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ORIGINAL ARTICLE

Resveratrol prevents AngII-induced hypertension via AMPK activation and RhoA/ROCK suppression in mice

Xia Cao, Tao Luo, Xi Luo and Zhiyi Tang

The purpose of this study was to determine the effects of resveratrol (RSV) and the molecular mechanisms by which it regulates vascular smooth muscle contraction and blood pressure in mice. In cultured human vascular smooth muscle cells (VSMCs), we found that the activation of AMP-activated protein kinase (AMPK) by RSV inhibited angiotensin II (AngII)-induced phosphorylation of myosin phosphatase-targeting subunit 1 (MYPT1) and myosin light chain (MLC). Inversely, AMPK inhibition with RNA interference and compound C, an AMPK inhibitor, abolished the inhibitory effect of RSV on AngII-induced MYPT1 and MLC phosphorylation. Thiazovivin, a Rho-associated kinase (ROCK) inhibitor, reversed AngII-induced MYPT1 and MLC phosphorylation, suggesting that ROCK functions as an upstream kinase for MYPT1/MLC. RSV reversed AngII-induced Ras homolog gene family member A (RhoA) and ROCK activity, whereas AMPK inhibition via pharmacological or genetic means abolished this effect. In addition, gene silencing of p190-guanosine triphosphatase-activating protein blocked the effects of RSV-induced AMPK activation on MLC, MYPT1 and RhoA in VSMCs. *Ex vivo* analyses demonstrated that AngII-induced aorta contractions were dramatically inhibited by RSV, and this effect was abolished by AMPK inhibition. Finally, daily chronic administration of RSVl alleviated hypertension in the experimental model of AngII-induced hypertensive mice, and these effects were accompanied by the activation of AMPK, significantly decreased RhoA activity and phosphorylation levels of MYPT1 and MLC in AngII-treated murine aortic VSMCs. More importantly, administration of compound C significantly abolished the effects of RSV. In conclusion, AMPK suppression of the p190-GAP-dependent RhoA/ROCK/MYPT1/MLC pathway contributes to the hypotensive effect of RSV in AngII-treated mice.

INTRODUCTION

Hypertension, or high blood pressure (BP), is a chronic medical condition in which the BP in the arteries is increased.[1] The World Health Organization estimates that ∼25% of adults worldwide suffer from hypertension.[2] Despite the continual developments in the treatment of hypertension, ∼20–30% of patients with hypertension are resistant to at least three anti-hypertensive drugs.[3] In addition, increasing numbers of individuals with metabolic syndrome contribute to the growing prevalence of hypertension. Hypertension is associated with severe complications, including myocardial infarction, end organ damage, aneurysms of the arteries (for example, aortic aneurysm), arteriosclerosis, peripheral arterial disease, left ventricular hypertrophy and stroke.[4] Even a modest increase in arterial BP is associated with a shortened life expectancy. Although pharmaceutical treatment is often essential in people for whom lifestyle changes prove ineffective, dietary and lifestyle alterations can improve BP control and diminish the risk of associated complications. Thus, the development of effective supplementary treatments that control BP and also prevent cardiovascular complications is urgent.

The polyphenol resveratrol (RSV) (2,3,40-trihydroxystilbene) is enriched in the skins of red grapes. It has attracted increasing scientific attention due to its cardiovascular benefits and potent antitumor activity.[5] In obese rodents, RSV treatment produces various health benefits, including enhanced vascular function, decreased steatosis and hypertension, reduced inflammation and a gene expression pattern resembling that which occurs during caloric restriction.[6–13] In a recent study, Kanamori *et al.*[14] reported that RSV reverses remodeling in hearts with large, old myocardial infarctions by enhancing the autophagy-activating AMP kinase pathway. In addition, recent clinical studies demonstrate that RSV also confers metabolic benefits in humans.[15,16] Despite the significance of RSV for protecting against cardiovascular diseases, the mechanisms mediating these effects have remained a mystery. Understanding how RSV exerts its effects will provide potential insights into the biological causes of cardiovascular diseases and allow the development of more effective and specific molecules.

AMP-activated protein kinase (AMPK) is a critical component of an energy-sensing system that allows cells to sense changes in energy status.[17,18] Activated AMPK phosphorylates several target proteins,

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leading to increased metabolism, glucose uptake and fatty acid oxidation. Thus, it is considered as a new target for the treatment of type 2 diabetes mellitus and obesity.[19] Recent studies found that AMPK has protective effects against cardiovascular diseases.[20–23] In addition, 5-aminoimidazole-4-carboxamide 1-D-ribofuranoside, an AMPK activator, has been reported to decrease BP in insulin-resistant and obese rats.[24,25] However, whether AMPK regulates BP by directly modulating vascular tone or improving insulin resistance and metabolic anomalies in these models remains unknown.

This study aimed to test whether RSV alleviates hypertension induced by the chronic infusion of angiotensin II (AngII) in C57BL6 mice and affects the Ang II-induced myosin phosphatase-targeting subunit 1 (MYPT1)/myosin light chain (MLC) vascular constriction pathway in cultured human smooth muscle cells. Our findings demonstrate for the first time that RSV activates AMPK in vascular smooth muscle cells (VSMCs) and that chronic treatment with RSV can prevent Ang II-induced hypertension, mainly through its AMPK activation properties, by suppressing the p190-GAP-dependent Ras homolog gene family member A (RhoA)/Rho-associated kinase (ROCK)/MYPT1/MLC pathway.

MATERIALS AND METHODS

Materials
Polyclonal or monoclonal antibodies against MYPT1, p-MYPT1 (Thr696), AMPK (α2), p-AMPK (Thr172), MLC, p-MLC (Ser19), ROCK, RhoA and β-actin were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA) or Cell Signaling Technology (Beverly, MA, USA). Secondary antibodies were obtained from Cell Signaling Technology. RSV was obtained from Sigma. Ang II was obtained from Bachem (Torrance, CA, USA). Thiazovivin was obtained from Stemgent. Compound C was obtained from Calbiochem (La Jolla, CA, USA). The specific AMPK-targeted SAMS peptide used in AMPK activity assays was from GenScript (Piscataway, NJ, USA). All reagent concentrations are expressed as final concentrations in the buffer. Human smooth muscle cells were obtained from Clonetics (Walkersville, MD, USA).

Cell culture
HSMCs were cultured in smooth muscle cell growth medium 2 (Lonza, Portland, OR, USA). All cells were incubated at 37 °C in a humidified atmosphere of 5% CO_2 and 95% air. The SMCs were grown to 60–70% confluence before being treated with different agents. The cells used in all experiments were between passages 3 and 6.

siRNA transfection
Transient transfection with AMPKα2 siRNA was carried out according to a protocol provided by Cell Signaling. Briefly, the siRNA was dissolved in a buffer containing 0.2 mM $MgCl_2$, 20 mM KCl and 6 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (pH 7.5) to prepare a 10 μM stock solution. SMCs cultured in 6-well plates were transfected with siRNA in liposomal transfection reagent (Lipofectamine 2000, Invitrogen). For each transfection, 100 μl medium containing 4 μl transfection reagent was gently mixed with 100 μl medium containing 4 μl siRNA stock solution. After incubation for 30 min at room temperature, siRNA-lipid complexes were added to the cultures in 1.0 ml transfection medium, followed by further incubation for 6 h at 37 °C. The transfection medium was then removed, and the SMCs were cultured in normal medium for 48 h.

Animals
The experiments were carried out in accordance with the guidelines and practices established by the Third Hospital of Chinese Medical University Animal Care and Use Committee. Male wild-type C57BL6 mice (14 weeks of age; 20–25 g body weight) (Jackson Laboratories, Bar Harbor, ME, USA) were housed in a temperature-controlled room under a 12-h light-dark cycle and given free access to water and food. The mice were kept in the facility for approximately 10 days before starting the experimental protocol.

The mice were subjected to chronic infusion of Ang II (2.2 ng min^{-1} per g body weight) for 2 weeks using osmotic minipumps (Alzet; Durect, Cupertino, CA, USA). During Ang II infusion, the animals were given either a standard diet or a diet supplemented with 0.4% (RSV) and allowed to drink freely. Some mice were treated with compound C (15 mg kg^{-1} body weight per day delivered intraperitoneally). Thus, the animals were grouped according to Ang II infusion and administered treatments as follows: (i) control mice, (ii) Ang II, (iii) Ang II + RSV and (iv) Ang II + RSV + compound C ($n = 10$–12 per each group). In this study, RSV was supplied in a diet supplemented with 0.4% RSV, which is consistent with previous studies[26–28] that showed the beneficial effects of RSV on hypertension, cardiac hypertrophy, cardiac function and diet-induced metabolic syndrome. As suggested by Dolinsky *et al.*,[26] the rodents were fed the standard chow (control) diet or a standard chow diet that contained 4 g RSV per kg diet (0.4%). For the spontaneously hypertensive rats, the dosage of RSV was equivalent to ~146 mg RSV kg^{-1} per day; for mice, the dosage was equivalent to approximately 320 mg RSV kg^{-1} per day.

Western blotting
Western blot analyses were performed according to standard procedures. The cultured VSMCs and endothelium-denuded aortic tissues were homogenized on ice in cell lysis buffer containing 20 mM Tris-HCl (pH 7.5), 1 mM Na_2EDTA, 1 mM ethylene glycol tetraacetic acid, 1% Triton, 150 mM NaCl, 1 mM glycerophosphate, 2.5 mM sodium pyrophosphate, 1 μg ml^{-1} leupeptin, 1 mM Na_3VO_4 and 1 mM phenylmethylsulfonyl fluoride. The protein concentration of the resulting lysate was assayed with the bicinchoninic acid protein assay reagent (Pierce, USA). Proteins (20 μg) were separated on 4–12% Tris-glycine gels and transferred to nitrocellulose membranes (Invitrogen). The membranes were then probed with antibodies against AMPK, phospho-AMPK, MYPT1, phospho-MYPT1, MLC, phospho-MLC and actin followed by incubation with the appropriate horseradish peroxidase-conjugated secondary antibodies before signals were visualized with the enhanced chemiluminescence detection system (enhanced chemiluminescence detection system, Amersham Biosciences). The intensity (area × density) of the individual bands was quantified using Scion Image software (Scion Corp, Frederick, MD, USA). The background was subtracted from the calculated area, and the control was set to 1.

RhoA activation assay
To determine RhoA activation, a Rhotekin pull-down assay was performed as described previously.[29] VSMCs were cultured in 100-mm dishes containing medium with 0.5% serum. After treatment as indicated, the cells were rapidly lysed on ice and processed for guanosine triphosphate (GTP)-bound RhoA quantification according to the manufacturer's instructions (Cell Biolabs).

Kinase assay
ROCK activity in cultured VSMCs was assayed with a ROCK assay kit in accordance with the manufacturer's instructions (Cell Biolabs). Briefly, each kinase reaction contained 40 μl of 1 × kinase buffer/ATP/substrate solution and ROCK immunoprecipitates. The reaction was allowed to proceed for 40 min at 30 °C with gentle stirring. The reaction products were detected by western analysis for MYPT1 phosphorylation at Thr696, which served as an index of ROCK activity. AMPK activity was assayed as described previously.[30] Briefly, VSMC lysates were incubated with (γ-^{32}P)ATP and SAMS peptide, and the catalytic activity of AMPK was measured by the incorporation of ^{32}P into SAMS peptide.

Vessel tension and BP measurement
Measurement of vessel tension in mice was performed as described previously.[29] Briefly, mice treated with the different factors were anesthetized with diethyl ether and euthanized by decapitation. The aortas were quickly removed, immersed in Krebs bicarbonate buffer (1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 118 mM NaCl, 2.5 mM $CaCl_2$, 4.7 mM KCl, 25 mM $NaHCO_3$ and 5 mM glucose) and gassed with a mixture of 95% O_2 and 5% CO_2. All fat and connective tissues were carefully cleaned. The endothelium was gently removed with a scraper. Artery rings were mounted between two hooks in a 5-ml organ bath perfused with Krebs buffer at 37 °C. After an equilibration period, the rings were contracted with a high-potassium salt solution (60 mM). After two

washes and a further 30-min equilibration period, the rings were then contracted with Ang II (1 μM). Mouse BP was measured using the carotid catheter method as described previously.[29] Blood was directed to a pressure transducer through the catheter to obtain computerized BP values (AD instruments). The mice were allowed to recover, and the systolic and diastolic BPs were measured for 30 min in conscious states.

Statistical analysis

The data are presented as the mean ± s.d. All data were analyzed with a one- or two-way analysis of variance followed by multiple t-tests. $P < 0.05$ was considered significant.

RESULTS

RSV increases AMPK phosphorylation and activity in cultured VSMCs

To test whether RSV can phosphorylate AMPK, VSMCs were treated with different concentrations of RSV for up to 24 h. As shown in Figure 1a, the phosphorylation of Thr172 of AMPK in VSMCs increased in a dose-dependent manner, with a peak at 50 μM. Treatment with RSV did not alter the levels of AMPK in VSMCs. The augmented AMPK phosphorylation coincided with increased AMPK activity, as measured by SAMS peptide assay (Figure 1b). In control cells, the addition of dimethylsulfoxide (RSV vehicle) at a dilution of 1:104 (vol/vol) did not enhance any of these phosphorylation events. 5-aminoimidazole-4-carboxamide 1-D-ribofuranoside,

an AMPK activator, was used as a positive control and was capable of stimulating AMPK. As shown in Figures 1c and d, AMPK phosphorylation and AMPK activity increased in an RSV-exposure-time-dependent manner.

RSV inhibits AngII-induced MLC phosphorylation via AMPK activation

The phosphorylation status of MLC at Ser19 determined vessel tone in VSMCs.[31] First, we determined whether RSV could affect AngII-induced phosphorylation of MLC. Human smooth muscle cells were treated with AngII in the presence or absence of RSV, and MLC phosphorylation was measured by immunoblot analysis. Treatment of HSMC with AngII (1 μM, 30 min) significantly increased the phosphorylation of MLC. As expected, pretreatment with RSV abolished AngII-induced AMPK-Thr172 phosphorylation in a dose- and time-dependent manner in HSMCs (Figures 2a and b). However, RNA interference and chemical inhibition of AMPK abolished the inhibitory effect of RSV on AngII-induced MLC phosphorylation (Figure 2c). Our results suggest that RSV inhibits AngII-induced MLC phosphorylation via AMPK activation in VSMCs.

RSV inhibits AngII-induced Thr696 phosphorylation of MYPT1, an upstream regulator of MLC, via AMPK activation

MLC phosphatase inhibits smooth muscle contractions by dephosphorylating MLC that is bound to myosin heavy chain. MYPT1, a
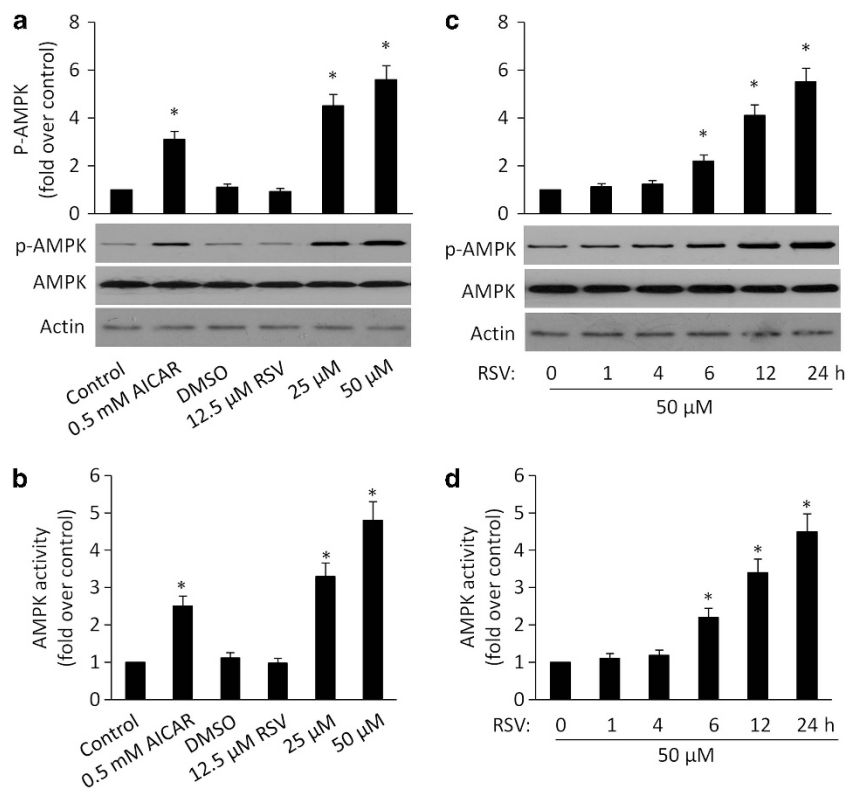

Figure 1 Resveratrol (RSV) activates AMP-activated protein kinase (AMPK) in cultured vascular smooth muscle cells (VSMCs). VSMCs were treated with (a) various concentrations of RSV for 24 h and (c) 50 μM RSV for the times indicated. 5-aminoimidazole-4-carboxamide 1–D-ribofuranoside (AICAR), an AMPK activator, was used as a positive control. The cells were then lysed, and 100 μg of the cell lysates was subjected to SDS-polyacrylamide gel electrophoresis (PAGE) followed by western analysis with various primary antibodies as indicated. The proteins were then visualized using the chemiluminescence system. The bar graphs above illustrate the densitometric analysis of the corresponding western blot bands from three independent experiments. The data are presented as the mean fold increases (± s.d.) in treated groups over basal values, which were arbitrarily set to 1. In separate sets of experiments shown in (b and d), 2 μg of protein extracts were subjected to AMPK activity assays with SAMS peptide and (γ-^{32}P)ATP used as substrates. The data are presented as the mean ± s.d. from three separate experiments. *$P < 0.05$ vs. non-treated controls.

subunit of the MLC phosphatase complex, suppresses the catalytic activity of the MLC phosphatase complex on myosin II. Here, western analysis revealed that AngII-induced MYPT1 phosphorylation at Thr696 in HSMCs (Figure 3a). Conversely, RSV treatment attenuated AngII-induced MYPT1 phosphorylation. However, RNA interference and chemical inhibition of AMPK ablated the inhibitory effect of RSV on AngII-induced MYPT1 phosphorylation (Figure 3a), suggesting that RSV, via AMPK activation, inhibits AngII-induced MYPT1 phosphorylation in VSMCs. The effects of RSV, compound C and AMPK siRNA on AMPK activation were further confirmed by AMPK kinase activity assessment, as measured by the SAMS peptide assay (Figure 3b). Taken together, these results demonstrate that RSV-induced AMPK activation negatively regulates the AngII-stimulated Thr696 phosphorylation of MYPT1, an upstream regulator of MLC.

ROCK mediates AngII-induced MYPT1 phosphorylation and the regulation of MYPT1 by AMPK

Previous studies have suggested that the ROCK-dependent signaling pathway is indispensable in the mediation of agonist-stimulated vessel smooth muscle contraction.[32,33] Here, we determined the effect of thiazovivin, a novel ROCK inhibitor, on MYPT1 phosphorylation. As shown in Figure 4a, thiazovivin treatment reversed AngII-induced MYPT1 phosphorylation in HSMCs, suggesting that ROCK mediates AngII-induced MYPT1/MLC activation. Next, we further determined the effects of RSV on ROCK activity and examined whether AMPK mediates this effect. For this purpose, ROCK activity was measured in VSMCs treated with RSV in the presence of compound C (an AMPK inhibitor) or after transfection with AMPK siRNA. As indicated in Figure 4b, RSV reversed AngII-induced ROCK activity, whereas co-incubation of compound C abolished this effect. Consistently, genetic
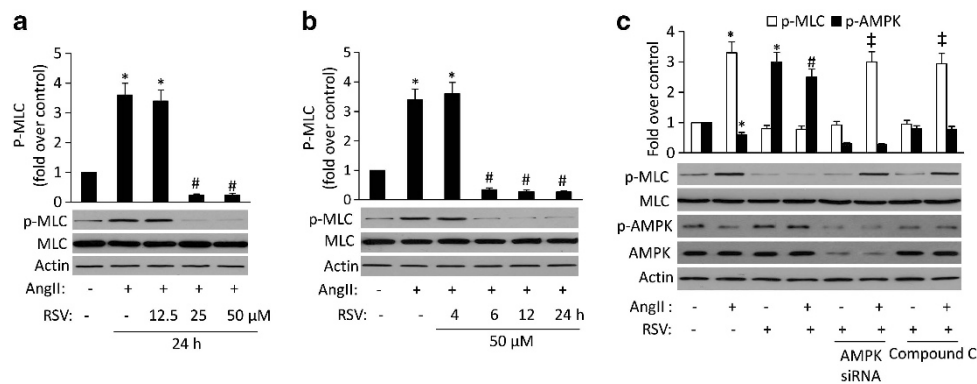

Figure 2 Resveratrol (RSV) inhibits angiotensin II (AngII)-induced myosin light chain (MLC) phosphorylation by activating AMP-activated protein kinase (AMPK) in vascular smooth muscle cells (VSMCs). (a and b), Western analysis of AMPK Thr172 phosphorylation and MLC Ser19 phosphorylation in AngII-stimulated human smooth muscle cells (HSMCs) pretreated with or without RSV. The cells were pretreated with varying concentrations of RSV (a) for various times (b) and then stimulated with AngII (1 μM) for 30 min. $N=3$, *$P<0.05$ vs. control, #$P<0.05$ vs. AngII alone. (c) MLC phosphorylation in cells treated with RSV for 24 h that were transfected or not with AMPK siRNA or exposed or not to compound C, an AMPK inhibitor, and subsequently treated with AngII for 30 min. $N=3$, *$P<0.05$ vs. control, #$P<0.05$ vs. AngII alone, ‡$P<0.05$ vs. AngII plus RSV.
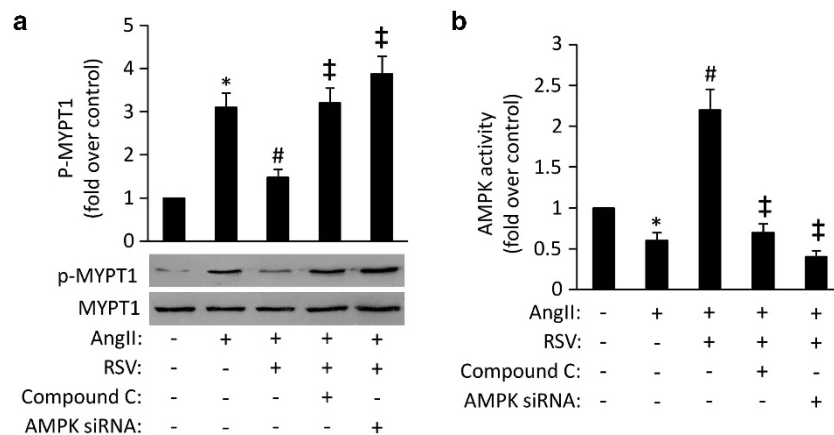

Figure 3 Resveratrol (RSV) inhibits angiotensin II (AngII)-induced Thr696 phosphorylation of myosin phosphatase-targeting subunit 1 (MYPT1), an upstream regulator of myosin light chain (MLC), by activating AMP-activated protein kinase (AMPK) vascular smooth muscle cells (VSMCs). (a) Western analysis of MYPT1 Thr696 phosphorylation and MYPT1 protein expression in human smooth muscle cells (HSMCs) treated with RSV for 24 h that were transfected or not with AMPK siRNA or exposed or not to compound C, an AMPK inhibitor, and subsequently treated with AngII for 30 min. (b) Protein extracts (2 μg) were used in AMPK activity assays; SAMS peptide and (γ-^{32}P)ATP were used as substrates. The data represent the mean ± s.d. from three separate experiments. *$P<0.05$ vs. control, #$P<0.05$ vs. AngII alone, ‡$P<0.05$ vs. AngII plus RSV.
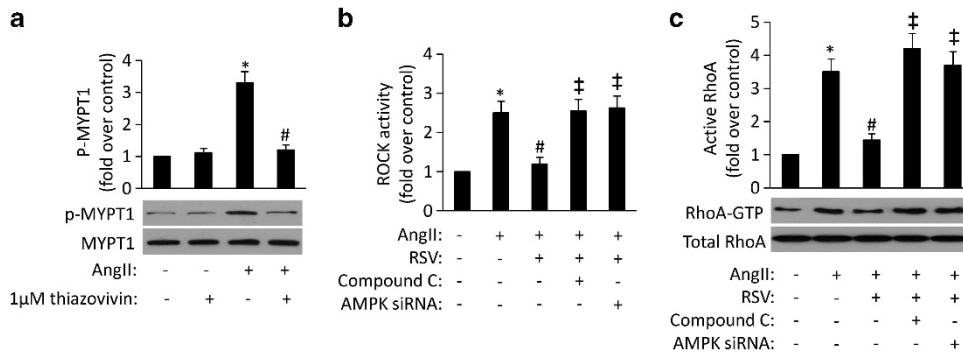
Figure 4 Resveratrol (RSV) negatively regulates angiotensin II (AngII)-upregulated Rho-associated kinase (ROCK) activity and Ras homolog gene family member A (RhoA) activation via AMP-activated protein kinase (AMPK) activation. (**a**) Western analysis of Thr696-phosphorylated myosin phosphatase-targeting subunit 1 (MYPT1) in cultured human smooth muscle cells (HSMCs) pretreated with thiazovivin, a novel ROCK inhibitor (1 μM), for 2 h and subsequently incubated with AngII for 30 min. $N=3$, *$P<0.05$ vs. control, #$P<0.05$ vs. AngII alone. (**b**) ROCK activity in HSMCs treated with RSV for 24 h that were transfected or not with AMPK siRNA or exposed or not to compound C and subsequently treated with AngII for 30 min. The kinase assay was performed as described in the Materials and methods section. (**c**) RhoA activation, as measured by Rhotekin pull-down, in HSMCs treated with RSV for 24 h that were transfected or not with AMPK siRNA or exposed or not to compound C and subsequently treated with AngII for 30 min. The data represent the mean ± s.d. from three separate experiments. *$P<0.05$ vs. control, #$P<0.05$ vs. AngII alone, ‡$P<0.05$ vs. AngII plus RSV.

suppression of AMPK by RNA interference significantly blocked the inhibitory effect of RSV on AngII-induced ROCK activation. Taken together, these data suggest that RSV-induced suppression of ROCK activation is AMPK dependent.

ROCK is the first and most well-characterized RhoA effector.[34] RhoA, a member of the Ras low-molecular weight G protein superfamily, activates ROCK by binding GTP, which in turn results in MYPT1 phosphorylation, MLC activation and smooth muscle cell contraction. As indicated in Figure 4c, AngII activated RhoA by increasing the amount of GTP-bound RhoA. RSV inhibited AngII-induced RhoA activation; however, compound C abolished this effect. These results were confirmed by transfection of cells with AMPK siRNA and measurement of the RhoA activation status. Therefore, these data suggest that RSV-induced suppression of RhoA activation is AMPK dependent.

RSV-induced AMPK activation enhances p190-GTP-activating protein interaction with RhoA in AngII-stimulated VSMCs
RhoA shuttles between a GTP-bound active state and a guanosine diphosphate-bound inactive state through nucleotide exchange, which is controlled by guanine nucleotide exchange factors[35] and p190-GTP-activating proteins. Next, we tested whether AMPK activation by RSV inactivates RhoA by stimulating the association of RhoA with p190-GAP. We demonstrated that the binding of p190-GAP to RhoA was dramatically increased by RSV in AngII-stimulated VSMCs, whereas inhibition of AMPK with compound C or AMPK knockdown using an AMPK-specific siRNA notably decreased RhoA/p190-GAP complex formation (Figure 5a). Together, our results suggest that AMPK activation by RSV promotes p190-GAP association with RhoA in AngII-stimulated HSMCs.

To further substantiate the role of p190-GAP in AMPK-mediated RhoA inactivation, VSMCs were transfected with control siRNA or p190-GAP–targeted siRNA. p190-GAP knockdown was confirmed by western blot analysis (Figure 5b). As expected, in AngII-stimulated VSMCs that were transfected with control siRNA, RSV treatment significantly decreased the levels of active RhoA, phosphorylated MYPT1 and phosphorylated MLC (Figure 5b). On the contrary, p190-GAP knockdown via RNA interference abolished the effects of

RSV, suggesting that p190-GAP is required for AMPK-mediated RhoA inhibition.

AMPK activation is responsible for the RSV-induced decrease in AngII-induced aortic vessel contraction in mice
Next, we determined the effect of RSV on AngII-induced contraction of aortic rings. Vessel rings were pretreated with RSV (50 μM) in the presence or absence of compound C (20 μM) for 6 h, and contractions were then induced by AngII in an organ bath. As shown in Figure 6a, AngII significantly increased artery contraction in control explants. Vessel contraction was dramatically inhibited by RSV, and this effect was abolished by compound C. These results suggest that the activation of AMPK by RSV suppresses AngII-induced vasoconstriction.

RSV inhibits AngII-induced hypertension via AMPK activation in vivo
To explore the physiological relevance, we measured systolic BP, diastolic BP and mean BP in mice subjected to chronic infusion of Ang II (2.2 ng min^{-1} per g body weight) that were fed with a diet supplemented with 0.4% RSV or treated with compound C (15 mg kg^{-1} body weight per day delivered intraperitoneally) for two weeks. BP was measured by left carotid catheter. As depicted in Figures 6b and c, all BP levels (systolic BP, diastolic BP and mean BP) as well as active RhoA, phosphorylation levels of MYPT1 and phosphorylation levels of MLC were higher in the endothelium-denuded aortas of AngII-treated mice compared with those of control mice. Notably, RSV administration significantly decreased the BP values, RhoA activity and levels of phosphorylated MYPT1 and MLC in AngII-treated mice. More importantly, compound C administration significantly abolished the effects of RSV. Taken together, our results demonstrate that AMPK suppression of the p190-GAP-dependent RhoA/ROCK/MYPT1/MLC pathway contributes to the hypotensive effect of RSV in AngII-treated mice.

DISCUSSION
The most important finding of the present study is that AMPK activation induced by RSV lowers BP by inhibiting VSMC contractility through the suppression of MYPT1/MLC phosphorylation. In
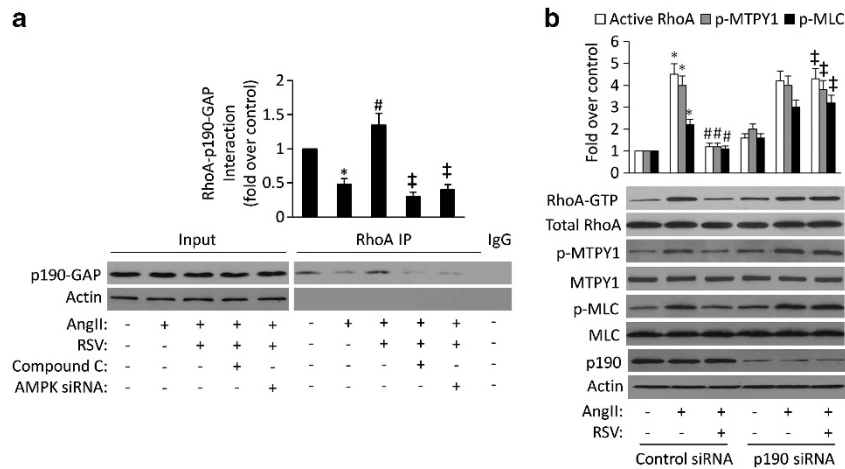
Figure 5 p190-GTP-activating protein (GAP) mediates AMP-activated protein kinase (AMPK) suppression of Ras homolog gene family member A (RhoA) induced by resveratrol (RSV). (**a**) Coimmunoprecipitation/western analysis of p190-GAP/RhoA binding in human smooth muscle cells (HSMCs) treated with RSV for 24 h that were transfected or not with AMPK siRNA or exposed or not to compound C and subsequently treated with angiotensin II (AngII) for 30 min. (**b**) HSMCs were treated with RSV for 24 h in the presence or absence of control siRNA or p190-GAP siRNA and subsequently treated with AngII for 30 min. Cell lysates were subjected to Rhotekin pull-down assays to measure RhoA activation, phosphorylated myosin phosphatase-targeting subunit 1 (MYPT1) or phosphorylated myosin light chain (MLC). The data represent the mean ± s.d. from three separate experiments. *$P<0.05$ vs. control, #$P<0.05$ vs. AngII alone, ‡$P<0.05$ vs. AngII plus RSV.

addition, we demonstrated that AMPK-activated RSV reduces VSMC contractility by suppressing p190-GAP-dependent RhoA activation.

One of the major findings of this study was that RSV activated AMPK and suppressed AngII-induced VSMC contractility by suppressing MYPT1/MLC phosphorylation. This finding is supported by several lines of evidence. First, activation of AMPK by RSV inhibited AngII-induced contraction in explanted murine aortic arteries, and inhibition of AMPK with compound C, a pharmacological inhibitor, abolished this effect. Second, RSV administration significantly decreased the BP values in AngII-treated mice. More importantly, compound C administration significantly abolished the effect of RSV. Finally, RSV treatment abolished AngII-induced RhoA activation, phosphorylation of MYPT1 at Thr696 and phosphorylation of MLC at Ser19 in vivo (mouse aortas) and in vitro (cultured VSMCs). However, these effects of RSV were ablated by RNA interference or chemical inhibition of AMPK. Taken together, our results provide evidence that RSV exerts a direct inhibitory effect on VSMC contractility by activating AMPK.

Although AMPK is traditionally considered as an intracellular fuel gauge, current studies suggest that it is also important in maintaining physiological processes in the cardiovascular system. In the heart, AMPK signaling modulates the bioenergetic status of cardiomyocytes and maintains the cardiac muscle and its electrical pacemakers in an optimal condition.[36] Previous studies demonstrated that AMPK mutations cause serious heart rhythm anomalies.[36] In the endothelium, AMPK restores the impaired redox balance associated with vascular pathology. In addition, it exerts anti-atherosclerotic effects by enhancing nitrogen oxide bioavailability, attenuating reactive oxygen species stress and activating proangiogenic factors. Some medicines and compounds that have good therapeutic effects on disorders such as cardiovascular disease (for example, the polyphenol RSV), diabetes (for example, metformin) and infectious diseases (for example, berberine) appear to elicit their therapeutic/protective effects through the regulation of AMPK signaling. Furthermore, AMPK activation in the endothelium is also related to endothelial control of vascular smooth muscle function,

particularly vasorelaxation induced by nitrogen oxide that appears to be partially regulated by AMPK activation.

In the present study, we report the novel finding that AMPK activation by RSV modulates vascular constriction signaling, resulting in attenuation of AngII-stimulated vessel constriction. It is well known that activation of the RhoA/ROCK cell signaling pathway has an indispensable role in hypertension.[34] Thus, inhibition of RhoA/ROCK by RSV-induced AMPK activation might be an essential mechanism for lowering BP via suppression of VSMC contractility. Consistent with our findings, metformin, a widely used antidiabetic drug that is capable of activating AMPK in vivo, has been reported to decrease BP in a variety of models of hypertensive animals as well as in patients with or without diabetes mellitus. In addition, RhoA/ROCK signaling plays a critical role in cell cycle progression, cell proliferation and cell survival after vascular injury.[37] Because AMPK activation potentiates the binding of p190-GAP to RhoA, leading to inhibition of RhoA activity, AMPK might also be essential for other physiological processes such as tube formation or angiogenesis after vascular damage, in which the RhoA/ROCK signaling pathway is involved.[38] This finding is consistent with a recent study indicating that 5-aminoimidazole-4-carboxamide 1-D-ribofuranoside, an AMPK activator, suppresses ROCK activity in osteoblastic cells.[39] Overall, our studies reveal a novel mechanism of RSV action whereby activation of AMPK attenuates the AngII-induced signaling pathway by inhibiting RhoA/ROCK activity via p190-GAP, which sequentially diminishes MYPT1/MLC phosphorylation in VSMCs, resulting in decreased vessel contraction. In combination, these effects directly facilitate the regulation of BP in mice.

In conclusion, the present results show that AMPK may be considered a novel therapeutic target for efficient treatment of hypertension. For the first time, we reveal that daily chronic administration of RSV alleviates hypertension in the experimental model of AngII-induced hypertensive mice and that these effects are accompanied by the activation of AMPK and inhibition of the RhoA/ROCK/MYPT1/MLC pathway in VSMCs. Our results substantiate the beneficial effects of RSV in the treatment of hypertension.
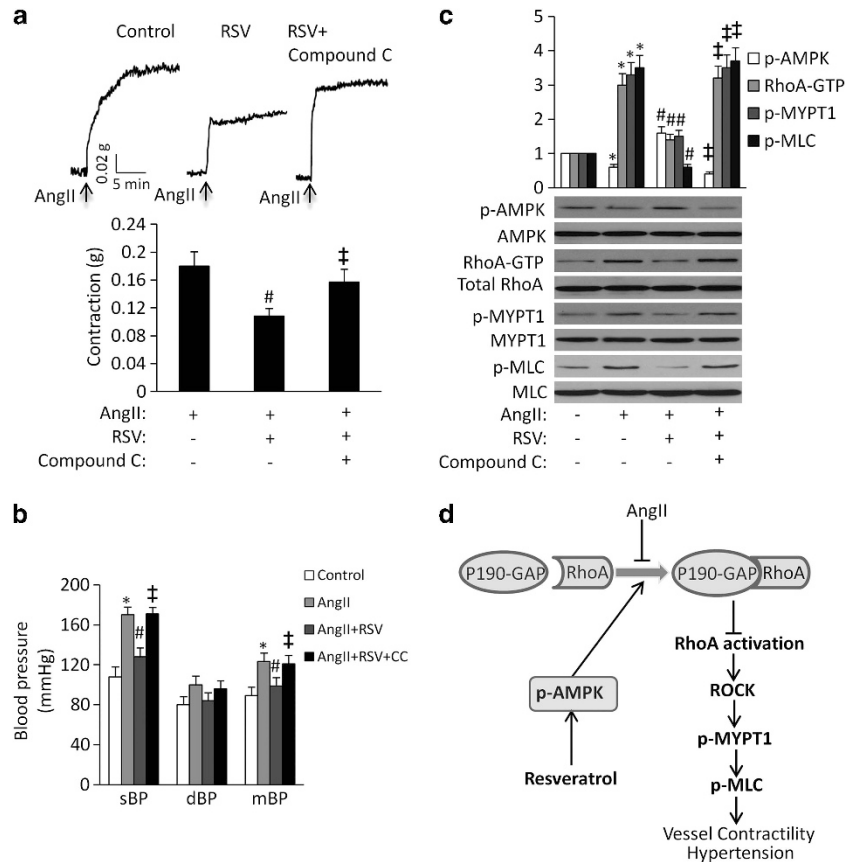
Figure 6 Resveratrol (RSV) acts through AMP-activated protein kinase (AMPK) to regulate angiotensin II (AngII)-induced mesenteric vessel contraction and blood pressure in mice by suppressing the p190-GTP-activating protein (GAP)/Ras homolog gene family member A (RhoA)/Rho-associated kinase (ROCK) pathway. (a) AngII-induced contraction of aortic artery rings isolated from mice treated with RSV (RSV) or compound C. The concentration of AngII was 1 μM. Contraction was recorded with software (AD Instruments) via a transducer connected to a computer. $n = 7$, #$P < 0.05$ vs. AngII, ‡$P < 0.05$ vs. AngII plus RSV. (b) The mice were subjected to chronic infusion of AngII (2.2 ng min^{-1} per g body weight) using osmotic minipumps. During AngII infusion, the animals were given either a standard diet or a diet supplemented with 0.4% RSV and either treated or not treated with compound C (15 mg kg^{-1} body weight per day delivered intraperitoneally). Two weeks later, the blood pressure was measured by left carotid catheter. (c) Endothelium-denuded aortic tissues were used for analysis of RhoA activation and phosphorylated AMPK, myosin phosphatase-targeting subunit 1 (MYPT1) and myosin light chain (MLC). $N = 6$, *$P < 0.05$ vs. control, #$P < 0.05$ vs. AngII alone, ‡$P < 0.05$ vs. AngII plus RSV. (d) Proposed mechanism by which RSV-induced AMPK activation regulates vessel contraction and blood pressure via the RhoA/ROCK/p-MYPT1/p-MLC pathway. A full color version of this figure is available at the Hypertension Research journal online.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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