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Metformin exaggerates phenylephrine-induced AMPK phosphorylation independent of CaMKK β and attenuates contractile response in endothelium-denuded rat aorta

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Abstract

Metformin, a widely prescribed antidiabetic drug, has been shown to reduce the risk of cardiovascular disease, including hypertension. Its beneficial effect toward improved vasodilation results from its ability to activate AMPK and enhance nitric oxide formation in the endothelium. To date, metformin regulation of AMPK has not been fully studied in intact arterial smooth muscle, especially during contraction evoked by G protein-coupled receptor (GPCR) agonists. In the present study, *ex vivo* incubation of endothelium-denuded rat aortic rings with 3 mM metformin for 2 hours resulted in significant accumulation of metformin (~600 pmoles/mg tissue), as revealed by LC-MS/MS MRM analysis. However, metformin did not show significant increase in AMPK phosphorylation under these conditions. Exposure of aortic rings to a GPCR agonist (e.g., phenylephrine) resulted in enhanced AMPK phosphorylation by ~2.5-fold. Importantly, in metformin-treated aortic rings, phenylephrine challenge showed an exaggerated increase in AMPK phosphorylation by ~9.7-fold, which was associated with an increase in AMP/ATP ratio. Pretreatment with compound C (AMPK inhibitor) prevented AMPK phosphorylation induced by phenylephrine alone and also that induced by phenylephrine after metformin treatment. However, pretreatment with STO-609 (CaMKK β inhibitor) diminished AMPK phosphorylation induced by phenylephrine alone but not that induced by phenylephrine after metformin treatment. Furthermore, attenuation of phenylephrine-induced contraction (observed after metformin

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treatment) was prevented by AMPK inhibition but not by CaMKK β inhibition. Together, these findings suggest that, upon endothelial damage in the vessel wall, metformin uptake by the underlying vascular smooth muscle would accentuate AMPK phosphorylation by GPCR agonists independent of CaMKK β to promote vasorelaxation.

Keywords

vascular smooth muscle; metformin; phenylephrine; AMPK; CaMKK β

1. Introduction

Metformin, a widely prescribed antidiabetic drug, has been shown to reduce the risk of cardiovascular disease [1]. With regard to hypertension, clinical studies reveal its blood pressure-lowering effects in some but not in all subjects with varying co-morbid conditions [cited in 2, 3]. Studies with different animal models demonstrate that its antihypertensive effects are attributed to endothelium-dependent and endothelium-independent relaxation of the underlying vascular smooth muscle in the vessel wall [2, 4-7]. Although the molecular mechanism of endothelium-dependent vasodilation by metformin has been extensively studied [8-10], it remains unclear as to how metformin regulates agonist-induced contractions in intact arterial smooth muscle.

In vascular endothelium, metformin activates AMP-activated protein kinase (AMPK), which phosphorylates endothelial nitric oxide synthase thereby increasing the production of nitric oxide, a potent vasodilator [10, 11]. In endothelium-denuded vessels, metabolic stress and 5-aminoimidazole-4-carboxamide-1- β -D-ribofuranoside (AICAR) have been shown to activate AMPK [12-15]. Importantly, activation of AMPK results in diminished myosin light chain kinase activity thereby attenuating vascular smooth muscle contraction [16]. Although metformin has been shown to inhibit vascular smooth muscle contraction [17], the intermediary role of AMPK remains unclear especially during agonist-induced contractions. For instance, metformin does not activate AMPK in endothelium-denuded porcine carotid artery [12], but it induces AMPK phosphorylation in porcine and rat aortic smooth muscle cells in culture [17, 18]. Hence, it is critically important to further investigate metformin regulation of contractile function and AMPK phosphorylation in intact arterial smooth muscle *ex vivo*.

AMPK is a heterotrimeric protein consisting of a catalytic α subunit and β and γ regulatory subunits [19]. Its activity is regulated by increases in adenosine diphosphate (ADP) and/or adenosine monophosphate (AMP) levels. In addition, increase in AMPK activity occurs through phosphorylation of Thr172 residue, inhibition of dephosphorylation of phosphorylated AMPK, and allosteric activation. The major upstream kinases that phosphorylate AMPK include liver kinase B1 (LKB1) and Ca²⁺/calmodulin-dependent protein kinase kinase- β (CaMKK β) [19, 20].

Metformin has been shown to inhibit mitochondrial respiratory chain complex 1 [21, 22], thereby diminishing ATP/ADP ratio [22] in hepatocytes. However, it does not affect AMP level or AMP/ATP ratio in skeletal muscle cells [23]. To date, metformin regulation of

nucleotide levels has not been examined in vascular smooth muscle during agonist-induced contractions. Previously, we and several other investigators have shown that, in vascular smooth muscle cells, vasoconstrictors evoke a rise in cytosolic free Ca^{2+} [24, 25] that would facilitate the activation of CaMKK β [20]. Accordingly, vasoactive peptides (e.g., GPCR agonists such as vasopressin, angiotensin II, and endothelin-1) promote an increase in AMPK phosphorylation through an intermediary activation of CaMKK β in rat aortic smooth muscle cells [16]. The objectives of the present study are to determine: i) the effects of metformin and/or phenylephrine on nucleotide levels and AMPK phosphorylation; and ii) the intermediary role of CaMKK β toward AMPK phosphorylation in intact arterial smooth muscle during isometric contractions.

Using endothelium-denuded rat aortic rings, we performed isometric tension measurements to determine how metformin regulates phenylephrine-induced smooth muscle contraction. Under the same conditions, we performed LC-MS/MS MRM analysis to determine the changes in nucleotide levels. In addition, we performed immunoblot analysis to determine metformin and/or phenylephrine regulation of AMPK phosphorylation before and after treatment with compound C (AMPK inhibitor) or STO-609 (CaMKK β inhibitor). To determine whether metformin regulation of contraction and AMPK phosphorylation occurs in a reversible manner, select studies included post-treatment washout protocols. To determine whether metformin treatment results in its uptake by smooth muscle, aortic tissue lysates were subjected to LC-MS/MS MRM analysis. The present findings demonstrate that metformin exaggerates phenylephrine-induced AMPK phosphorylation (independent of CaMKK β), which is associated with diminished smooth muscle contraction.

2. Materials and methods

2.1. Materials

Phenylephrine hydrochloride, serotonin (5-hydroxytryptamine hydrochloride), and acetylcholine chloride were purchased from Sigma-Aldrich (St. Louis, MO). Metformin hydrochloride, AICAR (5-aminoimidazole-4-carboxamide-1- β -D-ribofuranoside or acadesine), compound C (or dorsomorphin dihydrochloride), and STO-609 acetate were purchased from Tocris Bioscience (Minneapolis, MN). Phenformin hydrochloride and L-NMMA (L-N^G-monomethyl arginine acetate) were purchased from Cayman Chemical (Ann Arbor, MI). Adenosine 5'-triphosphate disodium (ATP), adenosine 5'-diphosphate monosodium (ADP), and adenosine 5'-monophosphate disodium (AMP) were purchased from EMD Millipore Chemicals (Billerica, MA). The primary antibodies for phospho-AMPK α^{Thr172} (2535) and AMPK α (2532) were purchased from Cell Signaling Technology (Danvers, MA). The primary antibody for CaMKK β (sc-50341) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The primary antibody for β -actin was purchased from Abcam (Cambridge, MA). HRP-conjugated goat anti-rabbit secondary antibody was from Bio-Rad (Hercules, CA). All other chemicals were from Fisher Scientific (Fair Lawn, NJ) or Sigma-Aldrich (St. Louis, MO).

2.2. Animals

All animal experiments were performed in accordance with the Charlie Norwood Veterans Affairs Medical Center Institutional Animal Care and Use Committee guidelines and were approved by the committee. Adult male Wistar rats (280 to 350 g, Charles River Laboratories, Inc., Wilmington, MA) were maintained in a room at a controlled temperature of 23°C with a 12:12-hr dark-light cycle. The rats had free access to water and standard rodent chow diet.

2.3. Preparation of aortic rings and isometric tension measurements

After sacrificing the rats, thoracic aorta was isolated and immediately placed in a petri-dish containing ice-cold oxygenated Krebs-Henseleit bicarbonate (KHB) buffer (118 mM NaCl, 4.7 mM KCl, 1.2 mM MgSO₄, 1.2 mM KH₂PO₄, 2.5 mM CaCl₂, 25 mM NaHCO₃, and 11 mM glucose; pH 7.4). The aorta was carefully cleaned free of adherent fat and connective tissue. Endothelium was removed by gently rubbing the luminal surface using a polyethylene tube. Endothelium-intact and endothelium-denuded aortas were then cut into 2-mm rings.

To ensure complete denudation of aortic rings, initial studies assessed acetylcholine-induced relaxation in both endothelium-intact and endothelium-denuded rings before or after treatment with nitric oxide synthase inhibitor, L-NMMA (300 µM, 30 min) [26]. In endothelium-intact rings pretreated with L-NMMA, addition of cumulative concentrations of acetylcholine (10⁻¹⁰ to 10⁻⁴ M) to phenylephrine (1 µM)-precontracted tissue resulted in vasorelaxation by 89%, compared with vehicle control. In endothelium-denuded rings, acetylcholine did not show any relaxant response with or without L-NMMA pretreatment (n = 4).

For long-term treatment studies with metformin (up to 18 hours), endothelium-denuded rings were maintained in vascular cell basal medium prior to isometric tension measurements as described in 2.4.1. For short-term treatment studies with metformin (up to 2 hours), rings were immediately mounted in organ baths for isometric tension measurements, as described in 2.4.2.

After passing stainless steel wires through the lumen, aortic rings were suspended from the isometric force displacement transducers (Model FT03; Grass Technologies, West Warwick, RI) and kept immersed in 10 ml of KHB buffer in the organ bath system (8 chambers, Radnoti Glass Technology, Monrovia, CA). The KHB buffer was bubbled continuously with a gas mixture of 95% O₂ and 5% CO₂ and maintained at 37°C. Isometric tension was measured as changes in millinewtons (mN) of force using Octal Bridge Amplifier and PowerLab 8/35 data-acquisition system and recorded using LabChart Pro V7 software (ADInstruments, Colorado Springs, CO).

Each aortic ring was gradually stretched to a basal tension of 19.6 mN (2 g) for ~1 hour and then equilibrated for additional 1.5 to 2 hours. During this equilibration period, KHB buffer was changed every 30 min. After equilibration, the maximal contractile response to 80 mM KCl was determined followed by washes with KHB buffer until the passive basal tension is restored. To verify endothelial denudation in all studies, aortic rings were precontracted with

phenylephrine (1 μ M) until the attainment of plateau phase, followed by the addition acetylcholine (1 μ M). The absence of relaxant response to acetylcholine confirmed the denudation of rings. The aortic rings were then washed with KHB buffer until the passive basal tension is restored.

2.4. Experimental protocols

2.4.1. Protocol for contractility studies (long-term treatment with metformin)—

Endothelium-denuded aortic rings were incubated in vascular cell basal medium (Cat # PCS-100-030; ATCC) supplemented with antibiotic/antimycotic solution in the presence of vehicle (control) or metformin (10 μ M, 100 μ M, or 1 mM) for 15 hours in a humidified atmosphere of 95% air and 5% CO₂ at 37°C, as described previously with minor modifications [27]. Subsequently, the respective aortic rings were mounted in the organ baths and maintained in KHB buffer containing vehicle or metformin (10 μ M, 100 μ M, or 1 mM). The aortic rings were gradually stretched to a passive basal tension of 19.6 mN (2 g) over a period of 1 hour and then equilibrated for additional 2 hours. Control and metformin-treated rings were then challenged with cumulative concentrations of PE (10⁻¹⁰ to 10⁻⁴ M) to determine the changes in contractility.

2.4.2. Protocol for contractility studies (short-term treatment with metformin)

—Endothelium-denuded aortic rings were treated with vehicle (control) or metformin (10 μ M to 3 mM) for 30 min or 2 hours. In select experiments, aortic rings were also treated with vehicle (control), phenformin (10 μ M to 3 mM) for 2 hours, or AICAR (1 mM) for 30 min. Subsequently, control and treated rings were challenged with cumulative concentrations of PE (10⁻¹⁰ to 10⁻⁴ M) to determine the changes in contractile response.

2.4.3. Protocol for contractility studies and immunoblot analysis—

Aortic rings were exposed to vehicle (control), 3 mM metformin or 100 μ M phenformin for 2 hours. In addition, aortic rings were exposed to vehicle (control) or 1 mM AICAR for 30 min. Subsequently, vehicle and treated rings were challenged with or without a fixed concentration of PE (1 μ M) for 6 min to determine the changes in basal tension and PE-induced contraction. After contractility studies, the respective aortic rings were subjected to extraction procedures for immunoblot analysis of phosphorylated AMPK and total AMPK, as described in 2.5. and 2.6. To determine the intermediary role of AMPK in metformin action, aortic rings were pretreated with compound C (40 μ M, 30 min). To determine whether CaMKK β mediates AMPK phosphorylation, rings were pretreated with a CaMKK β inhibitor, STO-609 (10 μ M, 30 min).

2.4.4. Protocol for quantification of nucleotides (ATP, ADP, and AMP) in aortic rings—

Aortic rings were exposed to vehicle (control) or 3 mM metformin for 2 hours. The rings were then challenged with or without PE (1 μ M) for 6 min. For each treatment condition, four aortic rings from 2 rats (two rings/rat) were pooled together to quantify nucleotides, as described in 2.7. In parallel, human aortic vascular smooth muscle cells (VSMCs) were maintained in culture under control conditions to analyze all three nucleotides, as described in 2.8.

2.4.5. Protocol for contractility studies: post-treatment washout of metformin

—Aortic rings were exposed to vehicle (control) or 3 mM metformin for 2 hours followed by challenge with cumulative concentrations of PE (10^{-10} to 10^{-4} M). In parallel, metformin-treated aortic rings were subjected to washout for 2 hours followed by challenge with cumulative concentrations of PE. In addition, rings were subjected to metformin treatment and washout protocols to determine the changes in contractile response to serotonin (5-HT, 10^{-9} to 10^{-3} M).

2.4.6. Protocol for contractility and immunoblot studies: post-treatment washout of metformin—Aortic rings were exposed to vehicle (control) or 3 mM metformin for 2 hours followed by challenge with or without 1 μ M PE for 6 min. In parallel, metformin-treated aortic rings were subjected to washout for 2 hours followed by PE challenge. After the contractility studies, the respective rings were subjected to extraction procedures for immunoblot analysis of AMPK.

2.4.7. Protocol for quantification of metformin in aortic rings—Aortic rings were exposed to vehicle (control), 3 mM metformin for 2 hours, or 3 mM metformin for 2 hours followed by its washout for 2 hours. The respective aortic rings were subjected to quantification of metformin, as described in 2.9.

2.5. Extraction and quantification of proteins in aortic tissues

After contractility studies, aortic rings were immediately rinsed in ice-cold fresh phosphate-buffered saline, blotted to dryness, snap-frozen in liquid nitrogen, and stored at -80°C until analysis. Aortic tissues were then thawed and homogenized in 100 μ l RIPA lysis buffer containing protease and phosphatase inhibitors (Thermo Scientific, Rockford, IL) using TissueLyser LT (Qiagen, Valencia, CA) at a setting of 50 Hz for 5 min with samples being placed on ice intermittently. The homogenates were incubated at 4°C for 1 hour on a rotator and centrifuged at $1000 \times g$ for 10 min at 4°C to remove tissue debris. The supernatants were mixed with 2x laemmli sample buffer at a ratio of 1:1 followed by heating at 67.5°C for 10 min. Proteins were quantified using Bio-Rad DC assay kit (Bio-Rad, Hercules, CA).

2.6. Immunoblot analysis

Aortic tissue samples (20 μ g protein each) were electrophoresed using pre-cast 4-12% NuPage mini-gels (Life Technologies, Carlsbad, CA), and the resolved proteins were transferred to nitrocellulose membranes (Hybond C, GE Healthcare Life Sciences, Piscataway, NJ) as described [28]. The membranes were blocked in 5% bovine serum albumin, and probed with the primary antibodies specific for phospho-AMPK α (Thr172), AMPK α , CaMKK β , or β -actin. After extensive washes, the immunoreactivity was detected using specific HRP-conjugated secondary antibodies followed by enhanced chemiluminescence (GE Healthcare Life Sciences). The protein bands were quantified by densitometric analysis using Image J.

2.7. Quantification of ATP, ADP, and AMP in aortic tissues using LC-MS/MS MRM

The respective tissues (from 2.4.4.) were rinsed in fresh ice-cold PBS, blotted to dryness, snap-frozen in liquid N_2 , and stored at -80°C until analysis. To increase detection threshold

for nucleotides, four aortic rings from each treatment condition were pooled together (as described in 2.4.4.) and pulverized to a fine powder. The tissue powder was transferred to 2.0 ml QIAcube tubes containing 200 μ l of ice-cold 5% perchloric acid and subjected to homogenization using Tissue Lyser LT (Qiagen, Valencia, CA). Homogenization was performed at a setting of 50 Hz (2 pulses, 30 seconds each) with samples being placed on ice intermittently. Each homogenized sample was centrifuged at $10,000 \times g$ (3 min, 4°C) to remove acid-insoluble material. Perchloric acid in the collected supernatant was extracted by three washes with 10% excess volume of a 1:1 mixture of tri-*n*-octylamine and 1,1,2-trichlorotrifluoroethane, as described [23]. The nucleotides remaining in the aqueous phase were then analyzed using liquid chromatography/tandem mass spectrometry (LC-MS/MS) with multiple reaction monitoring (MRM) on a 4000 QTRAP system (Applied Biosystems, Carlsbad, CA) [29]. The concentrations of ATP, ADP, and AMP in the samples were calculated using the standard curve prepared from serial dilutions of respective nucleotide stock solution (1 mM). The samples and standards were run on Amide XBridge HPLC column (Cat # 186004860; 3.5 μ M particle size; Waters, Milford, MA) using buffer A (20 mM ammonium hydroxide and 20 mM ammonium acetate in 5% acetonitrile, pH 9.0) and buffer B (100% acetonitrile) at a flow rate of 0.3 ml/min for 10 min. Mobile phase consisted of isocratic elution with 20% buffer B. The MRM transitions for nucleotides are described in Table 1.

2.8. Quantification of ATP, ADP, and AMP in human aortic VSMCs using LC-MS/MS MRM

Human aortic VSMCs, vascular cell basal medium (Cat # PCS-100-030), and VSMC growth kit (Cat # PCS-100-042) were purchased from ATCC (Manassas, VA). VSMCs (passages 3 to 6) were maintained in culture with vascular cell basal medium containing VSMC growth kit and antibiotic/antimycotic solution in a humidified atmosphere of 95% air and 5% CO_2 at 37°C . After the attainment of confluence, VSMCs were trypsinized, centrifuged, and ~3 million cells were seeded on to 150 mm petri dishes each, as described [28]. Subconfluent VSMCs were deprived of growth supplements for 2 days followed by incubation with KHB buffer for 2 hours. VSMCs were then washed twice with ice-cold PBS and collected in 1 ml ice-cold PBS. The cell suspension was centrifuged at $1000 \times g$ for 5 min at 4°C . After discarding the supernatant, the cell pellet was snap-frozen using liquid N_2 and stored at -80°C until analysis. On the day of nucleotide extraction, 200 μ l of 5% ice-cold perchloric acid was added to the cell pellet to lyse the cells and the samples were centrifuged at $10,000 \times g$ (3 min, 4°C) to remove acid-insoluble material. Perchloric acid extractions and nucleotide analyses were performed as described in 2.7.

2.9. Quantification of metformin in aortic tissues using LC-MS/MS MRM

The respective tissues (from 2.4.7.) were rinsed in fresh ice-cold PBS, blotted to dryness, snap-frozen in liquid N_2 , and stored at -80°C until analysis. Tissues were then thawed and homogenized in 100 μ l of HPLC-grade water using Tissue Lyser LT at a setting of 50 Hz for 5 min. Each homogenized sample was deproteinized with 400 μ l of acetonitrile, 500 μ l of methanol, and 1 μ l of internal standard (C17-sphingosine-1-phosphate, 1 mM). Samples were vortexed for 2 min and centrifuged at $16,000 \times g$ (15 min at 4°C). Supernatants were collected and analyzed using LC-MS/MS MRM [30, 31]. This system included the use of C18 HPLC column (Cat # 00D-4475-AN; Phenomenex, Torrance, CA) and mobile phase

gradient. Metformin concentrations in samples were calculated using the standard curve prepared from serial dilutions of metformin stock solution (10 μ M). Working standards for metformin (0.1 nM, 1 nM, 10 nM, 100 nM, and 1 μ M) were prepared by diluting stock solution using a mixture of 400 μ l of acetonitrile, 500 μ l of methanol, and 1 μ l of internal standard (C17-sphingosine-1-phosphate, 1 mM) and adjusting the final volume to 1000 μ l using HPLC-grade water. The MRM transitions for metformin and sphingosine-1-phosphate are described in Table 1.

2.10. Statistical analysis

Results are expressed as means $\pm$ SEM values. The n value represents the number of animals. To determine $E_{\max}$ and EC_{50} values ($-\log EC_{50}$ or pEC_{50}), nonlinear regression analysis was performed using GraphPad Prism software (version 6.01, GraphPad Software, Inc., La Jolla, CA). Statistical analyses of the data among groups were performed by one-way repeated measures ANOVA followed by Bonferroni t test. Values of $p < 0.05$, 0.01, 0.001 were considered statistically significant.

3. Results

3.1. Long-term metformin treatment, at 100 μ M or 1 mM concentration, inhibits PE-induced smooth muscle contractility in rat aorta *ex vivo*

Previous studies have shown that oral administration of metformin for up to 11 weeks results in diminished vascular reactivity in rats [32]. In particular, the maximal contractile response to norepinephrine is diminished in the mesenteric arteries (with or without endothelium) isolated from metformin-treated rats [32]. In the present study, we examined whether *ex vivo* incubation of endothelium-denuded aorta with metformin for an extended period of time (18 hours) alters vascular smooth muscle contractility. Since the therapeutically-relevant plasma metformin concentration is 10-50 μ M [33], we chose a concentration ranging from 10 μ M to 1 mM metformin for these *ex vivo* studies, as described in 2.4.1. As shown in **Fig. 1**, in control aortic rings, stimulation with PE led to an increase in smooth muscle contractility with an $E_{\max}$ value of 6.05 ± 0.3 mN/mm and pEC_{50} value of 6.6 ± 0.06 M. Treatment with 10 μ M metformin did not show significant changes in PE-induced contractility. However, upon incubation with 100 μ M or 1 mM metformin, there was a marked inhibition of PE-induced maximal contractile response without significant changes in pEC_{50} values. While 100 μ M metformin diminished PE-induced maximal contractility by 39.6% ($E_{\max}$: 3.65 ± 0.4 versus control value of 6.05 ± 0.3 mN/mm; $p < 0.05$), 1 mM metformin treatment led to a decrease in maximal contractility by 72.7% ($E_{\max}$: 1.65 ± 0.3 versus control value of 6.05 ± 0.3 mN/mm; $p < 0.01$). Thus, under the present *ex vivo* incubation conditions for up to 18 hours, metformin at 100 μ M or 1 mM concentration showed a marked attenuation of smooth muscle contraction independent of endothelium.

3.2. Short-term metformin treatment, at 3 mM concentration, inhibits PE-induced smooth muscle contractility *ex vivo*

Since long-term metformin treatment requires the maintenance of aortic ring preparations in tissue culture medium, we performed short-term treatment studies to determine metformin

regulation of smooth muscle contraction. Recent studies using endothelium-denuded rat aorta have shown that 2 mM metformin treatment for 30 min results in marked inhibition of PE-induced contractility [17]. Importantly, Zhou et al. have shown that in rat hepatocytes, metformin exhibits significant biological effects at 500 μ M to 3 mM concentrations upon incubation for 1 to 3 hours [34]. This has been attributed to its slow membrane permeability / transport in hepatocytes [34, 35]. In the present study, we therefore incubated aortic rings with metformin at 10 μ M to 3 mM concentrations for 30 min (**Fig. 2A**) or 2 hours (**Fig. 2B**).

For 30-min time interval studies (**Fig. 2A**), stimulation of control aortic rings with PE led to an increase in contractility with an $E_{\max}$ value of 7.14 ± 0.1 mN/mm and pEC_{50} value of 8.1 ± 0.05 M. Metformin treatment at 10 μ M, 100 μ M, or 1 mM concentrations did not result in significant changes in PE-induced contractility. 3 mM metformin treatment followed by PE challenge showed a trend toward a decrease in $E_{\max}$ value (6.7 ± 0.2 mN/mm), but it was not statistically significant. However, 3 mM metformin significantly diminished pEC_{50} value to 7.4 ± 0.06 M ($p < 0.001$). These data suggest that metformin uptake during the 30-min incubation period may not be sufficient enough to diminish PE-induced maximal contractility. Hence, we performed similar studies with metformin for a 2-hour incubation period.

For 2-hour time interval studies (**Fig. 2B**), stimulation of control aortic rings with PE led to an increase in contractility with an $E_{\max}$ value of 7.08 ± 0.2 mN/mm and pEC_{50} value of 8.1 ± 0.06 M. Metformin treatment at 10 μ M or 100 μ M concentrations did not significantly affect the $E_{\max}$ values and pEC_{50} values for PE-induced contractility. 1 mM metformin treatment followed by PE challenge showed a trend toward a decrease in $E_{\max}$ value (6.56 ± 0.11 mN/mm), but it was not statistically significant. However, 1 mM metformin significantly diminished the pEC_{50} value to 7.77 ± 0.04 M ($p < 0.05$). Notably, 3 mM metformin significantly diminished both $E_{\max}$ and pEC_{50} values for PE-induced contractility, and the respective values were 5.39 ± 0.2 mN/mm ($p < 0.05$) and 7.44 ± 0.07 M ($p < 0.001$). Together, these data suggest that short-term incubation of aorta with 3 mM metformin for 2 hours would allow its uptake sufficient enough to diminish PE-induced maximal contractility. Hence, we followed this protocol for all subsequent studies to determine how metformin regulates agonist-induced contractility and AMPK phosphorylation.

3.3. Short-term phenformin treatment, at 100 μ M, 1 mM, or 3 mM concentration, inhibits PE-induced smooth muscle contractility ex vivo

To determine whether metformin inhibition of PE-induced contractility is recapitulated by its closely related biguanide analog (e.g., phenformin) [36], we performed short-term treatment studies with phenformin at 10 μ M to 3 mM concentrations for 2 hours. Previously, phenformin has been used at a concentration of up to 10 mM to determine AMPK regulation in rat skeletal muscle [37]. In addition, phenformin has been shown to be rapidly transported in hepatocytes, compared with metformin [35]. In the present study (**Fig. 2C**), stimulation of control aortic rings with PE led to an increase in contractility with an $E_{\max}$ value of 7.15 ± 0.2 mN/mm and pEC_{50} value of 7.89 ± 0.07 M. 10 μ M phenformin did not significantly

affect the $E_{\max}$ and pEC_{50} values for PE-induced contractility. However, 100 μ M, 1 mM and 3 mM concentrations of phenformin significantly diminished the $E_{\max}$ values of PE-induced contractility to 4.6 ± 0.4 ($p < 0.05$), 1.86 ± 0.4 ($p < 0.01$), and 0.67 ± 0.15 ($p < 0.001$) mN/mm, respectively, compared with control. In addition, 100 μ M, 1 mM and 3 mM concentrations of phenformin significantly diminished the pEC_{50} values of PE-induced contractility to 6.45 ± 0.09 , 5.47 ± 0.19 , and 4.49 ± 0.24 M ($p < 0.001$), respectively. Together, these data suggest that short-term exposure of aortic rings to phenformin, even at 100 μ M concentration, would allow its uptake sufficient enough to diminish PE-induced maximal contractility. Hence, we used 100 μ M phenformin to compare its effects with 3 mM metformin regarding AMPK phosphorylation, as described in 3.5.

3.4. Short-term AICAR treatment, at 1 mM concentration, inhibits PE-induced smooth muscle contractility ex vivo

To determine whether metformin inhibition of PE-induced contractility is recapitulated by an AMPK activator (e.g., AICAR) [34, 38], we performed short-term treatment studies with 1 mM AICAR for 30 min. AICAR is an adenosine analog and is known to undergo phosphorylation to ZMP, an AMP mimetic that activates AMPK [38]. Previous studies have shown that AICAR, at 1 mM to 10 mM concentrations, increases AMPK phosphorylation and also promotes relaxation of endothelium-denuded mouse or rat aorta precontracted with PE [13, 14]. In the present study (**Fig. 2D**), stimulation of control aortic rings with PE led to an increase in contractility with an $E_{\max}$ value of 7.19 ± 0.14 mN/mm and pEC_{50} value of 8.11 ± 0.04 M. AICAR pretreatment (1 mM, 30 min) diminished the $E_{\max}$ and pEC_{50} values for PE-induced contractility to 5.24 ± 0.18 mN/mm ($p < 0.01$) and 7.28 ± 0.05 M ($p < 0.001$), respectively, compared with control. Together, AICAR addition to PE-contracted aorta [13, 14] or AICAR pretreatment followed by PE challenge (present study) shows vasorelaxant response independent of endothelium.

3.5. Metformin inhibition of PE-induced smooth muscle contractility is reciprocally associated with accentuation of AMPK phosphorylation: comparison with phenformin or AICAR

To determine how metformin regulates contractile function and AMPK phosphorylation, control aortic rings and metformin-treated rings (3 mM, 2 hours) were challenged with or without 1 μ M PE for 6 min. As shown in **Fig. 3A** (upper panel), metformin treatment resulted in a significant diminution of PE-induced contractility by 29.7% ($p < 0.05$), compared with control (+ PE). However, it did not produce significant changes in the basal tension (lower panel). Regarding AMPK phosphorylation, **Fig. 3B** shows that acute PE challenge but not 2-hour metformin treatment alone induced a significant increase in AMPK phosphorylation by ~2.5-fold ($p < 0.05$), compared with control (– PE) (upper and lower panels). Importantly, 2-hour metformin treatment followed by acute PE challenge induced a robust increase in AMPK phosphorylation. This accentuated increase in AMPK phosphorylation by metformin-plus-PE occurred to the extent of ~9.7-fold ($p < 0.001$), compared with control (– PE).

In comparison, similar protocols were followed for aortic rings treated with phenformin (100 μ M, 2 hours) or AICAR (1 mM, 30 min). As shown in **Fig. 3C** (upper panel), phenformin

treatment led to a significant decrease in PE-induced contractility by 49.6% ($p < 0.01$), compared with control (+ PE). **Fig. 3D** shows that phenformin increased AMPK phosphorylation by itself and it was further augmented by PE challenge (upper and lower panels).

As shown in **Fig. 3E** (upper panel), AICAR treatment led to a significant diminution of PE-induced contractility by 31.8 % ($p < 0.01$), compared with control (+ PE). **Fig. 3F** shows that AICAR treatment alone (unlike metformin) induced a marked increase in AMPK phosphorylation by ~7.1-fold ($p < 0.001$, compared with -PE control), which was marginally enhanced by PE challenge (upper and lower panels).

Together, biguanide (metformin/phenformin) or AICAR inhibition of smooth muscle contractility upon PE challenge is reciprocally associated with enhanced AMPK phosphorylation, as evidenced by assessment of these two parameters in the same aortic rings.

3.6. Metformin treatment followed by PE stimulation results in enhanced AMP level and AMP/ATP ratio: LC-MS/MS MRM analysis

To determine the relationship between AMPK phosphorylation and nucleotide levels in the aortic rings subjected to isometric contractions, we performed LC-MS/MS MRM analysis for the quantification of all three nucleotides, ATP, ADP, and AMP. To validate the assay procedure, we used human aortic VSMCs in culture. As described in 'Materials and methods', VSMCs were maintained under control conditions and then subjected to nucleotide analysis. **Fig. 4A** shows that the levels of ATP, ADP, and AMP in VSMCs were 2788.3 ± 105.1 , 1341.6 ± 57.5 , and 268.4 ± 21.1 picomoles/mg protein, respectively. Next, control aortic rings and metformin-treated aortic rings (3 mM, 2 hours) were challenged with or without 1 μ M PE for 6 min to determine the changes in nucleotide levels. **Fig. 4B** shows that under control conditions (in the absence of PE stimulation), ATP, ADP, and AMP levels were 7.5 ± 0.7 , 58.7 ± 5.9 , and 294.2 ± 4.6 picomoles/mg tissue weight, respectively. Compared with much higher ATP and ADP levels observed in VSMCs, the levels of ATP and ADP in aortic tissues were relatively less. The decreased levels of ATP and ADP in aortic tissues are likely due to their rapid hydrolysis by ectonucleotidases [39, 40] during the processing of tissues. Next, stimulation with PE alone did not result in significant changes in the respective nucleotide levels. In metformin-treated aortic rings, the changes in ATP, ADP, and AMP levels were 7.1 ± 0.8 , 64.9 ± 3.6 , and 343.1 ± 13.2 picomoles/mg tissue weight, respectively. However, these values were not significantly different compared with control aortic rings. Importantly, after metformin treatment followed by PE challenge, the changes in ATP, ADP, and AMP levels were 4.3 ± 0.7 ($p < 0.05$), 67.2 ± 5.7 (n.s.), and 648.9 ± 38.9 ($p < 0.01$) picomoles/mg tissue weight, respectively, compared with metformin treatment alone. Thus, metformin-plus-PE treatment led to a marked increase in AMP level. As shown in **Fig. 4C**, data analysis of nucleotides revealed a significant increase in AMP/ATP ratio to 163.2 ± 21.5 ($p < 0.05$) in metformin-plus-PE treated group, compared with AMP/ATP ratio of 49.4 ± 3.3 for metformin treatment alone. Table 1 shows the parameters used for the quantification of nucleotides using LC-MS/MS MRM analysis. Together, the data from 3.5. and 3.6. reveal that metformin

inhibition of PE-induced smooth muscle contractility is associated with enhanced AMPK phosphorylation with an accompanying increase in AMP/ATP ratio.

3.7. Compound C, an AMPK inhibitor, prevents metformin inhibition of PE-induced smooth muscle contractility

To determine whether metformin inhibition of PE-induced contractility is mediated by AMPK activation, aortic rings were pretreated with an AMPK inhibitor, compound C (40 μ M, 30 min), as described [34]. Subsequently, aortic rings were treated with or without 3 mM metformin for 2 hours followed by stimulation with PE (10^{-10} to 10^{-4} M). As shown in **Fig. 5**, in the control group, the $E_{\max}$ and pEC_{50} values for PE-induced contractility were 7.17 ± 0.2 mN/mm and 8.07 ± 0.05 M, respectively. After metformin treatment for 2 hours, the $E_{\max}$ and pEC_{50} values for PE-induced contractility decreased to 5.84 ± 0.12 mN/mm ($p < 0.05$) and 7.32 ± 0.06 M ($p < 0.001$), respectively, compared with control. Pretreatment with compound C (40 μ M) alone did not significantly affect PE-induced contractility. However, after pretreatment with compound C followed by metformin treatment, the $E_{\max}$ and pEC_{50} values for PE-induced contractility were 6.76 ± 0.13 mN/mm ($p < 0.05$) and 7.95 ± 0.07 M ($p < 0.001$), respectively, compared with metformin treatment alone. Thus, compound C abrogated the inhibitory effects of metformin on PE-induced contractility, suggesting AMPK as an intermediary signaling component for metformin action in aortic smooth muscle.

3.8. Compound C prevents metformin inhibition of smooth muscle contractility and accentuation of AMPK phosphorylation upon PE challenge

To further ascertain the reciprocal relationship between contractile function and AMPK phosphorylation, control aortic rings, metformin-, compound C-, and compound C-plus-metformin-treated aortic rings were challenged with or without 1 μ M PE for 6 min to induce isometric contractions. As shown in **Fig. 6A** (upper panel), metformin treatment resulted in a significant diminution of PE-induced contractility by 26.7% ($p < 0.05$), compared with control (+ PE). Compound C alone did not induce significant changes in PE-induced contractility. However, compound C pretreatment significantly prevented metformin inhibition of PE-induced contractility ($p < 0.05$), compared with metformin treatment (+ PE). Neither metformin nor compound C produced significant changes in the basal tension, compared with control (lower panel). Regarding AMPK phosphorylation, **Fig. 6B** shows that compound C treatment prevented PE-induced AMPK phosphorylation, compared with control (+PE). In addition, compound C pretreatment nearly abolished metformin-plus-PE-induced robust increase in AMPK phosphorylation, compared with metformin treatment (+ PE). Together, these studies provide evidence that accentuated AMPK phosphorylation is responsible for the inhibition of smooth muscle contractility observed upon PE challenge in metformin-treated vascular smooth muscle.

3.9. STO-609, a CaMKK β inhibitor, abolishes PE-induced AMPK phosphorylation but does not prevent metformin accentuation of AMPK phosphorylation observed upon PE challenge

To determine whether metformin accentuation of AMPK phosphorylation upon PE challenge is mediated in part by CaMKK β (an upstream kinase for AMPK), aortic rings were pretreated with a CaMKK β inhibitor, STO-609 (10 μ M, 30 min) [16]. STO-609 has been shown to inhibit AMPK phosphorylation and accelerate myosin light chain phosphorylation by vasoactive peptides in rat aortic smooth muscle cells [16], suggesting that inhibition of CaMKK β /AMPK signaling would enhance agonist-induced contraction. In the present study, control aortic rings, metformin-, STO-609-, and STO-609-plus-metformin-treated aortic rings were challenged with or without 1 μ M PE for 6 min. As shown in **Fig. 7A** (upper panel), metformin treatment resulted in a significant diminution of PE-induced contractility by 33.4% ($p < 0.05$), compared with control (+ PE). In parallel, STO-609 treatment alone induced a significant decrease in PE-induced contractility by 31.5% ($p < 0.05$), compared with control (+ PE). In addition, STO-609 pretreatment did not prevent metformin inhibition of PE-induced smooth muscle contractility but instead further promoted the inhibitory effects of metformin on PE-induced contractility ($p < 1.5$), compared with metformin treatment (+ PE). Neither metformin nor STO-609 produced significant changes in the basal tension (lower panel). Regarding AMPK phosphorylation, **Fig. 7B** shows that STO-609 treatment prevented PE-induced AMPK phosphorylation, compared with control (+PE). However, STO-609 did not prevent metformin-plus-PE-induced robust increase in AMPK phosphorylation, compared with metformin treatment (+ PE). In addition, there were no significant changes in CaMKK β expression under all treatment conditions ($n = 3$).

Notably, STO-609 inhibits vasoactive peptide-induced AMPK phosphorylation in rat aortic smooth muscle cells *in vitro* [16] and PE-induced AMPK phosphorylation in endothelium-denuded rat aorta *ex vivo* (present study). Although STO-609 accelerates myosin light chain phosphorylation in aortic smooth muscle cells suggesting enhanced contraction [16], it inhibits PE-induced contractility in aorta (present study). STO-609-mediated inhibition of smooth muscle contraction *ex vivo* may likely be due to inhibition of CaMKK/calmodulin kinase I/myosin light chain phosphorylation, as reported previously [41]. Nevertheless, the present findings reveal that, while CaMKK β mediates PE-induced AMPK phosphorylation, it does not contribute to accentuated AMPK phosphorylation upon PE challenge in metformin-treated aorta.

3.10. Post-treatment washout of metformin reveals attenuation of agonist-induced contractility

To determine whether metformin inhibition of PE-induced contractility occurs in a reversible manner, contractile responses were monitored under the following conditions: i) metformin treatment followed by PE; ii) post-treatment washout of drugs for 2 hours; and iii) subsequent PE challenge. Similar studies were also performed with another GPCR agonist, serotonin (5-HT).

As shown in **Fig. 8A**, exposure of control aortic rings to PE (10^{-10} to 10^{-4} M) led to an increase in contractility with an $E_{\max}$ value of 7.33 ± 0.27 mN/mm and pEC_{50} value of 8.05 ± 1.6 M. After 2-hour metformin treatment, there was an attenuation of PE-induced contractility ($E_{\max}$: 5.66 ± 0.22 mN/mm, $p < 0.05$; and pEC_{50} : 7.32 ± 0.06 M, $p < 0.001$). Select experiments included the washout of drugs for 2 hours. Within 25-30 minutes during this washout period, the contractile response returned to basal tension level. In parallel, metformin treatment (2 hours) followed by washout for 2 hours led to diminution of PE-induced contractility ($E_{\max}$: 5.17 ± 0.2 mN/mm, $p < 0.05$; and pEC_{50} : 7.56 ± 0.063 M, $p < 0.001$). Thus, PE-induced contractility was significantly inhibited after metformin treatment and under post-treatment washout conditions.

Fig. 8B shows that exposure of control aortic rings to 5-HT (10^{-9} to 10^{-3} M) led to an increase in contractility with an $E_{\max}$ value 8.09 ± 0.33 mN/mm and pEC_{50} value of 6.22 ± 0.06 M. After 2-hour metformin treatment, there was a decrease in 5-HT-induced maximal contractility ($E_{\max}$: 5.19 ± 0.34 mN/mm, $p < 0.01$) without significant changes in pEC_{50} value. Select experiments included the washout of drugs for 2 hours. Within 25-30 minutes during this washout period, the contractile response returned to basal tension level. In parallel, metformin treatment followed by washout for 2 hours led to a decrease in 5-HT-induced maximal contractility ($E_{\max}$: 4.7 ± 0.37 , $p < 0.01$) without significant change in pEC_{50} value. Thus, 5-HT-induced contractility was significantly inhibited after metformin treatment and under post-treatment washout conditions.

Together, these data suggest that, in conjunction with its slow membrane transport/uptake [34, 35], the efflux of metformin from vascular smooth muscle cells may also be a slow process. Hence, under the apparent washout conditions for metformin, there is a marked diminution in the contractile response to PE or 5-HT.

3.11. Post-treatment washout of metformin reveals accentuation of AMPK phosphorylation upon PE challenge

To determine the relationship between PE-induced contraction and AMPK phosphorylation under post-treatment washout conditions, the changes in these two parameters were monitored in the same aortic rings. Notably, previous studies by Rubin *et al.* have shown that enhanced AMPK phosphorylation by metabolic stress returns to the baseline within minutes in vascular smooth muscle due to dephosphorylation during the washout period [12].

In the present study (**Fig. 9A**, upper panel), metformin treatment for 2 hours resulted in a significant diminution of PE-induced contractility by 29.7% ($p < 0.05$), compared with control (+ PE). In addition, metformin treatment (2 hours) followed by the washout for 2 hours showed a significant decrease in PE-induced contractility by 29.4% ($p < 0.05$), compared with control (+ PE). Neither metformin treatment nor post-treatment washout conditions showed significant changes in the basal tension, compared with control (lower panel).

Fig. 9B shows that, in comparison with control (lane 1), PE challenge (lane 2) resulted in a significant increase in AMPK phosphorylation by ~2.5-fold ($p < 0.05$). Although metformin

treatment for 2 hours did not show a significant increase in AMPK phosphorylation (lane 3), metformin treatment followed by PE stimulation (lane 4) resulted in a robust increase in AMPK phosphorylation by ~9.7-fold ($p < 0.001$). Select experiments included the washout of drugs (metformin-plus-PE) for 2 hours. Under these washout conditions, the phosphorylation state of AMPK (data not shown) was comparable to that seen in lane 3, suggesting dephosphorylation of phosphorylated AMPK. In addition, metformin treatment (2 hours) followed by washout for 2 hours showed no change in AMPK phosphorylation (lane 5). However, under these conditions, subsequent PE challenge (lane 6) increased AMPK phosphorylation by ~9.1-fold ($p < 0.001$).

Together, these data suggest that, in conformity with the reported reversible phosphorylation of AMPK in vascular smooth muscle subjected to metabolic stress [12], the accentuated AMPK phosphorylation by metformin-plus-PE is reversible after washout of drugs. However, under the apparent washout conditions, subsequent PE challenge accentuates AMPK phosphorylation.

3.12. Metformin treatment results in its accumulation by aortic smooth muscle as evidenced by LC-MS/MS MRM analysis

Recent studies demonstrate high expression levels of organic cation transporter-3 (OCT-3) in skeletal muscle and smooth muscle [42]. Notably, OCT-3 has been shown to mediate metformin uptake in skeletal muscle [42]. To determine the likely uptake/accumulation of metformin in aortic smooth muscle, we performed LC-MS/MS MRM analysis. As shown in **Fig. 10A-C**, aortic rings were exposed to vehicle control, 3 mM metformin for 2 hours, and 3 mM metformin for 2 hours followed by washout of this drug for 2 hours. Subsequently, aortic tissues were subjected to LC-MS/MS MRM to obtain the respective chromatograms. **Fig. 10D** shows that under control conditions, metformin was undetectable. After metformin treatment and under post-treatment washout conditions, significant amounts of metformin were observed in the aorta to the extent of 578.8 ± 41.8 pmoles/mg tissue and 149.0 ± 9.3 pmoles/mg tissue, respectively.

Together, the present findings strongly suggest that metformin uptake in vascular smooth muscle would accentuate AMPK phosphorylation and inhibit contractile response upon agonist challenge.

4. Discussion

The principal findings of the present study using *ex vivo* endothelium-denuded rat aorta include: i) metformin accumulation in intact arterial smooth muscle; ii) a modest but significant increase in AMPK phosphorylation by phenylephrine but not by metformin *per se*; iii) an exaggerated increase in AMPK phosphorylation with an associated elevation of AMP/ATP ratio by phenylephrine after metformin treatment; iv) compound C (AMPK inhibitor) inhibition of AMPK phosphorylation induced by phenylephrine alone and also that induced by phenylephrine after metformin treatment; v) STO-609 (CaMKK β inhibitor) inhibition of AMPK phosphorylation induced by phenylephrine alone but not that induced by phenylephrine after metformin treatment; and vi) attenuation of phenylephrine-induced contraction (after metformin treatment), which is prevented by AMPK inhibition but not by

CaMKK β inhibition. Together, these findings suggest that, upon endothelial disruption in the vessel wall, metformin uptake by the underlying vascular smooth muscle would accentuate AMPK phosphorylation by α -adrenergic agonists independent of CaMKK β to promote vasorelaxation (**Fig. 11**).

In general, vascular smooth muscle contraction in response to G protein-coupled receptor agonists (e.g., α -adrenergic agonists, endothelin-1, and serotonin) is mediated by the sequential activation of signaling events. They include a rise in cytosolic free Ca²⁺, Ca²⁺-calmodulin complex formation, myosin light chain kinase (MLCK) activation, MLC phosphorylation, and subsequent myosin-actin interaction for cross-bridge cycling [43]. In addition to MLCK-mediated MLC phosphorylation, activation of RhoA/Rho-associated kinase and the consequent phosphorylation of myosin phosphatase targeting subunit 1 (MYPT1) are known to maintain MLC phosphorylation state. Previous studies have shown that AMPK activation in vascular smooth muscle inhibits MLCK or RhoA/Rho-associated kinase activity, thereby diminishing agonist-induced MLC phosphorylation and contractile response [16, 44]. The significance of AMPK activation in vascular smooth muscle has been evidenced by genetic, metabolic, and pharmacological approaches. For instance, in AMPK α_1 - or AMPK α_2 -deficient mice, phenylephrine-induced contraction is enhanced in endothelium-denuded aorta [16, 44] and mesenteric arteries [44], suggesting AMPK regulation of vascular tone independent of endothelium in conduit as well as resistance arteries. Previous studies by Rubin *et al.* demonstrate that metabolic stress-induced increase in AMPK α_1 activity (by 2-deoxyglucose and anoxia) inhibits endothelin-induced contraction in porcine carotid artery in an endothelium-independent manner [12]. Goirand *et al.* have shown that pharmacological activation of AMPK by AICAR results in vasorelaxation in endothelium-denuded aorta, and these effects are abolished in AMPK α_1 - but not AMPK α_2 -deficient mice [13]. In addition, studies by Ford *et al.* reveal that AMPK activation by AICAR mediates endothelium-independent vasorelaxation in the aorta or mesenteric arteries from normotensive and hypertensive rats [14, 15]. Furthermore, AICAR increases AMPK phosphorylation by ~7-fold in endothelium-denuded rat aorta, which is marginally increased by phenylephrine challenge (present study). Notably, AICAR does not affect cellular energy state, as revealed by no significant changes in AMP/ATP or ADP/ATP ratio [14, 38, 45]. Metformin promotes endothelium-independent vasorelaxation in the normal rat aorta [17] (present study) and the mesenteric arteries from normotensive and hypertensive rats [32]. In particular, the present study shows that metformin treatment exaggerates phenylephrine-induced AMPK phosphorylation in intact vascular smooth muscle to induce vasorelaxation. Together, previous studies [12-15] and the present study provide direct evidence for AMPK activation in arterial smooth muscle. Nevertheless, in accordance with the recent report by Hardie and co-workers [45], AMPK activation upon exposure to metabolic stress, AICAR or metformin would occur through different mechanisms.

First, the uptake of 2-deoxyglucose (a key component of metabolic stress) and AICAR (an adenosine analog) in vascular smooth muscle cells is mediated by plasma membrane-localized glucose transporter-1 [28, 46] and presumably equilibrative nucleoside transporter-1 [47, 48], respectively. The present findings from LC-MS/MS MRM analysis

show significant amounts of metformin in arterial smooth muscle (~600 pmoles/mg aortic tissue) following *ex vivo* exposure to a high concentration of metformin (3 mM for 2 hr); however, loss of metformin from the aortic tissue is rapid with a $t_{1/2}$ of approximately 1 hr. Whether significant metformin uptake by vascular smooth muscle occurs at therapeutically relevant concentrations (10-50 μ M) was not determined in the present study. It is likely that the uptake of metformin, which is positively charged at physiological pH, is mediated by the organic cation transporter-3 (OCT-3). In this regard, recent studies demonstrate high expression levels of OCT-3 in vascular smooth muscle and skeletal muscle [42, 49], in comparison with the predominant OCT-1 and OCT-2 isoforms that mediate metformin uptake in the liver and kidney, respectively. Thus, after uptake through distinct transporters in vascular smooth muscle cells, 2-deoxyglucose, AICAR, and metformin would utilize different mechanisms to activate AMPK. For instance, 2-deoxyglucose undergoes rapid phosphorylation to form 2-deoxyglucose-6-phosphate, whereas AICAR requires its conversion to ZMP (an AMP mimetic) that binds to AMPK γ subunit to activate AMPK [38]. With regard to metformin, it is not likely to undergo conversion to intermediary metabolites in vascular smooth muscle based on the fact that it does not undergo hepatic metabolism and is excreted unchanged in urine [50, 51]. Previous studies on [14 C]metformin distribution in hepatocytes show a greater amount of metformin in the cytosol (~80%) with a modest radioactivity (7-10%) in the mitochondrial/lysosomal fraction [52]. Notably, metformin at 2-10 mM concentration inhibits mitochondrial respiratory chain complex 1 [21, 22] thereby diminishing ATP/ADP ratio [22] in hepatocytes. However, in skeletal muscle cells, metformin at 2 mM concentration does not affect mitochondrial energy metabolism as revealed by the absence of detectable increase in AMP level or AMP/ATP ratio [23]. Consistent with the findings observed in skeletal muscle cells [23], exposure of aortic smooth muscle to 3 mM metformin does not result in significant changes in AMP level in the present study.

Second, although metformin activates AMPK in mouse skeletal muscle cells without an increase in AMP/ATP ratio [23], the present study shows that in endothelium-denuded rat aorta metformin *per se* at 3 mM concentration produces a modest but insignificant increase in AMPK phosphorylation in the absence of any increase in AMP/ATP ratio. Our findings with aortic smooth muscle are in agreement with previous studies by Rubin *et al.* where metformin at 2 mM concentration does not activate AMPK during isometric contractions in endothelium-denuded porcine carotid artery [12]. However, in porcine and rat aortic smooth muscle cells [17, 53] and also in human aortic smooth muscle cells in culture (unpublished observations), metformin *per se* at 2 to 3 mM concentration significantly enhances AMPK phosphorylation. The differences in metformin regulation of AMPK phosphorylation between *ex vivo* arterial smooth muscle and *in vitro* cultured VSMCs/skeletal muscle cells may be attributed to: i) distinct differences in phenotypic characteristics; and ii) innate differences in energy state between these phenotypes.

Third, the present findings reveal that phenylephrine induces AMPK phosphorylation by ~2.5-fold in intact rat aortic smooth muscle. Moreover, after short-term metformin treatment (3 mM, 2 hours) in vascular smooth muscle, phenylephrine challenge that is known to elevate cytosolic free Ca^{2+} leads to an exaggerated increase in AMPK phosphorylation by

~9.7-fold. Parallel studies with phenformin (100 μ M, 2 hours) reveal that this biguanide increases AMPK phosphorylation by ~3.9-fold, which is further augmented upon phenylephrine challenge. At this juncture, it is important to note that a combination of phenformin and Ca^{2+} ionophore (A23187) induces a robust increase in AMPK phosphorylation in HeLa cells [20], even though phenformin *per se* does not enhance AMPK phosphorylation in these cells [20, 54]. Together, these findings suggest that biguanides have the potential to exaggerate AMPK activation in select tissues/cells that respond to $[\text{Ca}^{2+}]_i$ elevating agents. As noted in the Introduction, AMPK activation by metformin and vasoconstrictors occurs through distinct signaling events. Regardless of the stimuli for AMPK activation, the use of an ATP-competitive inhibitor of AMPK (e.g., compound C) should prevent an increase in AMPK phosphorylation [34]. Accordingly, compound C not only abrogates the increase in AMPK phosphorylation by phenylephrine *per se* but also the exaggerated increase in AMPK phosphorylation by phenylephrine after metformin treatment.

Fourth, in conformity with previous reports showing CaMKK β -dependent AMPK activation by vasoactive peptides such as angiotensin II and endothelin-1 in rat aortic smooth muscle cells in culture [16], the present findings reveal that AMPK phosphorylation by phenylephrine in rat aortic smooth muscle *ex vivo* is subject to inhibition by STO-609 (CaMKK β inhibitor). The rise in $[\text{Ca}^{2+}]_i$ by vasoconstrictors is a key event in vascular smooth muscle cells and it triggers the activation of not only CaMKK β /AMPK signaling in the cytoplasm [16, 55] but also several downstream signaling events including ATP-driven Ca^{2+} pumps localized in the sarcoplasmic reticulum and plasma membrane [56]. The apparent refractoriness of exaggerated AMPK phosphorylation to the CaMKK β inhibitor in vascular smooth muscle *ex vivo* (present study) may be explained by the notion that, under the conditions of metformin treatment that may inhibit mitochondrial ATP synthesis, phenylephrine challenge has the potential to enhance ATP consumption through the activation of ATP-driven Ca^{2+} pumps. Indeed, in the present study, although metformin or phenylephrine *per se* does not affect the energy state in vascular smooth muscle, metformin treatment followed by phenylephrine challenge leads to a significant increase in AMP/ATP ratio consistent with previous observations by Fogarty *et al.* [20]. In these earlier studies, Fogarty *et al.* have pointed out that inhibition of ATP synthesis (by phenformin) and increased ATP consumption/turnover (by A23187, a Ca^{2+} ionophore) in HeLa cells would act additively to increase intracellular ADP/ATP ratio (a surrogate for AMP/ATP ratio), thereby enhancing AMPK activation [20]. On this premise, earlier observations of enhanced AMPK activity by metabolic stress in porcine carotid artery smooth muscle, as reported by Rubin *et al.*, could also be explained by the additive effects of anoxia (diminution of ATP synthesis) and 2-deoxyglucose (increased ATP consumption due to its rapid phosphorylation to 2-deoxyglucose-6-phosphate) [12]. Thus, although ATP consumption rates for the maintenance of steady-state contractile force are >300-fold less in vascular smooth muscle compared with skeletal muscle, as observed by several investigators more than two decades ago [57], coordinated events that inhibit/diminish ATP synthesis and increase ATP consumption in vascular smooth muscle would enhance AMPK activation to promote vasorelaxation. Interestingly, while AMPK activation results in inhibition of

smooth muscle contraction, enhanced AMPK activity in skeletal muscle in response to contraction/exercise is implicated in GLUT4-mediated glucose transport [58].

In conclusion, although the energy needs of vascular smooth muscle is several fold less compared with skeletal muscle [57], intracellular environment that compromises ATP levels by inhibiting ATP synthesis (e.g., increased metformin uptake) and enhancing ATP consumption (e.g., Ca^{2+} elevating agents) would promote vasorelaxation. Future studies are clearly warranted that should examine the likely regulatory effects of OCT-3 on metformin action in conduit and resistance vessels. In view of the likely increase in metformin uptake/action due to enhanced OCT-1 expression in adipose tissue in obese subjects [59], future studies should also determine how metformin uptake in perivascular adipose tissue [60] influences vascular tone in health and disease.

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Fig. 1.

Effects of long-term metformin treatment on PE-induced smooth muscle contractility. Endothelium-denuded rat aortic rings were maintained in vascular cell basal medium with vehicle control (°) or metformin (Met) at 10 μ M (■), 100 μ M (▲), or 1 mM (▼) concentrations for 15 hours. Subsequently, the aortic rings were transferred to the organ bath system and maintained in Krebs buffer with the respective treatments for an additional 3 hours, as described under 'Materials and methods'. Control and metformin-treated aortic rings were then challenged with cumulative concentrations of PE (10^{-10} to 10^{-4} M) to determine the changes in contractile response. The $E_{\max}$ and pEC_{50} values for PE-induced contractility were then calculated as described. The data shown are the means $\pm$ SEM values obtained with aortic rings from 3 different animals. # p < 0.05; ## p < 0.01 compared with control (+ PE).

Fig. 2.

Effects of short-term metformin treatment on PE-induced smooth muscle contractility. Endothelium-denuded rat aortic rings were maintained under control conditions (○) or subjected to treatments with metformin (Met) at 10 μ M (■), 100 μ M (▲), 1 mM (▼), or 3 mM (●) concentrations for two different time intervals, 30 min (**A**) or 2 hours (**B**). In

parallel, aortic rings were treated with phenformin (Phen) at 10 μ M (■), 100 μ M (▲), 1 mM (▼), or 3 mM (●) concentrations for 2 hours (C). In addition, aortic rings were treated with AICAR at 1 mM (●) concentration for 30 min (D). Subsequently, control and treated aortic rings were challenged with cumulative concentrations of PE (10^{-10} to 10^{-4} M) to determine the changes in contractile response. The $E_{\max}$ and pEC_{50} values for PE-induced contractility were then calculated as described under 'Materials and methods'. The data shown are the means $\pm$ SEM values obtained with aortic rings from 4 different animals. # $p < 0.05$; ## $p < 0.01$; ### $p < 0.001$ compared with control (+ PE); n.s., not significant.

Fig. 3.

Effects of metformin (*verses* phenformin or AICAR) on basal and PE-induced changes in smooth muscle contractility and AMPK phosphorylation. Endothelium-denuded rat aortic rings were maintained under control conditions or treated with 3 mM metformin (Met) for 2 hours. In parallel, aortic rings were treated with 100 μ M phenformin (Phen) for 2 hours or 1 mM AICAR for 30 min. Subsequently, control and treated aortic rings were challenged with or without a fixed concentration of PE (1 μ M) for 6 min. (**A, C, E**) For both control and treated groups, the extent of increases in PE-induced smooth muscle contractility was determined (upper panels). In addition, the extent of changes in basal tension was determined prior to PE challenge (lower panels). (**B, D, F**) Immediately after contractility studies, the respective aortic rings were snap-frozen in liquid nitrogen and subjected to immunoblot analysis using primary antibodies specific for phospho-AMPK α (Thr172) and total AMPK α , as described under 'Materials and methods'. β -actin was used as an internal control. The extent of changes in AMPK phosphorylation is illustrated by the representative immunoblots (upper panel) and p-AMPK/AMPK ratio (lower panel). The data shown in the bar graphs are the means $\pm$ SEM values obtained with aortic rings from 3 different animals. [#] $p < 0.05$; ^{##} $p < 0.01$; ^{###} $p < 0.001$ compared with control (+ PE); ^{*} $p < 0.05$; ^{***} $p < 0.001$ compared with control (- PE); ^{££} $p < 0.01$; ^{£££} $p < 0.001$ compared with metformin/phenformin treatment (- PE).

Fig. 4.

Effects of metformin on basal and PE-induced changes in nucleotides (ATP, ADP, and AMP) and AMP/ATP ratio. Endothelium-denuded rat aortic rings were maintained under control conditions or treated with 3 mM metformin (Met) for 2 hours. Control and metformin-treated aortic rings were then challenged with or without 1 μ M PE for 6 min. After determining the changes in PE-induced smooth muscle contractility, the aortic rings were blotted dry, snap-frozen in liquid nitrogen, and stored at -80°C until analysis. In parallel, human aortic VSMCs were maintained under control conditions, snap-frozen in liquid nitrogen, and stored at -80°C until analysis, as described under 'Materials and methods'. VSMCs and aortic tissues were then subjected to quantification of nucleotides by LC-MS/MS MRM analysis, as described. The bar graphs show the nucleotide levels in VSMCs (**A**); and the changes in ATP, ADP, and AMP levels (**B**) and AMP/ATP ratio (**C**) in aortic rings under different treatment conditions. The data shown are the means $\pm$ SEM values obtained with aortic rings from 6 different animals, as described. * $p < 0.05$; ** $p < 0.01$ compared with control ($-$ PE); # $p < 0.05$; ## $p < 0.01$ compared with control ($+$ PE); £ $p < 0.05$; ££ $p < 0.01$ compared with metformin treatment ($-$ PE).

Fig. 5.

Effects of compound C, an AMPK inhibitor, on metformin inhibition of PE-induced smooth muscle contractility. Endothelium-denuded rat aortic rings were treated with vehicle control (○) or 40 μ M compound C (△) for 30 min. After 30 min, a parallel set of vehicle-treated aortic rings (●) and compound C-pretreated (▲) aortic rings were subjected to treatments with 3 mM metformin for 2 hours. Subsequently, control and treated aortic rings were challenged with cumulative concentrations of PE (10^{-10} to 10^{-4} M) to determine the changes in contractile response. The $E_{\max}$ and pEC_{50} values for PE-induced contractility were then calculated as described under 'Materials and methods'. The data shown are the means $\pm$ SEM values obtained with aortic rings from 4 different animals. # $p < 0.05$ compared with control (+ PE); \$ $p < 0.05$ compared with metformin treatment (+ PE).

Fig. 6.

Effects of metformin on basal and PE-induced changes in smooth muscle contractility and AMPK phosphorylation before or after pretreatment with compound C. Endothelium-denuded rat aortic rings were maintained under four different conditions: i) control group; ii) treatment with 3 mM metformin for 2 hours; iii) treatment with 40 μ M compound C for 30 min plus 2 hours; and iv) pretreatment with 40 μ M compound C for 30 min followed by treatment with 3 mM metformin for 2 hours. Subsequently, each group of aortic rings was challenged with or without 1 μ M PE for 6 min. (A) For all four groups, the extent of increases in PE-induced smooth muscle contractility was determined (upper panel). In addition, the extent of changes in basal tension was determined prior to PE challenge (lower panel). (B) Immediately after contractility studies, the respective aortic rings were snap-frozen in liquid nitrogen and subjected to immunoblot analysis using primary antibodies specific for phospho-AMPK α (Thr172) and total AMPK α , as described under 'Materials and methods'. β -actin was used as an internal control. The extent of changes in AMPK phosphorylation is illustrated by the representative immunoblots (upper panel) and p-AMPK/AMPK ratio (lower panel). The data shown in the bar graphs are the means $\pm$ SEM values obtained with aortic rings from 3 different animals. [#] $p < 0.05$; ^{###} $p < 0.001$ compared with control (+ PE); ^{*} $p < 0.05$; ^{***} $p < 0.001$ compared with control (– PE); ^{\$} $p < 0.05$; ^{\$\$\$} $p < 0.001$ compared with metformin treatment (+ PE).

Fig. 7.

Effects of metformin on basal and PE-induced changes in smooth muscle contractility and AMPK phosphorylation before or after pretreatment with STO-609. Endothelium-denuded rat aortic rings were maintained under four different conditions: i) control group; ii) treatment with 3 mM metformin for 2 hours; iii) treatment with 10 μ M STO-609 for 30 min plus 2 hours; and iv) pretreatment with 10 μ M STO-609 for 30 min followed by treatment with 3 mM metformin for 2 hours. Subsequently, each group of aortic rings was challenged with or without 1 μ M PE for 6 min. For all four groups, (A) PE-induced smooth muscle contractility and basal tension, and (B) AMPK phosphorylation and p-AMPK/AMPK ratio were determined as described under 'Materials and methods'. The data shown in the bar graphs are the means $\pm$ SEM values obtained with aortic rings from 3 different animals. $\#p < 0.05$; $##p < 0.01$; $###p < 0.001$ compared with control (+ PE); $*p < 0.05$; $***p < 0.001$ compared with control (- PE); $\$p < 0.05$ compared with metformin treatment (+ PE).

Fig. 8.

Effects of metformin on PE- or 5-HT-induced smooth muscle contractility under post-treatment washout conditions. Endothelium-denuded rat aortic rings were maintained under control conditions (○), treated with 3 mM metformin for 2 hours (●), or treated with 3 mM metformin for 2 hours followed by washout for 2 hours (□). Subsequently, all three groups of aortic rings were challenged with: **(A)** cumulative concentrations of PE (10^{-10} to 10^{-4} M); or **(B)** cumulative concentrations of 5-HT (10^{-9} to 10^{-3} M) to determine the changes in contractile response. The $E_{\max}$ and pEC_{50} values for PE- or 5-HT-induced contractility were then calculated as described under 'Materials and methods'. The data shown are the means $\pm$ SEM values obtained with aortic rings from 4 different animals. # $p < 0.05$; ## $p < 0.01$ compared with control (+ PE/+5-HT).

Fig. 9.

Effects of metformin on basal and PE-induced changes in smooth muscle contractility and AMPK phosphorylation under post-treatment washout conditions. Endothelium-denuded rat aortic rings were maintained under control conditions, treated with 3 mM metformin for 2 hours, or treated with 3 mM metformin for 2 hours followed by washout for 2 hours. Subsequently, each group of aortic rings was challenged with or without 1 μ M PE for 6 min. For all three groups, (A) PE-induced smooth muscle contractility and basal tension, and (B) AMPK phosphorylation and p-AMPK/AMPK ratio were determined as described in the legend to Fig. 5. The data shown in the bar graphs are the means $\pm$ SEM values obtained with aortic rings from 4 different animals. [#] $p < 0.05$; ^{###} $p < 0.001$ compared with control (+ PE); ^{*} $p < 0.05$; ^{***} $p < 0.001$ compared with control (- PE).

Fig. 10.

Quantification of metformin uptake in rat aortic rings by LC-MS/MS MRM analysis. Endothelium-denuded rat aortic rings were maintained under control conditions, treated with 3 mM metformin for 2 hours, or treated with 3 mM metformin for 2 hours followed by post-treatment washout of this drug for 2 hours. Subsequently, aortic rings were rinsed in ice-cold PBS, blotted dry, snap-frozen in liquid nitrogen, and stored at -80°C until analysis. Aortic tissue lysates were then subjected to quantification of metformin by LC-MS/MS MRM analysis, as described under 'Materials and methods'. (A-C) The representative chromatograms for metformin in the respective treatment groups are shown in comparison with control. IS, internal standard; CPS, counts per second. (D) The data shown in the bar graph are the means $\pm$ SEM values obtained with aortic rings from 3 different animals. * $p < 0.05$, compared with control.

Fig. 11.

Metformin inhibits phenylephrine (PE)-induced smooth muscle contraction through exaggerated AMPK phosphorylation (independent of CaMKKβ), which would occur via ↑AMP/ATP ratio upon coordinated inhibition of ATP synthesis (by metformin) and increase in ATP consumption (by $[Ca^{2+}]_i$ elevating agents that activate ATP-driven Ca^{2+} pumps).

Table 1

MRM transitions for nucleotides (ATP, ADP, and AMP), metformin and spingosine-1-phosphate.

	Q1 (Da)	Q3 (Da)	Dwell Time (msec)	CE (V)	DP (V)	EP (V)	CXP (V)
ATP	506	159	50	-50	-93	-10	-5
	506	79	50	-40	-93	-10	-5
ADP	426	134	50	-35	-93	-10	-5
	426	79	50	-65	-93	-10	-5
AMP	346	97	50	-36	-93	-10	-5
	346	79	50	-60	-93	-10	-5
Metformin	130	71	50	21	45	10	10
	130	60.1	50	32	45	10	10
Spingosine -1-phosphate	366.4	250.1	50	24	60	10	10

Note: The respective nucleotides (ATP, ADP, and AMP) and metformin were monitored using the parent mass (Q1) and fragment mass (Q3). CE, collision energy; DP, declustering potential; EP, entrance potential; CXP, collision cell exit potential; Da, Daltons; V, Volts.