYPT1 at Thr696 and MLC at Ser19. Conversely, AMPK-DN or compound C treatment potentiated PE-induced activation of MYPT1 and MLC. Taken together, our results support a direct inhibitory effect of AMPK on VSMC contractility.

RhoA/ROCK signaling plays an important role in cell proliferation, cell cycle progression, and cell survival after vascular injury.25 Because AMPK activation promotes the binding of p190-GAP to Rhoa and in this way blocks Rhoa activity, AMPK might also be important for other physiological processes, such as angiogenesis or tube formation after vascular damage, in which RhoA/ROCK signaling is involved.26 This finding is consistent with a recent study demonstrating that the AMPK activator, AICAR, inhibits ROCK activity in osteoblastic cells in a dose-dependent manner.27 The regulation of the RhoA/ROCK pathway by AMPK appears to be cell type dependent. It has been shown recently that AMPK activation enhances MLC phosphorylation through the RhoA/ROCK signaling pathway in epithelial cells.28 Taken together, these studies support a novel mechanism of AMPK action whereby activation of AMPK suppresses the PE-induced signaling pathway by reducing RhoA/ROCK activity via p190-GAP. This, in turn, inhibits MYPT1/MLC phosphorylation in smooth muscle cells, leading to decreased vessel contraction (Figure S5). In combination, these effects contribute to the regulation of BP in mice.

**Perspectives**

Activation of the RhoA/ROCK cell signaling pathway is known to play an essential role in hypertension.29 Thus, inhibition of RhoA/ROCK by AMPK might be an important mechanism for lowering BP. In addition to its suppression of VSMC contractility, AMPK can also lower BP by improving NO bioactivity and endothelial cell function. AMPK might increase NO release by increasing phosphorylation and activation of endothelial NO synthase at Ser1177 and Ser633 or by decreasing NO inactivation by reactive oxygen species. Indeed, there is a growing body of evidence that supports the notion that AMPK suppresses reactive oxygen species from mitochondria and/or NADPH oxidase.9,30 Interestingly, depressed AMPK activation, or AMPK deregulation, has been observed in arteries of rodent models where vascular dysfunction exists, including in streptozotocin-induced diabetes, Zucker diabetic fatty rats, aged rats, and Otsuka Long Evans Tokushima Fatty rats.31 In addition, adiponectin-AMPK signaling becomes depressed in insulin-sensitive tissues of hypertensive rats.32 Consistently, AMPK activation by AICAR or resveratrol lowered BP in insulin-resistant and obese rats.11,12 Importantly, metformin, one of the mostly widely used antidiabetic drugs that is reported to activate AMPK in vivo, is reported to lower BPs in various models of hypertensive animals and in patients with or without diabetes mellitus. Interestingly, recent studies report that telmisartan and losartan, selective angiotensin II receptor blockers, are reported to inhibit VSMC proliferation53 and glucose uptake by activating AMPK.34 Taken together, AMPK inhibition might contribute to the initiation and progression of hypertension, and AMPK is a novel therapeutic target for effective treatments of hypertension, especially in metabolic syndromes such as obesity and diabetes mellitus.

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Disclosures
None.

References
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Supplementary Materials and Methods

Inhibition of the AMP-activated Protein Kinase Alpha 2 Accentuates Agonist-Induced Vascular Smooth Muscle Contraction and High Blood Pressure in Mice

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Materials and Methods

**Animals.** Male wild-type (WT, C57BL6) mice (12–16 weeks of age; 20–25 g body weight) were obtained from The Jackson Laboratory (Bar Harbor, ME), and both AMPKα1⁻/⁻ and AMPKα2⁻/⁻ mice were presented by Benoit Viollet kindly. Mice were housed in temperature-controlled cages under a 12-h light-dark cycle and given free access to water and food. The animal protocol was reviewed and approved by the University of Oklahoma Institute Animal Care and Use Committee.

**Materials.** Polyclonal or monoclonal antibodies against AMPK (α, α1, and α2), pAMPK (Thr¹⁷²), MLC, pMLC (Ser¹⁹), MYPT1, pMYPT1 (Thr⁶⁹⁶), RhoA, ROCK, anti-serine phosphorylation, and β-actin were obtained from Cell Signaling Technology or Santa Cruz Biotechnology. Secondary antibodies were from Cell Signaling Technology. Phenylephrine (PE), 9,11-dideoxy-11-9-epoxymethanoprostaglandin F2 (U46619), and [1S-(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 (IBOP) were obtained from Cayman Chemical. All drug concentrations are expressed as final concentrations in the buffer. Human smooth muscle cells (HSMCs) were obtained from Clonetics, Inc. (Walkersville, MD).

**Cell culture.** HSMCs were grown in M231 medium (Cascade Biologics, Portland, USA) supplemented with 10% FBS, penicillin (100 u/ml), and streptomycin (100 µg/ml). All cells were incubated at 37°C in a humidified atmosphere of 5% CO₂ and 95% air. Cells were grown to 70-80% confluence before being treated with different agents. The cells used in all experiments were between passages 3 and 8.

Primary murine aortic smooth muscle cells (MSMCs) were isolated from aortas of WT, AMPKα1⁻/⁻, or AMPKα2⁻/⁻ mice. Aortas were washed twice with PBS at 4°C, carefully freed from all fat and connective tissue, and cut into 3-mm-long sections. The endothelium was removed by rubbing. These sections were then incubated in a 0.2% collagenase solution at 37°C with frequently shaking to detach smooth muscle cells from the aorta. MSMCs were pelleted from solution through centrifugation at 1,000 rpm for 15 min at 4°C,
washed with PBS, and seeded onto culture plates containing M231. The purity of MSMC cultures was confirmed through positive staining for actin. Experiments were performed with MSMCs at passages 3 – 5.

**Gene transfection of cells.** Ad-GFP, a replication-defective adenoviral vector expressing green fluorescence protein (GFP), served as control. An adenoviral vector expressing a dominant-negative mutant of AMPK (AMPK-DN) was constructed from AMPK bearing a Lys\(^{45}\)-to-Arg mutation (K45R). To generate an adenoviral vector expressing a constitutively active mutant of AMPK (AMPK-CA), we subcloned a rat cDNA encoding residues 1 – 312 of AMPK and bearing a Thr\(^{172}\)-to-Asp mutation (T172D) into a shuttle vector (pShuttle CMV [cytomegalovirus]). HSMCs were infected with GFP, AMPK-DN, or AMPK-CA in medium with 2% FCS overnight. The cells were then washed and incubated in fresh endothelium growth medium without FCS for an additional 12 h before experimentation. Using these conditions, infection efficiency was typically >80%, as determined by GFP expression.

**Transfection of siRNA into cells.** Transient transfection of siRNA was carried out according to Santa Cruz’s protocol. Briefly, the siRNAs were dissolved in siRNA buffer (20 mM KCl; 6 mM HEPES, pH 7.5; 0.2 mM MgCl\(_2\)) to prepare a 10 µM stock solution. Cells grown in 6-well plates were transfected with siRNA in transfection medium containing liposomal transfection reagent (Lipofectamine 2000, Invitrogen). For each transfection, 100 µl transfection medium containing 4 µl siRNA stock solution was gently mixed with 100 µl transfection medium containing 4 µl transfection reagent. After a 30-min incubation at room temperature, siRNA-lipid complexes were added to the cells in 1.0 ml transfection medium, and cells were incubated with this mixture for 6 h at 37°C. The transfection medium was then replaced with normal medium, and cells were cultured for 48 h.

**Western blot analysis.** Aortic tissues were homogenized on ice in cell lysis buffer (20 mM Tris-HCl, pH 7.5; 150 mM NaCl; 1 mM Na\(_2\)EDTA; 1 mM EGTA; 1% Triton; 2.5 mM sodium pyrophosphate; 1 mM -glycerophosphate; 1 mM Na\(_3\)VO\(_4\); 1 µg/ml leupeptin) and 1 mM PMSF. The protein content of the resulting lysate was determined using the BCA protein assay reagent (Pierce, USA). Twenty micrograms of protein was separated by SDS-PAGE and then transferred to a membrane. The membrane was incubated with a 1:1,000 dilution of primary antibody and a 1:2,000 dilution of horseradish peroxidase-conjugated secondary antibody. Protein bands were visualized by ECL (GE Healthcare). The intensity (area × density) of the individual bands on western blots was measured by densitometry (model
GS-700, Imaging Densitometer; Bio-Rad). The background was subtracted from the calculated area. The control was set to 100%.

**Rhotekin pull-down assay for RhoA activation.** Vascular smooth muscle cells (VSMCs) were plated in 10-cm culture dishes containing 0.5% serum medium. After treatment, cells were rapidly lysed on ice and processed for quantification of GTP-bound RhoA. Assays were performed according to the manufacturer's instructions (Cell Biolabs, Inc.).

**ROCK activity assay.** Cell lysates were assayed for ROCK activity using a ROCK assay kit (Cell Biolabs, Inc.) according to the manufacturer's instructions. Briefly, each kinase assay contained 50 μl of 1×Kinase buffer/ATP/Substrate Solution and ROCK immunoprecipitates. The reaction was allowed to proceed for 30 – 60 min at 30°C with gentle agitation. The reaction products were then analyzed by western blot to detect Thr⁶³⁹-phosphorylated MYPT1, which served as an index of ROCK activity.

**Measurement of vessel tension in mice.** Mice were anesthetized with diethyl ether and killed by decapitation. Aortas or mesenteric arteries were rapidly removed, immersed in Krebs bicarbonate buffer (118 mM NaCl, 4.7 mM KCl, 25 mM NaHCO₃, 1.2 mM KH₂PO₄, 1.2 mM MgSO₄, 2.5 mM CaCl₂, and 5 mM glucose), gassed with a mixture of 95% O₂ and 5% CO₂, and carefully cleaned of all fat and connective tissue. The endothelium was gently removed using a cotton stick. Artery rings were mounted between two hooks in a 5-ml, 37°C organ bath perfused with Krebs buffer. After undergoing an equilibration period, rings were contracted with 60 mM high-potassium salt solution. The rings were then washed, subjected to an additional equilibration period (30 min), and contracted with PE (1 μM) or U46619 (30 nM).

**Blood pressure measurement.** Blood pressure was determined using the left carotid catheter method. Mice were anesthetized with a mixture of ketamine and xylazine (70:6 mg/kg, intramuscular injection) and placed under warm light (37°C). A catheter was inserted into the left common carotid artery with the aid of a dissecting microscope to measure arterial blood pressure. For catheter insertion, the left common carotid artery was carefully exposed via a 0.5- to 1.0-cm midline incision in the ventral neck region. The tip of the artery toward the head was ligated with a suture (5–0 silk), and the tip toward heart was occluded with a microclip (no. 18055–03; Fine Science Tool, Foster City, CA). A small cut was then made in the vessel wall using microscissors (no. 15000–08, Fine Science Tool). A 60-cm catheter (PE10 tubing, A-M Systems) containing a sterile 10% heparin-90% saline
solution was inserted into the artery a distance of 0.65 cm toward the thorax. The arterial clip was removed, and the catheter was tied in place. Blood was directed to a pressure transducer through the catheter to obtain computerized blood pressure measurements (AD instruments). The mice were allowed to recover, and the systolic and diastolic blood pressures were monitored for at least 30 min in conscious states.

**Statistical analysis.** Data are reported as mean ± S.E. All data were analyzed with the use of a one- or two-way ANOVA followed by multiple t-tests. *P*<0.05 was considered significant.
Supplementary Figures

Figure S1. Effect of agonist and AICAR on AMPK phosphorylation in VSMCs. Western blot analysis of Thr^{172}-phosphorylated AMPK in PE-stimulated human VSMCs (1 μM PE, 30 min) that had been pretreated with (A) varying concentrations of AICAR for (B) varying times. n=3.
Figure S2. Effect of adenovirus or siRNA transfection on the targeted gene expression in VSMCs. (A) AMPK levels in human VSMCs transfected with control siRNA, AMPKα1 siRNA, or AMPKα2 siRNA for 48 h and then incubated with PE (1 μM) for 30 min. n=3. (B) p190-GAP protein expression in cells transfected with control siRNA and p190-GAP siRNA for 48 h and then incubated with PE (1 μM) in presence of AICAR (2 mM) for 30 min. n=3.
Figure S3. AMPK acts through ROCK to regulate agonist-induced mesenteric vessel contraction in mice. (A) PE- (1 μM) or (B) U46619- (30 nM) induced contraction of mesenteric artery rings isolated from WT, AMPKα1−/−, and AMPKα2−/− mice in the presence or absence of Y27632 (2 μM, 1 h). Contraction was recorded through software (chart 5 for windows, AD Instruments) via a transducer connected to the computer. n=6, *P<0.05 vs. WT, #P<0.05 vs. control AMPKα1−/− or AMPKα2−/−.
Figure S4. Loss of AMPK promotes agonist-induced contraction of murine aortas. (A) Contraction of aortic rings from WT, AMPKα<sup>1</sup>−/−, or AMPKα<sup>2</sup>−/− mice. Contraction was induced by U46619 (30 nM) or PE (1 μM). Contractions were recorded through software (chart 5 for windows, ADInstruments) via a transducer connected to the computer. The trace is representative of six independent experiments. (B) Contraction of a WT aortic ring pretreated with AICAR (2 mM) or compound C (20 μM) for 2 h and then challenged with PE (1 μM) for 30 min. The trace is a representative of six independent experiments. (C) Contraction of aortas (WT, AMPKα<sup>1</sup>−/−, and AMPKα<sup>2</sup>−/−) pre-incubated with Y27632 (2 μM) for 1 h and then exposed to PE. The trace is representative of 6 independent experiments. *P<0.05 vs. WT.
Figure S5. Proposed mechanism by which AMPK regulates vessel contraction and BP via RhoA. Under physiological conditions, vasoconstrictor via its receptor promotes the activation of RhoA, resulting in the activation of the ROCK/MYPT1/MLC pathway and causing vasoconstriction. AMPK activation with AICAR or metformin may phosphorylate serine of p190-GAP and act through association of p190-GAP/RhoA to suppress the shift of RhoA-GDP to RhoA-GTP, inactivate the RhoA/ROCK/MYPT1 pathway. This, in turn, results in increased activity of MLCP, dephosphorylation of MLC, and inhibition of agonist-induced contraction of smooth muscle. In AMPK-deficient mice, the absence of AMPK suppression of vessel contraction produces hypertension.