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 (Thr^{172}), MLC, pMLC (Ser^{19}), MYPT1, pMYPT1 (Thr^{696}), 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 [1*S*-(1α,2β(5*Z*), 3α(1*E*,3*R*),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% CO_2 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_2EDTA; 1 mM EGTA; 1% Triton; 2.5 mM sodium pyrophosphate; 1 mM □-glycerophosphate; 1 mM Na_3VO_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 Thr^{696}-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 $NaHCO_3$, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 2.5 mM $CaCl_2$, and 5 mM glucose), gassed with a mixture of 95% O_2 and 5% CO_2, 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

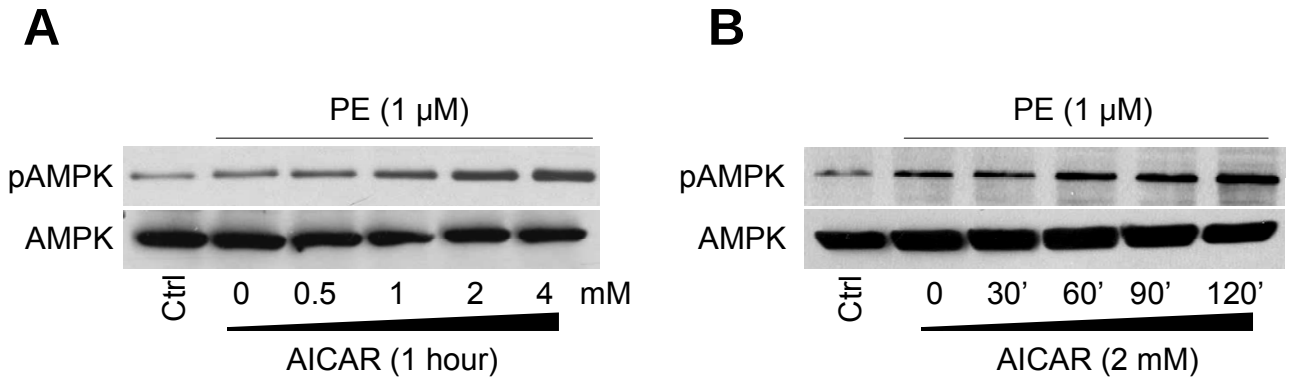

Figure S1. Effect of agonist and AICAR on AMPK phosphorylation in VSMCs. Western blot analysis of Thr^{172}-phosphorylated AMPK in PE-stimulated human VSMCs (1 μM PE, 30 min) that had been pretreated with (**A**) varying concentrations of AICAR for (**B**) varying times. n=3.

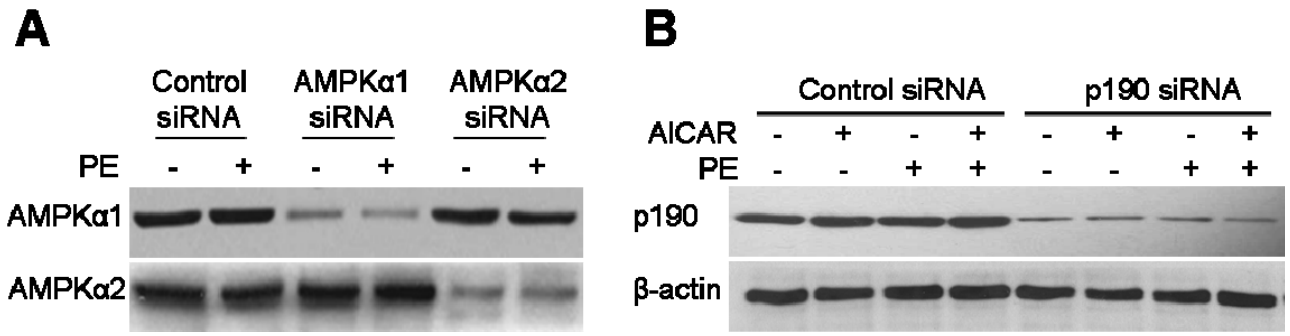

Figure S2. Effect of adenovirus or siRNA transfection on the targeted gene expression in VSMCs. (**A**) AMPK levels in human VSMCs transfected with control siRNA, AMPKα1 siRNA, or AMPKα2 siRNA for 48 h and then incubated with PE (1 μM) for 30 min. n=3. (**B**) p190-GAP protein expression in cells transfected 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.

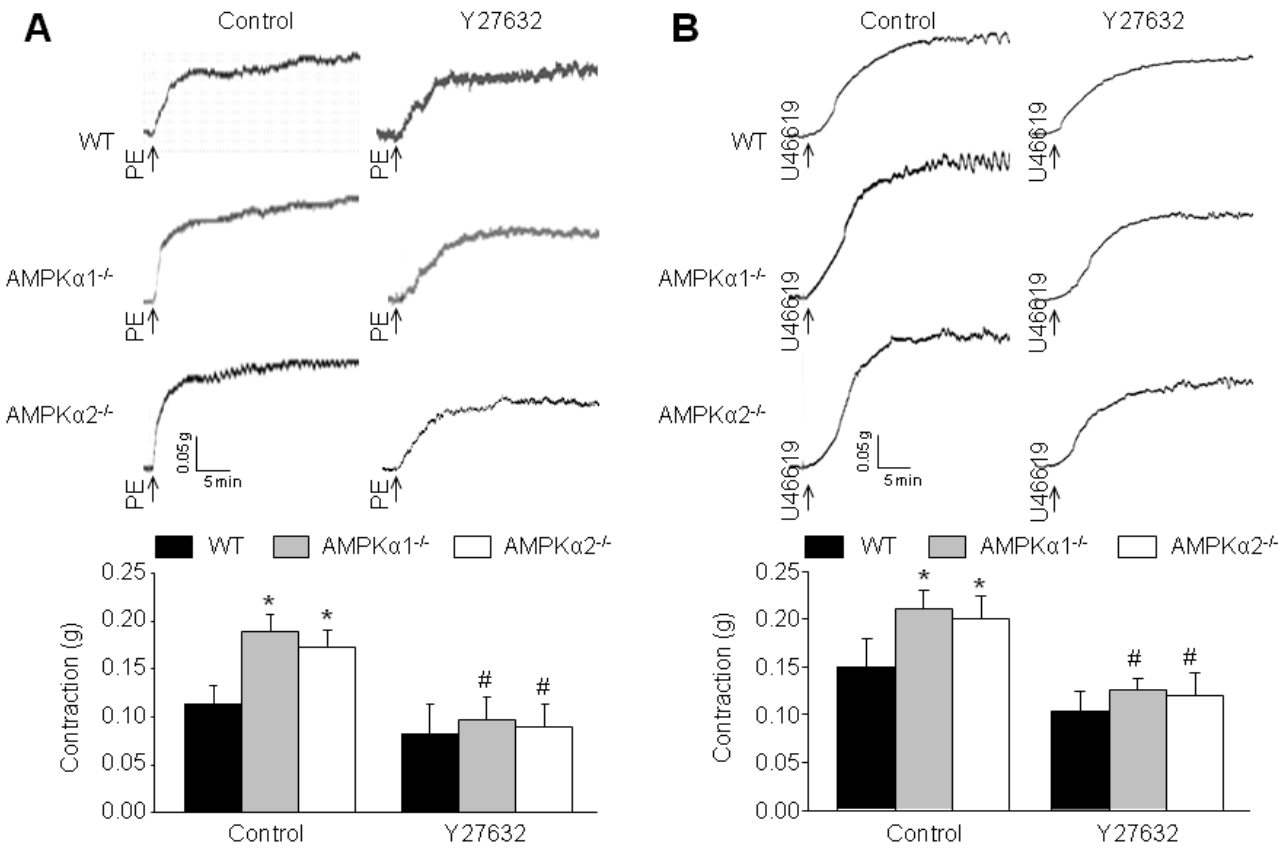

Figure S3. AMPK acts through ROCK to regulate agonist-induced mesenteric vessel contraction in mice. (**A**) PE- (1 μM) or (**B**) U46619- (30 nM) induced contraction of mesenteric artery rings isolated from WT, AMPKα1$^{-/-}$, and AMPKα2$^{-/-}$ mice in the presence or absence of Y27632 (2 μM, 1 h). 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$^{-/-}$.

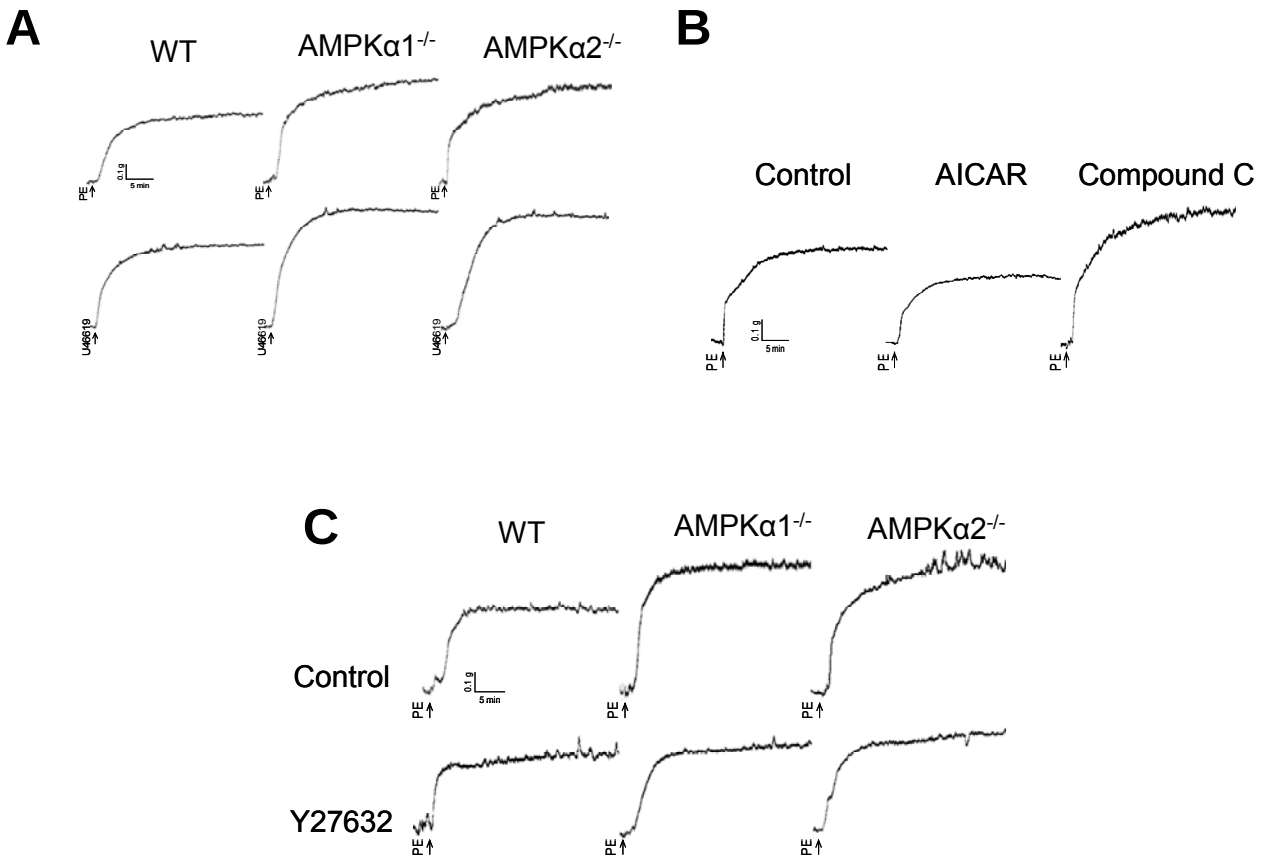

Figure S4. Loss of AMPK promotes agonist-induced contraction of murine aortas. (**A**) Contraction of aortic rings from WT, AMPKα1$^{-/-}$, or AMPKα2$^{-/-}$ mice. Contraction was induced by U46619 (30 nM) or PE (1 μM). Contractions were recorded through software (chart 5 for windows, ADInstruments) 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, AMPKα1$^{-/-}$, and AMPKα2$^{-/-}$) 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.

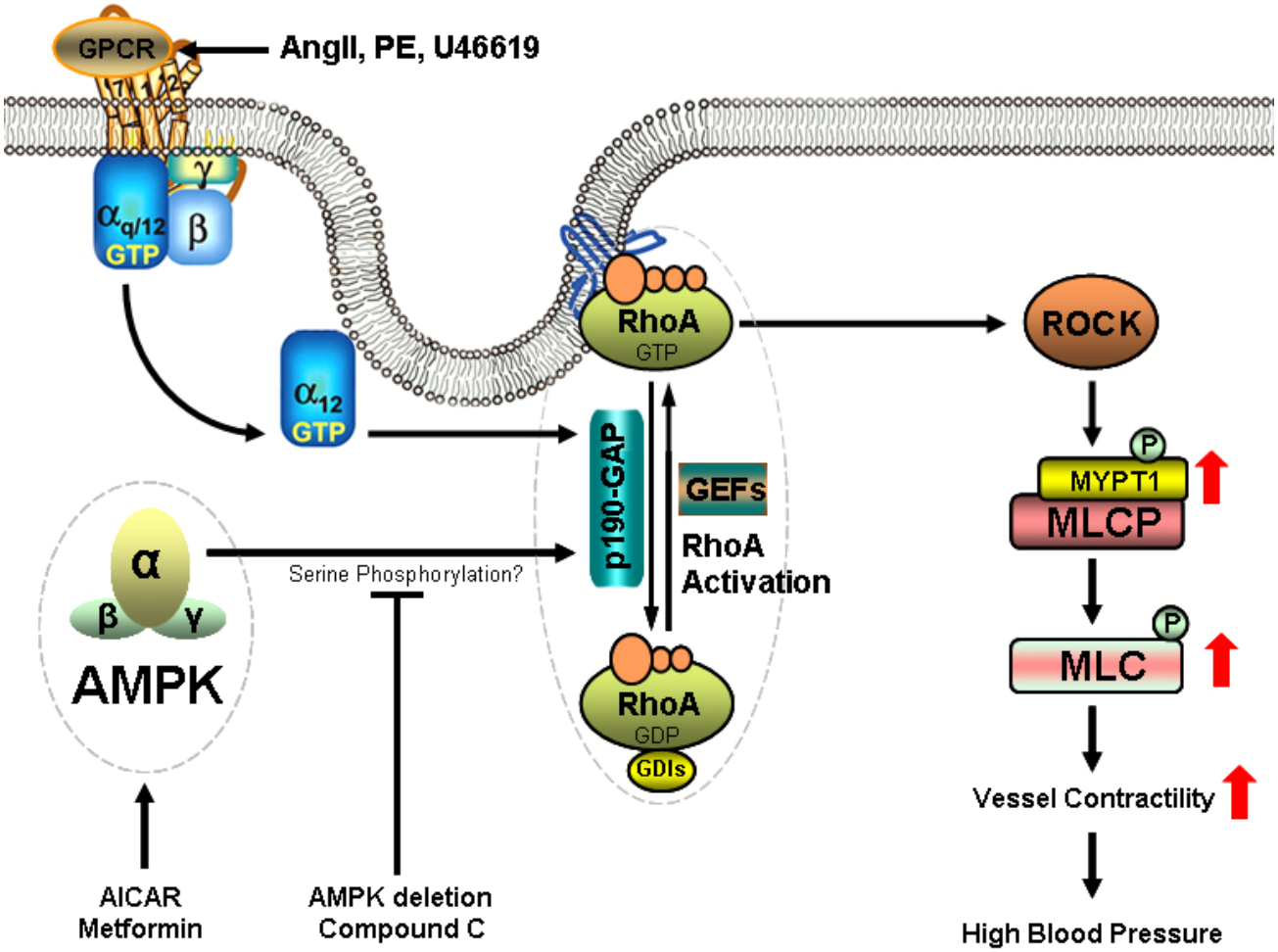

Figure S5. Proposed mechanism by which AMPK regulates vessel contraction and BP via RhoA. Under physiological conditions, vasoconstrictor via its receptor promotes the activation of RhoA, resulting in the activation of the ROCK/MYPT1/MLC pathway and causing vasoconstriction. AMPK activation with AICAR 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.