

Arteriosclerosis, Thrombosis, and Vascular Biology



JOURNAL OF THE AMERICAN HEART ASSOCIATION

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Arterioscler Thromb Vasc Biol. 2013;33:595-604; originally published online January 3, 2013;
doi: 10.1161/ATVBAHA.112.300606

Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272
Greenville Avenue, Dallas, TX 75231

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Print ISSN: 1079-5642. Online ISSN: 1524-4636

The online version of this article, along with updated information and services, is located on the
World Wide Web at:

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Aberrant Endoplasmic Reticulum Stress in Vascular Smooth Muscle Increases Vascular Contractility and Blood Pressure in Mice Deficient of AMP-Activated Protein Kinase- α 2 In Vivo

Bin Liang, Shuangxi Wang, Qilong Wang, Wencheng Zhang, Benoit Viollet, Yi Zhu, Ming-Hui Zou

Objective—The endoplasmic reticulum (ER) plays a critical role in ensuring proper folding of newly synthesized proteins. Aberrant ER stress is reported to play a causal role in cardiovascular diseases. However, the effects of ER stress on vascular smooth muscle contractility and blood pressure remain unknown. The aim of this study was to investigate whether aberrant ER stress causes abnormal vasoconstriction and consequent high blood pressure in mice.

Methods and Results—ER stress markers, vascular smooth muscle contractility, and blood pressure were monitored in mice. Incubation of isolated aortic rings with tunicamycin or MG132, 2 structurally unrelated ER stress inducers, significantly increased both phenylephrine-induced vasoconstriction and the phosphorylation of myosin light chain (Thr18/Ser19), both of which were abrogated by pretreatment with chemical chaperones or 5-Aminoimidazole-4-carboxamide ribonucleotide and metformin, 2 potent activators for the AMP-activated protein kinase. Consistently, administration of tauroursodeoxycholic acid or 4-phenyl butyric acid, 2 structurally unrelated chemical chaperones, in AMP-activated protein kinase- α 2 knockout mice lowered blood pressure and abolished abnormal vasoconstrictor response of AMP-activated protein kinase- α 2 knockout mice to phenylephrine. Consistently, tunicamycin (0.01 μ g/g per day) infusion markedly increased both systolic and diastolic blood pressure, both of which were ablated by coadministration of 4-phenyl butyric acid. Furthermore, 4-phenyl butyric acid or tauroursodeoxycholic acid, which suppressed angiotensin II infusion-induced ER stress markers in vivo, markedly lowered blood pressure in angiotensin II-infused mice in vivo.

Conclusion—We conclude that ER stress increases vascular smooth muscle contractility resulting in high blood pressure, and AMP-activated protein kinase activation mitigates high blood pressure through the suppression of ER stress in vivo. (*Arterioscler Thromb Vasc Biol.* 2013;33:595-604.)

Key Words: AMPK ■ ER stress ■ hypertension ■ vascular smooth muscle

The endoplasmic reticulum (ER) is a membranous intracellular network where a wide range of proteins are synthesized, processed, and prepared for trafficking. Under stress conditions, proteins become misfolded and accumulated within the ER to provoke the unfolded protein response.¹⁻⁴ After detecting such accumulation, the ER activates transcriptional and translational pathways leading to the increased expression of ER-resident chaperones, inhibition of protein translation, and acceleration of protein degradation.⁵ However, prolonged ER stress is considered a pathogenic mechanism in human diseases, including aging,⁶⁻¹¹ obesity,¹²⁻¹⁴ atherosclerosis,¹⁵⁻¹⁸ and diabetes mellitus.¹⁹⁻²¹ However, a causal link between ER stress and hypertension remains to be established.

The AMP-activated protein kinase (AMPK) is an evolutionarily conserved serine/threonine protein kinase, which is a heterotrimeric complex composed of α , β , and γ subunits. The α subunit of AMPK contains the catalytic domain and has 2 isoforms, α 1 and α 2, which are phosphorylated at threonine 172 on enzyme activation. AMPK is ubiquitously expressed in tissues. It is activated by multiple stress signals including nutrient deprivation, hypoxia, and oxidants. On its activation, AMPK exerts multiple effects other than its conventional effects on energy metabolism.

Recent studies from us and others have established that AMPK is essential in maintaining vascular homeostasis. In vascular smooth muscle cells (VSMCs), AMPK activation is reported to suppress VSMC proliferation,²² migration,²³ and

Received on: July 17, 2012; final version accepted on: December 17, 2012.

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The online-only Data Supplement is with this article at <http://atvb.ahajournals.org/lookup/suppl/doi:10.1161/ATVBAHA.112.300606/-DC1>.

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DOI: 10.1161/ATVBAHA.112.300606

hypertrophy.²⁴ AMPK is also reported to exert a direct vaso-relaxing effect in isolated aortic rings.²⁵ Indeed, AMPK α 2 knockout mice (AMPK α 2^{-/-}) are hypertensive exhibiting increased responses to vasoconstrictors.²⁶ In endothelial cells, AMPK α 2 deficiency causes aberrant ER stress resulting in endothelial dysfunction and atherosclerosis *in vivo*.^{17,18} ER stress is reported to play causative roles in cardiovascular diseases, including diabetes mellitus, atherosclerosis, and heart failure. AMPK is reported to be essential in maintaining ER homeostasis,²⁷ and AMPK inhibition causes aberrant ER stress in endothelial cells, hepatocytes,^{18,28} and other cell types. 5-Aminoimidazole-4-carboxamide ribonucleotide (AICAR) is reported to cause both endothelium-dependent and endothelium-independent relaxation.^{29,30} Whether ER stress contributes to the initiation and progression of hypertension is unknown. In this study, we found that aberrant ER stress increases phenylephrine (PE)-induced vascular contractility and hypertension, both of which were suppressed by AMPK activation. Conversely, we found that AMPK α 2 deletion caused aberrant ER stress, abnormal vascular contractility, and high blood pressure *in vivo*.

Materials and Methods

The ER stress marker, vascular smooth muscle contractility, and blood pressure were monitored in cultured vascular cells and wild-type (WT) or mice deficient in AMPK α 1 or AMPK α 2. A full description of materials, animals, and methods used, including cell culture, blood pressure measurement, osmotic pump implantation, organ chamber, and Western blots, can be found in the online-only Data Supplement.

Results

Infusion of Tunicamycin Induces ER Stress in Mouse Aortas *In Vivo*

Tunicamycin is reported to trigger ER stress in cultured cells. Whether Tunicamycin caused ER stress in mice was unknown. We first determined whether Tunicamycin caused ER stress *in vivo*. To this end, C56BL/6 wild-type (WT) mice were infused with Tunicamycin (10 μ g/g per day) for 7 days. Mouse aortas were isolated for immunohistochemical staining of p-elf2 α , spliced XBP-1 (XBP-1s), and KDEL, 3 well-characterized ER stress markers. The staining with p-elf2 α , XBP-1s, or KDEL was barely detectable in vehicle-infused WT aortas (Figure 1A). However, Tunicamycin infusion markedly increased the levels of p-elf2 α , XBP-1s, and KDEL (Figure 1A), indicating that Tunicamycin is a potent ER stress inducer in vascular walls.

4-Phenyl butyric acid (4-PBA) is a potent ER chaperone. Infusion of 4-PBA did not alter the levels of p-elf2 α , spliced XBP-1, and KDEL (Figure 1A) in WT mice. However, 4-PBA markedly reduced Tunicamycin-increased ER stress markers (p-elf2 α , XBP-1s, and KDEL) in the aortas (Figure 1A).

We further validated the markers of ER stress in Tunicamycin-infused mouse aortas in Western blots. In line with increased immunohistochemical stainings shown in Figure 1A, Tunicamycin infusion markedly increased the levels of p-elf2 α and XBP-1s (Figure 1B). Consistently, 4-PBA administration ablated Tunicamycin-enhanced levels of p-elf2 α and XBP-1s (Figure 1B).

Tunicamycin Infusion Increases Systolic and Diastolic Blood Pressures *In Vivo*

We next evaluated whether Tunicamycin infusion altered systolic (sBP) and diastolic blood pressure (dBP) in mice *in vivo*. Blood pressure was continuously monitored in vehicle- or Tunicamycin-infused mice for 16 days by using telemetry transmitters. Tunicamycin infusion lowered sBP at days 5 to 7 (Figure 1C). After this initial reduction, Tunicamycin caused a gradual elevation of sBP from day 7 to 9 (Figure 1C). After day 10, sBPs in Tunicamycin-infused mice were significantly higher than those in vehicle-infused mice (Figure 1C). sBP in Tunicamycin-treated mice reached maximal at day 13 and remained elevated until the end of the experiments (day 16; Figure 1C). Tunicamycin infusion did not alter heart rate (data not shown). Thus, Tunicamycin-induced systolic hypertension appeared to be independent of heart rates (data not shown).

We also monitored the effects of Tunicamycin on dBP. Similar to sBP, after an initial drop in pressure between days 5 and 7, dBP in Tunicamycin-infused mice gradually increased at day 8 and reached maximal at day 13 (Figure 1C). The dBP remained significantly elevated at day 16 (Figure 1C).

4-PBA Abolishes Tunicamycin-Enhanced sBP and dBP in Mice

To establish the causal link of ER stress and hypertension, we treated Tunicamycin-infused mice with ER chaperone 4-PBA. As shown in Figure 1C and 1D, 4-PBA alone did not affect either sBP or dBP in vehicle-treated WT mice. 4-PBA alone did not alter heart rate in mice (data not shown). Interestingly, 4-PBA administration partially but significantly reduced Tunicamycin-induced sBP increase (Figure 1C). In contrast to a partial reduction of sBP, 4-PBA completely normalized dBP in Tunicamycin-infused mice (Figure 1D), suggesting that ER stress might preferentially cause the elevation of dBP *in vivo*.

Exposure of Isolated Aortic Rings to Tunicamycin or MG132 Induces ER Stress *Ex Vivo*

Next, we assayed whether exposure of isolated aortas to Tunicamycin or MG132 caused ER stress in isolated aortas. To this end, isolated aortas were exposed to Tunicamycin or MG132 with or without the addition of 4-PBA or AICAR. As expected, exposure of aortic rings to either Tunicamycin or MG132 markedly increased the levels of p-elf2 α , GRP78, and XBP-1s (Figure 2A and 2B). In addition, 4-PBA attenuated the levels of p-elf2 α , GRP78, and XBP-1s enhanced by Tunicamycin or MG132 (Figure 2A and 2B). Furthermore, preincubation of aortic rings with AICAR, an AMPK activator, abolished ER stress markers enhanced by Tunicamycin or MG132 (Figure 2A and 2B). These results suggest that AICAR and 4-PBA can effectively lower Tunicamycin- and MG132- induced ER stress in vessels.

ER Stress Inducers Accentuate PE-Induced Vasoconstriction *Ex Vivo*

dBP is mainly determined by the contractility of peripheral resistant arteries. Increased vasocontractility was found to be similar in the aortas and peripheral resistant arteries from AMPK α 2^{-/-} mice.²⁶ Although mouse aortas are not resistant

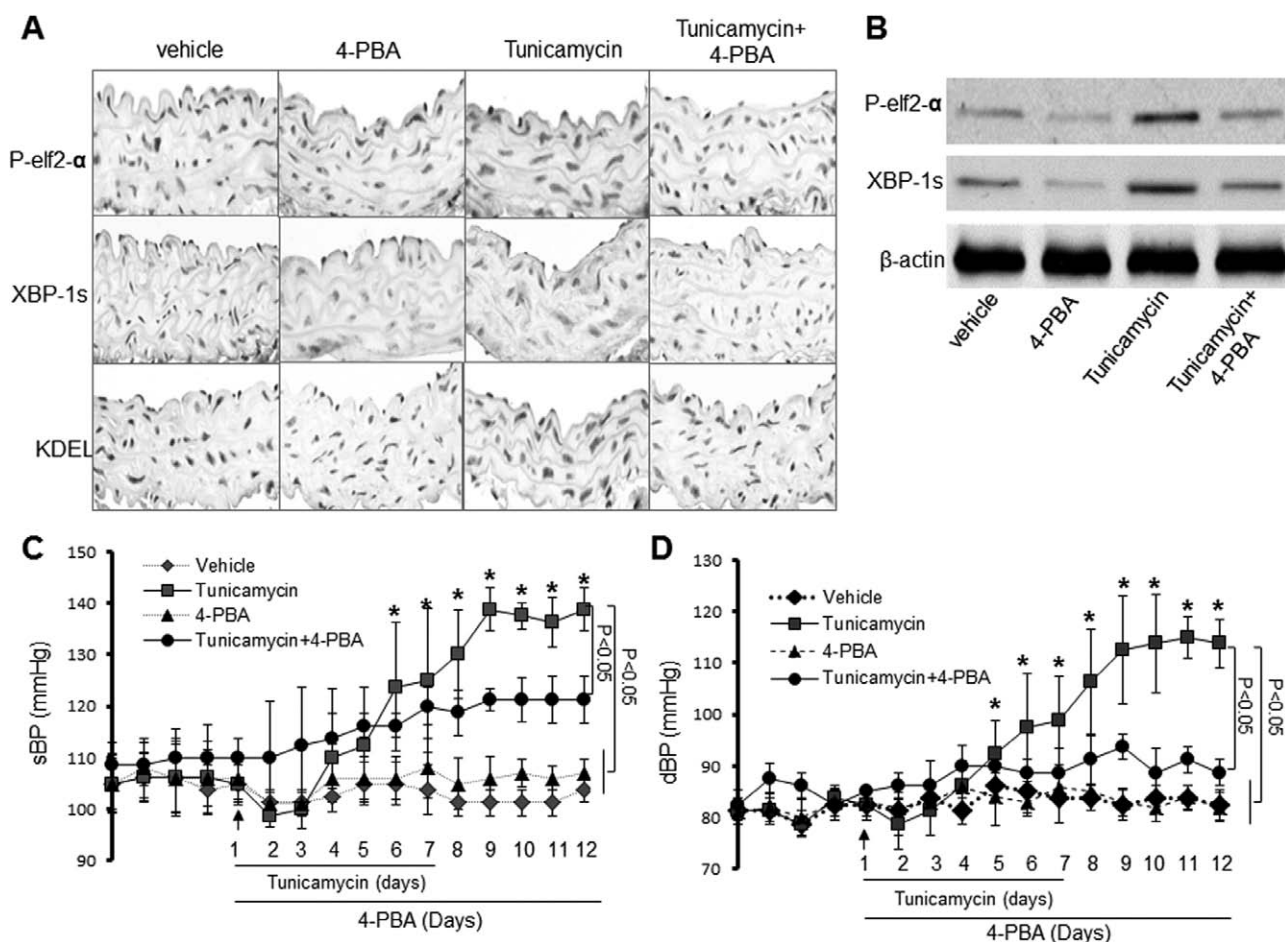


Figure 1. Acute infusion of Tunicamycin causes a 4-Phenyl butyric acid (4-PBA)-inhibitable high blood pressure in vivo. Mice were infused with Tunicamycin with or without 4-PBA. Blood pressures and heart rates were monitored by telemetry, as described in Materials and Methods. **A**, Effects of 4-PBA on Tunicamycin-induced endoplasmic reticulum (ER) stress in aortas in vivo. The image is a representative of 5 images from ≥ 5 mice. **B**, 4-PBA reduces ER stress markers in Tunicamycin-induced ER stress in aortas in vivo. The blot is a representative of 6 blots from 6 individual experiments. **C**, Effects of 4-PBA on Tunicamycin-induced elevation of systolic blood pressure (sBP); $n=12$ to 16 in each group, $*P<0.05$. **D**, Effects of 4-PBA on Tunicamycin-induced changes of diastolic blood pressure (dBP). $n=12$ to 16 in each group, $*P<0.05$. The data from **C** and **D** were analyzed using the 2-way ANOVA for repeated measures followed by the Bonferroni post hoc test.

arteries, we used isolated mouse aortas to assay the effects of ER stress inducers on vasocontractility. As depicted in Figure 2C and 2D, PE caused vasoconstriction in a dose-dependent manner. Incubation of aortic rings with either Tunicamycin or MG132 markedly accentuated PE-induced vasoconstriction (Figure 2C and 2D). In addition, coinubation with 4-PBA, an ER stress inhibitor, markedly lowered vasoconstriction enhanced by either 4-PBA or MG132 (Figure 2C and 2D), implying that ER stress potentiates PE-dependent vasoconstriction.

AICAR Attenuates ER Stress-Accentuated Vasoconstriction

Because AICAR reduced Tunicamycin- and MG132-induced ER stress (Figure 2A and 2B), we hypothesized that AICAR might suppress Tunicamycin-enhanced vasoconstriction in response to PE. As shown in Figure 2C, AICAR abrogated the Tunicamycin-enhanced PE response in isolated aortas. Similarly, AICAR abolished MG132-potentiated PE-induced vasoconstriction (Figure 2D).

ER Stress-Enhanced PE-Induced Vasoconstriction Is Endothelium Independent

We had reported previously that AMPK activation suppressed ER stress in endothelial cells.^{17,18} It was important to determine whether ER stress in the endothelium contributed to ER stress-enhanced vasoconstriction in response to PE. To this end, endothelium-denuded aortas were incubated with Tunicamycin before its incubation with PE. As shown in Figure 2E, Tunicamycin-induced PE response was comparable between endothelium-intact aortas and the endothelium-denuded aortas.

One of the most important factors in maintaining vasotone is nitric oxide (NO). We next investigated the effects of Tunicamycin in the aortas isolated from endothelial NO synthase (eNOS)-deficient mice (eNOS^{-/-}). PE caused a dose-dependent vasoconstriction in the aortas from WT and eNOS^{-/-} mice (Figure 2F). Exposure of mouse aortas to Tunicamycin markedly increased PE-triggered vasoconstriction in WT and eNOS^{-/-} mice (Figure 2F), suggesting that ER stress in the endothelium was not involved in Tunicamycin-enhanced PE response.

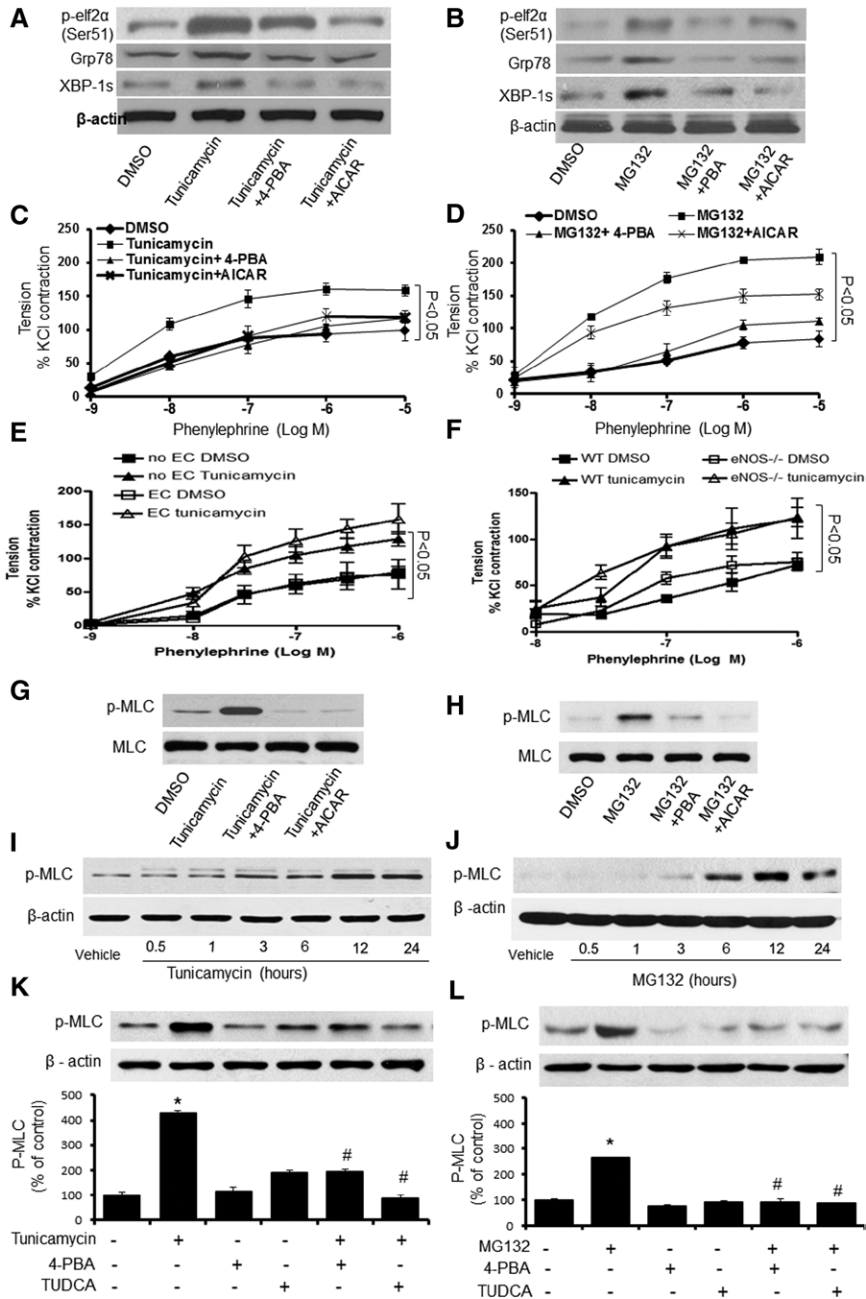


Figure 2. Endoplasmic reticulum (ER) stress enhances vasoconstriction and the phosphorylation of myosin light chain (MLC) in isolated vessels. **A**, Effects of 4-phenyl butyric acid (4-PBA) and 5-Aminoimidazole-4-carboxamide ribonucleotide (AICAR) on Tunicamycin-induced ER stress in isolated mouse aortas; n=24 measurements from 6 mice per group. **B**, Effects of 4-PBA and AICAR on MG132-induced ER stress in isolated mouse aortas; n=24 measurements from 6 mice per group. **C**, Effects of 4-PBA and AICAR on Tunicamycin-induced vasoconstriction. Results are mean±SEM (n=24 from 6 mice per group). **D**, Effects of 4-PBA and AICAR on MG132-induced vasoconstriction; vessel contraction in isolated aortas from WT mice treated with 4-PBA and AICAR after MG132 incubation for 2 hours; n=24 measurements from 6 mice per group. **E**, Effects of Tunicamycin on phenylephrine (PE)-induced vasoconstriction in endothelium-denuded aortic rings. Contraction to phenylephrine in aortic segments of 8- to 10-week-old wild-type (WT) mice scraped endothelial cells and treatment with Tunicamycin. Results are mean±SEM (n=6 from 6 mice per group). *P<0.05 vs Tunicamycin. **F**, Effects of endothelial nitric oxide synthase (eNOS) deficiency on Tunicamycin-induced vasoconstriction in isolated aortas. Aortic segments of 10-week-old WT mice or eNOS^{-/-} mice were exposed to PE after being treated with Tunicamycin; n=24 measurements from 6 mice per group. *P<0.05 vs Tunicamycin. **G** and **H**, Effects of AICAR on Tunicamycin- or MG132-induced phosphorylation of MLC in isolated mouse aortas. **I** and **J**, Immunoblots showing the levels of MLC phosphorylation in human smooth muscle cells (HSMCs) treated with Tunicamycin or MG132 for indicated hours. The blot is a representative of 6 blots from 6 mice; n=6. **K** and **L**, Immunoblots showing the levels of phosphorylated (p-)MLC in HSMCs treated with 4-PBA or tauroursodeoxycholic acid (TUDCA) for 3 hours after incubation with Tunicamycin (**G**) or MG132 (**H**) for 2 hours; n=5. *P<0.05 Tunicamycin- or MG132-treated vs vehicle-treated; #P<0.05 Tunicamycin- or MG132-treated vs 4-PBA plus Tunicamycin or 4-PBA plus MG132. The data from **A** to **F** were analyzed using the 2-way ANOVA for repeated measures followed by the Bonferroni post hoc test. Statistical analysis for **I** to **L** was performed using a 2-tailed Student *t* test between 2 groups.

ER Stress-Dependent Phosphorylation of Myosin Light Chain in Isolated Aortas

The phosphorylation of myosin light chain (MLC) facilitates the stimulation of myosin ATPase activity by α -actin, leading to cross-bridge cycling and contraction. Thus, it was important to evaluate the effects of ER stress on MLC phosphorylation in isolated aortas. As expected, exposure of isolated aortic rings to Tunicamycin or MG132 markedly increased the levels of phosphorylated (p-)MLC (Figure 2G and 2H). Importantly, inhibition of ER stress with 4-PBA abolished the effects of Tunicamycin and MG132 on p-MLC (Figure 2G and 2H), implying that ER stress mediated the increase of p-MLC. Similar to 4-PBA, AICAR attenuated Tunicamycin- or MG132-mediated MLC phosphorylation (Figure 2G and 2H), suggesting that the effects of AICAR on p-MLC are likely via its inhibition on ER stress.

ER Stress Triggers MLC Phosphorylation in Cultured VSMCs

Because vasocontractility is mainly dictated by VSMCs in vasculature, we next determined whether Tunicamycin or MG132 altered p-MLC in cultured VSMCs. As depicted in Figure 2I, Tunicamycin-enhanced MLC phosphorylation occurred at 3 hours and reached maximal at 12 hours (Figure 2I). Similarly, MG132 (500 nmol/L) markedly increased the

levels of p-MLC at 2 hours and reached a peak at 12 hours (Figure 2J). Importantly, pretreatment of tauroursodeoxycholic acid (TUDCA) or 4-PBA for 2 hours attenuated the effects of Tunicamycin or MG132 on MLC phosphorylation in VSMCs (Figure 2L and 2K), suggesting an ER stress-dependent MLC phosphorylation in VSMCs.

AMPK Activation Suppresses ER Stress-Enhanced MLC Phosphorylation in VSMCs

We next determined whether AMPK activators, AICAR and metformin, altered ER stress-induced MLC phosphorylation. As depicted in Figure 3A and 3B, treatment with AICAR or metformin markedly reduced Tunicamycin-induced ER stress in cultured VSMCs. Pretreatment with metformin or AICAR for 1 hour attenuated the levels of p-elf2 α , Grp78, and XBP-1s (Figure 3A and 3B). The maximal effect of metformin was observed at 2 hours. The maximal effect of AICAR was seen at 4 hours after the treatment (data not shown). Concomitantly, AICAR and metformin also markedly suppressed Tunicamycin- or MG132-enhanced MLC phosphorylation in VSMCs (Figure 3C and 3D).

p-MLC is regulated through myosin light-chain kinase and myosin light-chain phosphatase. Thus, it was interesting to determine whether ER stress affected MLC phosphatase. Because RhoA and Rho kinase, components of

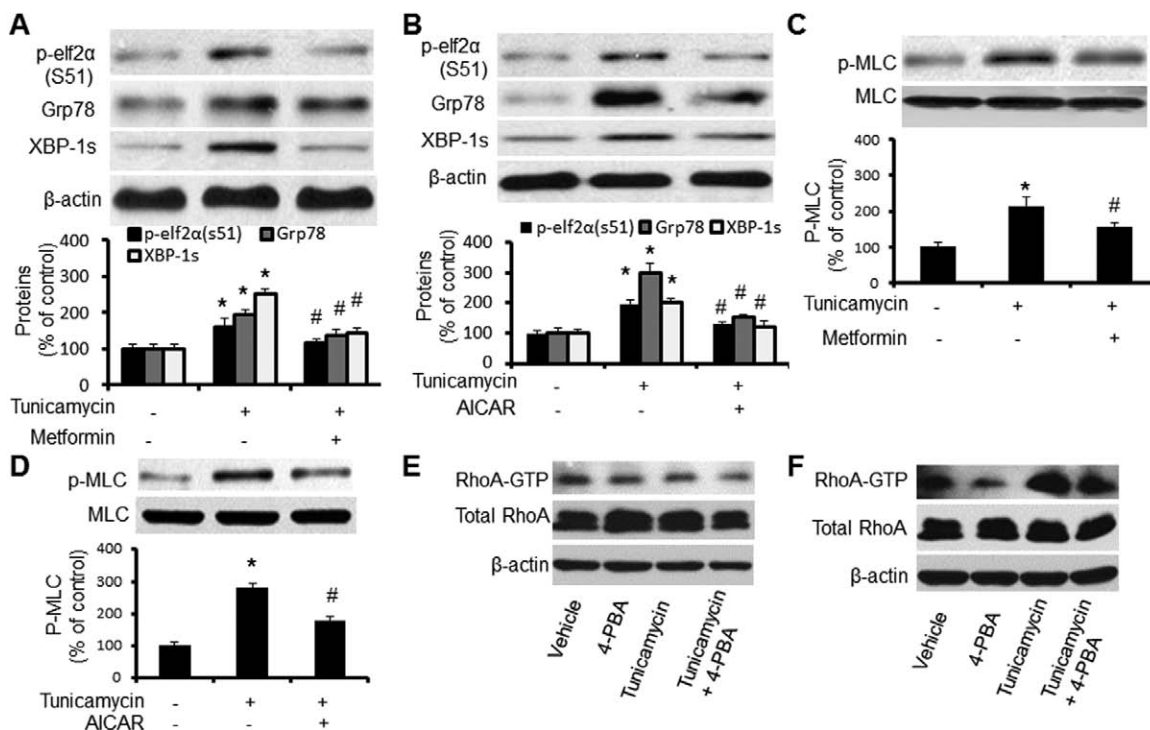


Figure 3. AMP-activated protein kinase (AMPK) activation suppresses endoplasmic reticulum (ER) stress-potentiated myosin light chain (MLC) phosphorylation in cultured vascular smooth muscle cells (VSMCs). **A** and **B**, Immunoblots showing the levels of ER stress relative protein P-elf2 α (s51), Grp78, and spliced XBP-1 in human smooth muscle cells (HSMCs) treated with 5-aminoimidazole-4-carboxamide ribonucleotide (AICAR; **A**) and metformin (**B**) after Tunicamycin incubation for 6 hours; $n=5$. * $P<0.05$, Tunicamycin vs vehicle; # $P<0.05$, Tunicamycin vs Tunicamycin plus AICAR or metformin. **C** and **D**, Effects of AICAR and metformin on Tunicamycin-induced MLC phosphorylation in VSMCs. VSMCs were incubated with indicated concentrations of Tunicamycin for 6 hours; $n=5$. * $P<0.05$ Tunicamycin vs vehicle; # $P<0.05$ Tunicamycin vs Tunicamycin plus AICAR or metformin. **E** and **F**, Effects of Tunicamycin on RhoA kinase. Confluent VSMCs were treated with tunicamycin for 4 hours (**E**) or 6 hours (**F**) with or without 4-phenyl butyric acid (4-PBA). After the incubation, cells were collected and GTP-bound RhoA was pull-down/detected by total RhoA antibody. The blot is representative of 5 blots from 5 independent experiments. Statistical analysis for **A** to **C** was performed using a 2-tailed Student t test between 2 groups.

the Ca^{2+} -independent pathway for maintaining muscle contraction, inhibited MLC phosphatase activity, we further determined whether RhoA kinase was required for ER stress-enhanced MLC phosphorylation in VSMCs. Cultured VSMCs were stimulated with Tunicamycin with or without 4-PBA, and GTP-bound RhoA was pulled down and Western blotted with the antibody against RhoA antibody. Short incubation of Tunicamycin (<6 hours) did not alter RhoA-GTP contents (Figure 3E). Thus, increased p-MLC was before RhoA activation by Tunicamycin. Longer incubation of Tunicamycin (>6 hours) markedly increased the level of RhoA-GTP without altering total RhoA levels, which were partially attenuated by 4-PBA (Figure 3F). Taken together, RhoA activation unlikely contributed to early phosphorylation of p-MLC caused by ER stress.

AMPK Activation Suppresses ER Stress–Enhanced Intracellular Calcium but Increases Calcium in ER

Previous studies^{31,32} had demonstrated that elevation of intracellular Ca^{2+} is a common mechanism for aberrant ER stress and unfolded protein response activation. Importantly, p-MLC is regulated through MLC kinase via intracellular Ca^{2+} elevation. Therefore, we tested whether inhibition of AMPK increased ER stress by altering intracellular Ca^{2+} levels. As expected, Tunicamycin caused a rapid increase of cytosolic Ca^{2+} in parallel with a reduction of Ca^{2+} in ER (Figure 4A); pretreatment with AICAR or metformin significantly reduced Tunicamycin-induced rise of intracellular Ca^{2+} , whereas they increased the levels of Ca^{2+} in ER (Figure 4A). Consistently,

inhibition of AMPK with compound C markedly increased the levels of intracellular Ca^{2+} (Figure 4B).

To exclude the potential off-target effects of compound C, we assayed the intracellular Ca^{2+} concentration in VSMCs isolated from WT and $\text{AMPK}\alpha 2^{-/-}$ mice. As depicted in Figure 4C, intracellular Ca^{2+} levels in $\text{AMPK}\alpha 2^{-/-}$ VSMCs were significantly elevated when compared with VSMCs isolated from WT mice (Figure 4C). Consistently, ER Ca^{2+} levels were significantly lower in VSMCs from $\text{AMPK}\alpha 2^{-/-}$ mice, as compared with those obtained from WT mice ($P<0.05$; Figure 4C). Finally, free cytoplasmic calcium [Ca^{2+}] /relative fluorescence units were significantly elevated in VSMCs from $\text{AMPK}\alpha 2^{-/-}$ mice when compared with those from WT mice (Figure 4D). Taken together, these results suggest that reduction of AMPK activity increased the intracellular Ca^{2+} levels but exhibited reduced levels of Ca^{2+} in the ER.

Aberrant ER Stress Contributes to Elevated Levels of MLC Phosphorylation in $\text{AMPK}\alpha 2^{-/-}$ Mice

It was interesting to determine whether aberrant ER stress altered the MLC phosphorylation in $\text{AMPK}\alpha 2^{-/-}$ mice in vivo. As depicted in Figure 5A, $\text{AMPK}\alpha 2^{-/-}$ mice exhibited higher levels of p-elf2 α (Ser51), Grp78, and XBPs than those from WT mice, which were attenuated by the pretreatment of TUDCA or 4-PBA. Importantly, the levels of p-MLC were higher in $\text{AMPK}\alpha 2^{-/-}$ mice than those in WT mice (Figure 5B). Furthermore, inhibition of ER stress with TUDCA or 4-PBA normalized the levels of p-MLC in $\text{AMPK}\alpha 2^{-/-}$ mice, although it did not alter the levels of p-MLC in WT mice

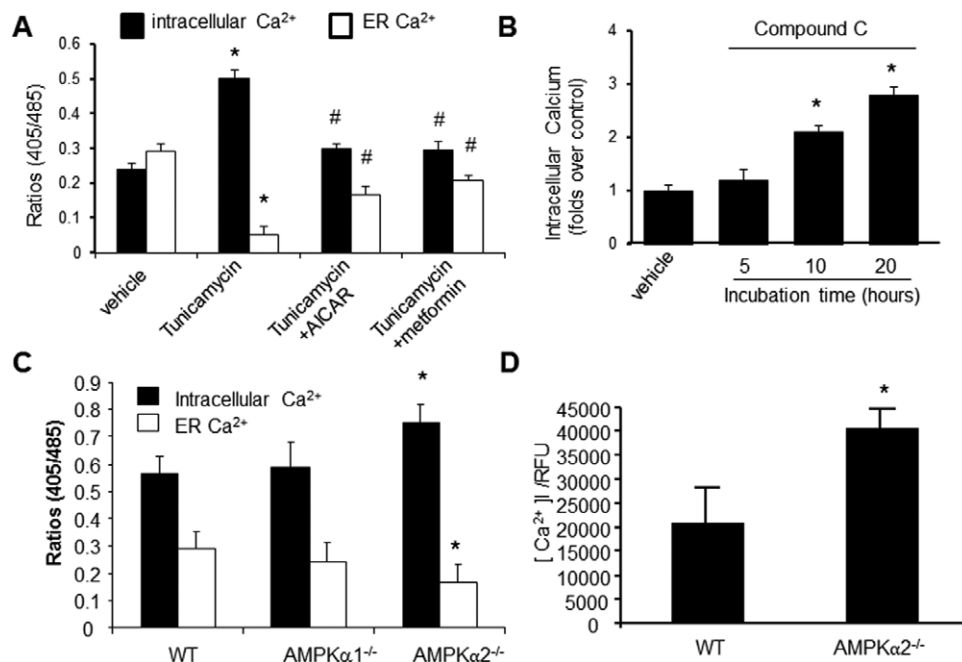


Figure 4. AMP-activated protein kinase (AMPK) activation on intracellular Ca^{2+} and endoplasmic reticulum (ER) Ca^{2+} in cultured vascular smooth muscle cells (VSMCs). After being loaded with Indo-1/AM dye, the cells were stimulated with ionomycin (10 $\mu\text{mol/L}$), which triggers Ca^{2+} release from the ER. **A**, 5-Aminoimidazole-4-carboxamide ribonucleotide (AICAR) and metformin suppress the rise of intracellular [Ca^{2+}]_i, whereas they increase Ca^{2+} in the ER. * $P<0.05$ Tunicamycin vs vehicle; # $P<0.05$ AICAR or metformin plus Tunicamycin vs Tunicamycin alone; n=5. * $P<0.05$ Tunicamycin vs vehicle; # $P<0.05$ Tunicamycin vs Tunicamycin plus AICAR or metformin. **B**, Effects of compound C on ionomycin-induced Ca^{2+} release and intracellular Ca^{2+} store; * $P<0.05$ compound C vs vehicle; n=6, * $P<0.05$ compound C vs vehicle. **C**, Increased levels of intracellular Ca^{2+} and decreased Ca^{2+} in ER in VSMCs derived from $\text{AMPK}\alpha 2^{-/-}$; $\text{AMPK}\alpha 2^{-/-}$ vs wild-type (WT); n=7; * $P<0.05$ WT vs $\text{AMPK}\alpha 2^{-/-}$. **D**, Increased [Ca^{2+}]_i/relative fluorescence units (RFUs) in VSMCs from $\text{AMPK}\alpha 2^{-/-}$ mice. $\text{AMPK}\alpha 2^{-/-}$ vs WT. n=8. Statistical analysis was performed by using a 2-tailed Student *t* test between 2 groups.

(Figure 5A and 5B). These results suggested that ER stress in AMPK $\alpha 2^{-/-}$ mice caused increased MLC phosphorylation.

Inhibition of ER Stress Normalizes PE-Induced Vasoconstriction in AMPK $\alpha 2^{-/-}$ Mice

We next determined whether aberrant ER stress was responsible for increased PE-induced vasoconstriction in AMPK $\alpha 2^{-/-}$ mice. As depicted in Figure 5C and 5D, PE caused greater vasoconstriction responses in AMPK $\alpha 2^{-/-}$ mice than those from WT mice. Incubation of 4-PBA or TUDCA alone did not alter the vasoconstriction response in WT aortas (data not shown). However, either 4-PBA or TUDCA markedly suppressed PE-induced vasoconstriction (Figure 5C and 5D), suggesting that heightened ER stress was likely responsible for increased vasoconstriction responses in AMPK $\alpha 2^{-/-}$ mice.

Inhibition of ER Stress Lowers High Blood Pressure in AMPK $\alpha 2^{-/-}$ Mice In Vivo

We had found previously that genetic deletion of AMPK $\alpha 2$ led to elevated blood pressure in C57BL6 mice²⁶ and that this also caused an elevated level of ER stress and disturbed intracellular Ca²⁺ balance. We therefore reasoned that inhibition of ER stress might lower high blood pressure observed in AMPK $\alpha 2^{-/-}$ mice. To this end, WT and AMPK $\alpha 2^{-/-}$ mice were administered 4-PBA for 2 weeks. As expected, the levels of ER stress markers (p-elf2 α , XBP-1s, and KDEL) were higher in AMPK $\alpha 2^{-/-}$ mice when compared with WT mice (Figure 6A). In addition, 4-PBA markedly decreased the levels of ER stress markers (p-elf2 α , XBP-1s, and KDEL) in AMPK $\alpha 2^{-/-}$ mice in vivo (Figure 6A). As shown in Figure 6B and 6C, 4-PBA significantly lowered both sBP and dBP in AMPK $\alpha 2^{-/-}$ mice in vivo.

Inhibition of ER Stress Lowers High Blood Pressure in Mice Infused With Angiotensin II In Vivo

The renin-angiotensin system is essential in maintaining blood pressure. Thus, it was important to determine whether ER stress contributes to angiotensin II (Ang II)-induced hypertension in vivo. As reported previously,³³ Ang II infusion caused an increase in sBP and dBP in WT animals (Figure 7A and 7B). As expected, 4-PBA lowered both sBP and dBP at day 7 after Ang II infusion (Figure 7A and 7B). The effects of 4-PBA reached a maximal effect at day 11 for both parameters (Figure 7A and 7B). In contrast, administration of Ang II in AMPK $\alpha 2^{-/-}$ mice resulted in a more marked rise in sBP and dBP (Figure 7C and 7D). Similarly, 4-PBA cotreatment in AMPK $\alpha 2^{-/-}$ mice resulted in a partial normalization of both sBP and dBP starting at day 7. The effects of 4-PBA on both parameters reached maximal at day 11 (Figure 7C and 7D). The effects of 4-PBA in AMPK $\alpha 2^{-/-}$ mice were smaller than those in WT mice (Figure 7E and 7F). The pressures in AMPK $\alpha 2^{-/-}$ mice remained ≈ 5 - to 10-mmHg higher than controls (4-PBA only; Figure 7C and 7D). These results might be explained by heightened ER stress levels in AMPK $\alpha 2^{-/-}$ mice.

Discussion

In this study, we have provided the first evidence that ER stress can significantly elevate blood pressure in vivo and that this effect is ameliorated by AMPK activation. ER stress inducer Tunicamycin increases VSMC phosphorylation of MLC, vasocontractility, and blood pressure. Importantly, chronic administration of ER stress chaperone lowers blood pressure in Ang II-infused mice and AMPK $\alpha 2^{-/-}$ mice, suggesting that AMPK inhibition-triggered ER stress in VSMCs might play a causative role in the development and progression of high blood pressure in vivo.

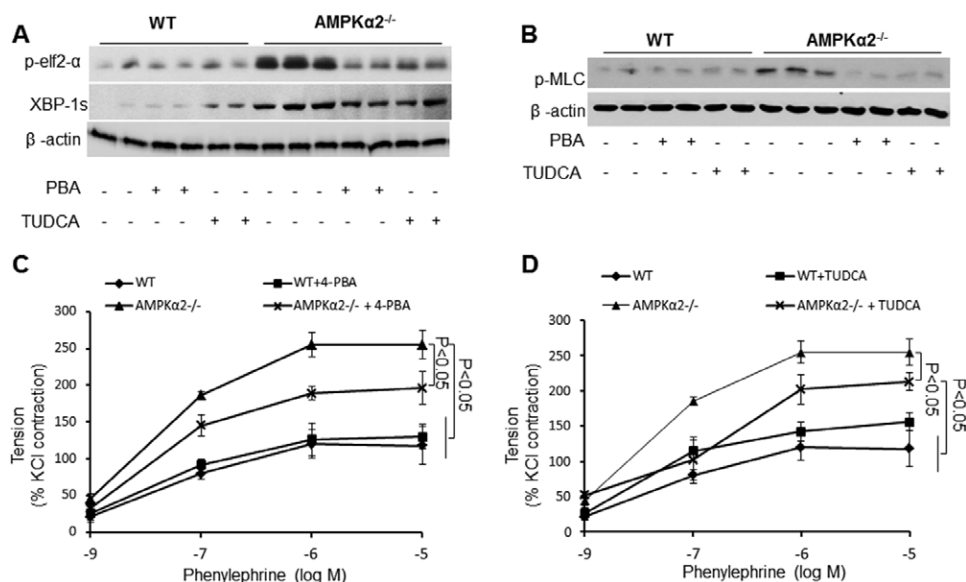


Figure 5. Inhibition of aberrant endoplasmic reticulum (ER) stress normalizes vasoconstriction and myosin light chain (MLC) phosphorylation in AMP-activated protein kinase (AMPK) $\alpha 2^{-/-}$ mice. Vessels were pretreated with tauroursodeoxycholic acid (TUDCA) or 4-phenyl butyric acid (4-PBA) for 2 hours. **A**, Immunoblots of P-elf2 α (Ser51), Grp78, and spliced XBP-1 in aortas from mice treated with 4-PBA or TUDCA for 3 weeks. The blot is a representative of 3 blots from ≥ 3 mice. **B**, Immunoblots of phosphorylated (p-) MLC in aortas from mice treated with 4-PBA or TUDCA for 3 weeks. The blot is a representative of 3 blots from ≥ 3 mice. **C**, Vessel contraction in isolated aortas from wild-type (WT) and AMPK $\alpha 2^{-/-}$ mice pretreated with 4-PBA for 2 hours; $n=6$. **D**, Effects of TUDCA on vessel contraction in isolated vessels from WT and AMPK $\alpha 2^{-/-}$ mice; $n=6$. The data from **C** and **D** were analyzed by using the 2-way ANOVA for repeated measures followed by Bonferroni post hoc test.

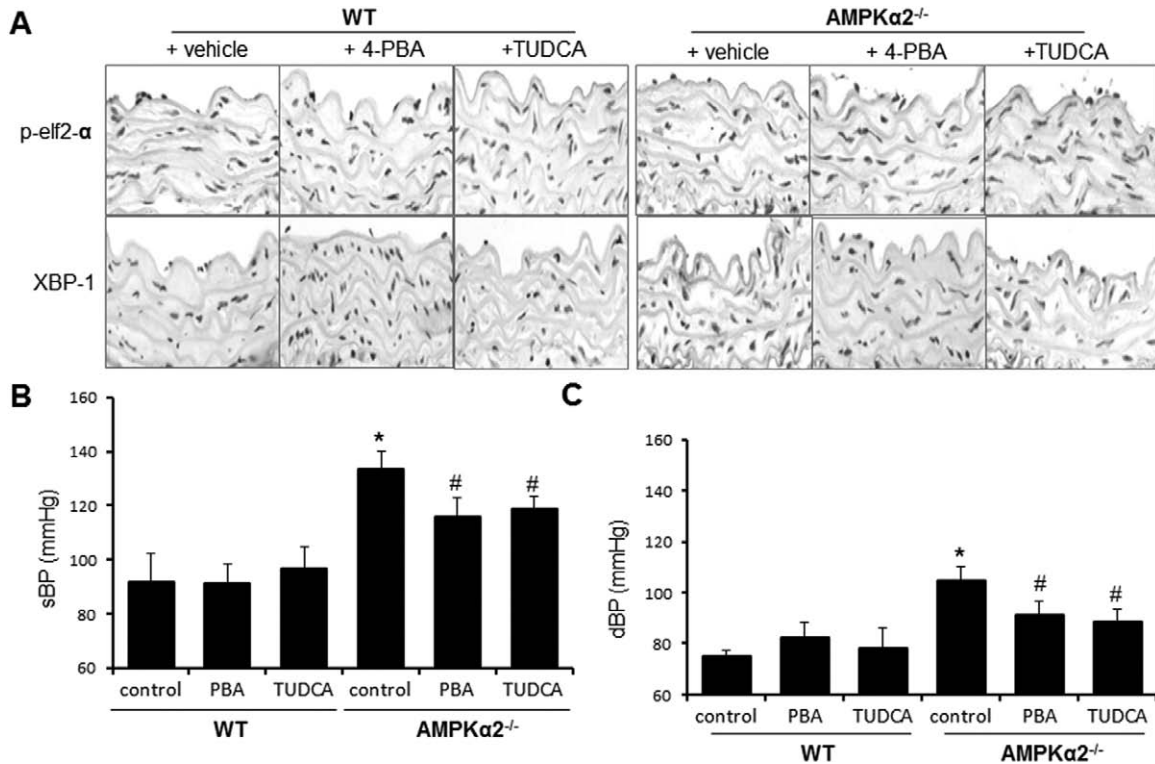


Figure 6. Endoplasmic reticulum (ER) stress chaperones normalize blood pressure in AMP-activated protein kinase (AMPK) α 2^{-/-} mice. Wild-type (WT) mice and AMPK α 2^{-/-} mice were orally administered with 4-phenyl butyric acid (4-PBA; 1 g/kg per day) or tauroursodeoxycholic acid (TUDCA; 0.5 g/kg per day) for 3 weeks, as described in Materials and Methods. **A**, 4-PBA and TUDCA suppress aberrant ER stress in AMPK α 2^{-/-} mice in vivo. ER stress markers were detected in immunohistochemical staining with the specific antibodies. The image is a representative of 6 images from 6 mice. **B**, Effects of 4-PBA and TUDCA on systolic blood pressure (sBP); n=12 in each group. * P <0.05 WT vs AMPK α 2^{-/-}. # P <0.05 vs AMPK α 2^{-/-} vs AMPK α 2^{-/-} mice treated with 4-PBA or TUDCA. **C**, Effects of 4-PBA and TUDCA on diastolic blood pressure (dBP); n=12 in each group. * P <0.05 WT vs AMPK α 2^{-/-}. # P <0.05 vs AMPK α 2^{-/-} vs AMPK α 2^{-/-} mice treated with 4-PBA or TUDCA. Statistical analysis for **B** and **C** was performed by using a 2-tailed Student *t* test between 2 groups.

Blood pressure is maintained by both cardiac output and peripheral vascular resistance.³² ER stress might cause hypertension by increasing cardiac output and peripheral vascular resistance. Indeed, increased sBP and dBP were found in mice infused with Tunicamycin and in AMPK α 2^{-/-} mice in which aberrant ER stress was observed. Importantly, administration of ER chaperones significantly reduced both sBP and dBP in AMPK α 2^{-/-} mice and in mice infused with Ang II. The most provocative finding in this study is that ER stress causes abnormal contractility of vascular smooth muscle, resulting in increased blood pressure. This conclusion is supported by the following findings. First, induction of ER stress by Tunicamycin or MG132 markedly increased PE-mediated vasoconstriction, which was further confirmed by increased detection of p-MLC. These effects of Tunicamycin were ablated by ER chaperones such as 4-PBA. Second, acute infusion of Tunicamycin caused a marked elevation of both sBP and dBP. Coadministration of 4-PBA, which alone had no effect on blood pressure, abolished the effects of Tunicamycin, suggesting an ER stress-dependent elevation of blood pressure. Third, coinfusion of 4-PBA significantly lowered both sBP and dBP in mice infused with Ang II, suggesting that ER stress might, at least partially, contribute to high blood pressure caused by Ang II infusion, a well-characterized model of hypertension. Finally, AMPK α 2^{-/-} mice, which exhibited aberrant levels of ER stress, were hypertensive with increased contractility. Importantly, administration of 4-PBA and TUDCA

markedly lowered sBP and dBP in AMPK α 2^{-/-} mice but not in WT mice, providing further support that ER stress is essential in maintaining normal blood pressure. Indeed, aberrant ER stress is also reported in hypertensive patients,^{32,33} or animals³⁴ with metabolic syndrome and in hypertensive animals such as high salt intake-induced hypertensive rats³⁵ and Ang II-infused hypertensive mice.³⁶ Taken together, our results strongly suggest that 4-PBA via ER stress inhibition inhibited vascular contractility and high blood pressure. However, our results have not established a causative relation among ER stress, vascular contractility, and high blood pressure. Although we consider that this possibility is low, we cannot exclude the possibility that 4-PBA might have myriad effects on blood pressure that are independent of ER stress or vascular contractility. Further study on a causative effect of ER stress, vascular contractility, and blood pressure is warranted.

Recent studies have found that activation of AMPK is an important defensive response to ER stress in cardiomyocytes.^{37,38} Our own previous studies have also found that activation of AMPK exerts protective effects in endothelial cells by suppressing ER stress in the genesis of atherosclerosis.^{17,18} Here we have extended these observations in VSMCs and found that AMPK, at least in part via its suppression on ER stress in VSMCs, maintains normal vascular tones. AMPK α 2 mice exhibited increased vasoconstriction to PE and exhibited high blood pressure, which were sensitive to ER chaperones. The molecular mechanisms by which AMPK inhibits vascular contractility and lowers

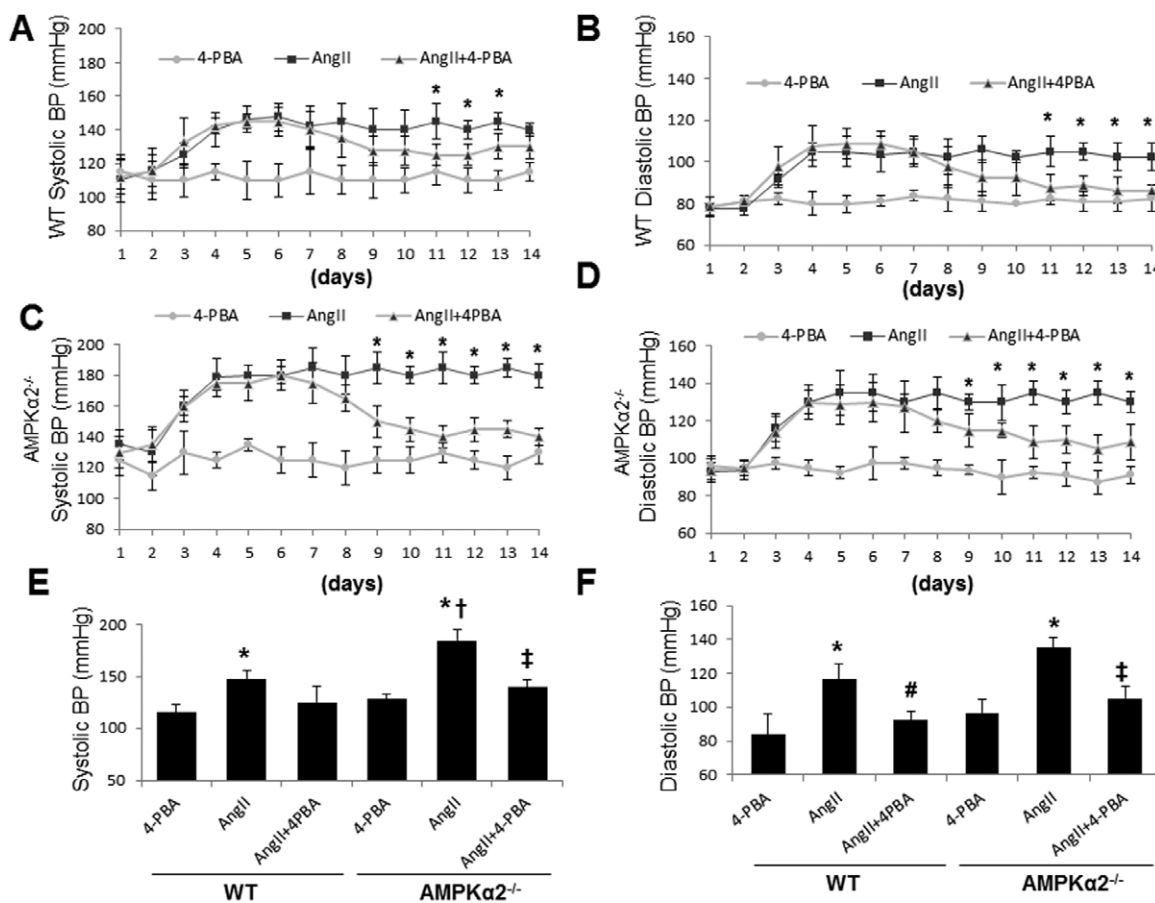


Figure 7. Suppression of endoplasmic reticulum (ER) stresses by 4-phenyl butyric acid (4-PBA) lowers angiotensin II (Ang II)-induced high blood pressure. The systolic blood pressure and diastolic blood pressure were monitored by telemetry system. Means±SEMs for each group (n=12) are presented for both AMP-activated protein kinase (AMPK) $\alpha 2^{-/-}$ and wild-type (WT) mice. * $P < 0.05$ Ang II vs 4-PBA or 4-PBA plus Ang II. **A** and **B**, WT mice were treated with 4-PBA for 2 weeks after Ang II treatment for 5 days; n=10 in each group. * $P < 0.05$ Ang II vs 4-PBA or 4-PBA plus Ang II. **C** and **D**, AMPK $\alpha 2^{-/-}$ mice were treated with 4-PBA for 2 weeks after Ang II treatment for 5 days; n=10 in each group. * $P < 0.05$ Ang II vs 4-PBA or 4-PBA plus Ang II. **E** and **F**, Systolic and diastolic blood pressures in AMPK $\alpha 2^{-/-}$ and WT mice treated with or without 4-PBA. * $P < 0.05$ Ang II vs 4-PBA, # $P < 0.05$ Ang II vs 4-PBA plus Ang II, † $P < 0.05$ Ang II in WT vs Ang II in AMPK $\alpha 2^{-/-}$, and ‡ $P < 0.05$ Ang II vs Ang II plus 4-PBA. The data were analyzed by using the 2-way ANOVA for repeated measures followed by Bonferroni post hoc test.

blood pressure might be multiple. In this study, we found that ER stress promotes the phosphorylation of MLC, likely via the reduction of intracellular Ca^{2+} . This finding is consistent with our previous study, which demonstrates that AMPK functions as a physiological suppressor of ER stress by maintaining sarco/ER Ca^{2+} -ATPase activity and intracellular Ca^{2+} homeostasis in endothelial cells.¹⁸ In addition, AMPK has been shown to lower blood pressure by improving NO bioactivity and endothelial cell function.²⁶ This has been mechanistically attributed to increasing NO release by enhancing the phosphorylation and activation of eNOS at Ser1177 and Ser633 and by blocking NO inactivation by reactive oxygen species.²⁶ Although the contribution of the AMPK-eNOS-ER stress axis in maintaining normal blood pressure remains to be investigated, the present study provides new evidence in support of an ER stress-AMPK-MLC axis that partly explains the hypertensive phenotype of mice deficient in AMPK $\alpha 2$, thereby establishing a novel role of AMPK in the regulation of blood pressure.

Clinical data strongly support that AMPK might be a therapeutic target in treating hypertension. Metformin, one of mostly used antidiabetic drugs, is a potent activator of AMPK. AMPK-lowering effects are supported by blood-lowering

effects of metformin.³⁹⁻⁴¹ In women with polycystic ovarian syndrome, metformin reduced sBP, hyperinsulinemia, and insulin resistance and facilitated menstrual regulation and pregnancy.⁴² Metformin also improves blood pressure in hypertensive and obese women.⁴³

In summary, our results have demonstrated that AMPK is essential in maintaining ER function in VSMCs, and aberrant ER stress might play a causative role in the development and progression of hypertension.

Sources of Funding

This study was supported by funding from the National Institutes of Health RO1 (HL074399, HL079584, HL080499, HL08920, HL096032, HL105157, and HL110488), the American Diabetes Association, and the Warren Endowed Chair of the University of Oklahoma Health Science Center (all to M. Zou). Part of this work was also supported by an international cooperation grant from the Chinese National Science Foundation (to Y. Zhu and M. Zou). M. Zou is a recipient of the National Established Investigator Award of the American Heart Association.

Disclosures

None.

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Online supplement

Aberrant Endoplasmic Reticulum Stress in Aortic Vascular Smooth Muscle Increases Vascular Contractility and Blood Pressure in Mice Deficient of AMP-activated Protein Kinase alpha 2 *in vivo*

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Materials and methods

Materials

Tunicamycin, 4-PBA, Ionomycin, Pluronic F-127 and Indo-1 were purchased from Sigma-Aldrich (St. Louis, MO). TUDCA, Compound C was from Calbiochem (San Diego, CA). AICAR was purchased from Toronto Research Chemicals Inc. (Toronto, Canada). All other chemicals, if not indicated, were purchased from Sigma-Aldrich (St. Louis, MO). Antibodies against total AMPK α , phosphor-AMPK α (T172), phospho-myosin light chain 2 (Thr18/Ser19), phospho-eIF2 α , were obtained from Cell Signaling Biotechnology (Danvers, MA). Antibodies against AMPK α 1, AMPK α 2 and XBP1 were obtained from Santa Cruz Biotechnology Inc (Santa Cruz, CA). Antibody against GRP78 was from Abcam (Cambridge, MA). Antibody against KDEL (which recognizes GRP78 and GRP94) was purchased from Stressgen (San Diego, CA). Osmotic mini-pumps were purchased from DURECT Corporation (Cupertino, CA).

Animals

Mice deficient of AMPK α 1 (AMPK α 2^{-/-}) or α 2 (AMPK α 1^{-/-}) were generated as described previously¹. Mice deficient of endothelial nitric oxide synthase were purchased from Jackson Laboratory. Mice were housed in temperature-controlled cages under a 12-h light-dark cycle and given free access to water and a regular rodent diet. The experimental procedures were approved by the Institutional Animal Care and Use Committee of the University of Oklahoma Health Science Center.

Cell culture

Human VSMCs 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 mouse VSMCs from wild-type (WT), AMPKα1KO, or AMPKα2KO mice were isolated and cultured as described previously². Briefly, aortas from WT, AMPKα1^{-/-}, or AMPKα2^{-/-} mice 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.

Assays of blood pressure

Blood pressure (BP) was measured by using both the carotid catheter method and the radiotelemetry technique, as described previously^{3, 4}. For the insertion of catheter into mice carotid, mice were anesthetized with a ketamine and xylazine mixture (70:6 mg/kg, intramuscular injection) and placed under warm light (37°C). 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 catheter (PE10 tubing) containing a sterile 10% heparin-90% saline solution or sterile gel was inserted into the common carotid. For radiotelemetry technique, the catheter was connected to a TA11PA-C10 radiotelemetry transmitter (Data Sciences, Laurel, MD) and then the radiotelemetry was implanted under peritoneum. The signal was received a receiver

which was connected to a computer. The signal (blood pressure and heart rate) was recorded by a radiotelemetry data-acquisition program (Dataquest ART 3.1, Data Sciences). For carotid catheter method, blood was directed to a pressure transducer through the catheter to obtain computerized blood pressure measurements (AD instruments) after removing the clip. The mice were allowed to recover and the mean, systolic, diastolic blood pressure and heart rates were monitored for at least 30 min in conscious states every day in the late afternoon (5 pm).

Implantation of osmotic pump

Osmotic minipumps (Alzet®) filled with tunicamycin or vehicle in total 200 µl by a sterile syringe were kept in 37°C sterile PBS overnight for activation. Mice were anesthetized and then pumps were implanted under the skin in the back of the mice, as described previously⁵.

Infusion of tunicamycin and treatment with 4-PBA in mice

Five days after the catheter implantation, the mice were implanted osmotic minipumps (Alzet®), DURECT Corporation) filled tunicamycin or vehicle (DMSO). The mice were continuously infused with tunicamycin (0.01 µg/g/day, 7 days) or vehicle (DMSO).

For mice treated with 4-PBA treatment, 4-PBA was dissolved in drinking water. 4-PBA was given at the same days at a dose of 1 g/kg/day when tunicamycin infusion started (day 1). 4-PBA was continuously administered until day 12.

Preparation of aortic rings

Mice were inhaled with isopropyl alcohol to anesthesia and sacrificed by cervical dislocation. The thoracic aorta was removed carefully, and clean the fat and adherent connective tissue to the vessel carefully without hurt the vessel wall in cold Krebs's buffer (NaCl 118.3 mM, KCl 4.7 mM, MgSO₄ 1.2 mM, KH₂PO₄ 1.2 mM, CaCl₂ 2.5 mM, NaHCO₃ 25.0 mM, EDTA 0.026 mM and glucose 11.0 mM) saturated with O₂ and clean the blood in the aorta. The aorta was cut into segments of 3 mm in length with special care not to damage the endothelium. Vascular rings were suspended in an organ bath

filled with 5 mL kreb's buffer ventilated continuously with 95%O₂ and 5% CO₂ at 37°C. The contraction was measured with a force transducer (AD Instruments) connected to a multi-channels acquisition and analysis system (AD Instruments). The basal tension of the ring was 0.8 g and the vascular was allowed to equilibrate for 60 min and changed the Kreb's buffer every 15 minutes.

Measurement of aortic contraction

The 60 mM KCl were injected into organ bath to induced aorta contraction. After the contraction reached the peak, pre-warmed and oxygenated Kreb's solution was replaced and kept another 30 min equilibration period. The contractile response was elicited by pheylephrine (final Conc. 1 µM) to produce contraction response of aorta. At the plateau of contraction, accumulative tectonrigen or tectoridin samples (10-100 µM) were added into the organ bath to induce relaxation.

Western blot analysis

Cell lysates or tissue homogenates were subjected to western blot analysis, as described previously⁶. The protein content was assayed by BCA protein assay reagent (Pierce, USA). Protein of 20 µg was loaded to SDS-PAGE and then transferred to membrane. Membrane was incubated with a 1:1000 dilution of primary antibody, followed by a 1:5000 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).

Immunohistochemistry

The aortic arch was dissected, fixed in 4% paraformaldehyde for 16 h, and embedded in paraffin. Sections were deparaffinized, rehydrated, and microwaved in citrate buffer for antigen retrieval. Sections were successively incubated in endogenous peroxidase and alkaline phosphatase block buffer (Dako, Glostrup, Denmark), protein block buffer, and primary antibodies, which were incubated with sections overnight at 4°C. After rinsing in wash buffer, sections were incubated with

labeled polymer–horseradish peroxidase anti-mouse or anti-rabbit antibodies and DAB chromogen. Alternatively, they were incubated with polymer–alkaline phosphatase anti-mouse or anti-rabbit antibody and Permanent Red chromogen (EnVision G 2 Doublestain System, Dako). After the final wash, the sections were counterstained with hematoxylin.

Statistical Analysis

Values are reported as mean \pm SEM with sample sizes indicated in each legend. Distribution normality was assessed by using GraphPad Prism 4 analysis software (La Jolla, CA) and all data were normally distributed. Statistical comparisons of vasodilation (organ chamber experiments) were performed with two-way ANOVA, and intergroup differences were tested with Bonferroni inequality. All other results were analyzed by using a two-tailed student's t-test between two groups. Significance was accepted at P-value <0.05 . All statistical analyses were performed using GraphPad Prism 4 analysis software (La Jolla, CA).

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