Endothelium-dependent vasorelaxation to the AMPK activator AICAR is enhanced in aorta from hypertensive rats and is NO and EDCF dependent

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Ford RJ, Rush JW. Endothelium-dependent vasorelaxation to the AMPK activator AICAR is enhanced in aorta from hypertensive rats and is NO and EDCF dependent. Am J Physiol Heart Circ Physiol 300: H64–H75, 2011. First published October 22, 2010; doi:10.1152/ajpheart.00597.2010.—Activation of AMP-activated protein kinase (AMPK) induces vasorelaxation in arteries from healthy animals, but the mechanisms coordinating this effect are unclear and the integrity of this response has not been investigated in dysfunctional arteries of hypertensive animals. Here we investigate the mechanisms of relaxation to the AMPK activator 5-aminomimidazole-4-carboxamide 1-β-D-ribofuranoside (AICAR) in isolated thoracic aorta rings from spontaneously hypertensive rats (SHR) and normotensive Wistar-Kyoto rats (WKY). Although AICAR generated dose-dependent (10^{-6}–10^{-3} M) relaxation in precontracted WKY and SHR aortic rings with E^+ (e.g., noradrenaline, angiotensin) or without E^+ (e.g., endothelium, relaxation was enhanced in E^+ rings. Relaxation in SHR E^+ rings was also enhanced at low [AICAR] (10^{-6} M) compared with that of WKY (57 ± 8% vs. 3 ± 2% relaxation in SHR vs. WKY E^+), but was similar and near 100% in both groups at high [AICAR]. Pharmacological dissection showed that the mechanisms responsible for the endothelium-dependent component of relaxation across the dose range of AICAR are exclusively nitric oxide (NO) mediated in WKY rings, but partly NO dependent and partly cyclooxygenase (COX) dependent in SHR vessels. Further investigation revealed that ACh-stimulated COX-endothelium-derived contracting factors (EDCF)-mediated contractions were suppressed by AICAR, and this effect was reversed in the presence of the AMPK inhibitor Compound C in quiescent E^+ SHR aortic rings. Western blots demonstrated that P(Thr^{172})-AMPK and P(Ser^{79})-acetyl-CoA carboxylase (indexes of AMPK activation) were elevated in SHR versus WKY E^+ rings. Together these findings suggest that AMPK-mediated inhibition of EDCF-dependent contraction and elevated AMPK activation may contribute to the enhanced sensitivity of SHR E^+ rings to AICAR. These results demonstrate AMPK-mediated vasorelaxation is present and enhanced in arteries of SHR and suggest that activation of AMPK may be a potential strategy to improve vasomotor dysfunction by suppressing enhanced endoperoxide-mediated contraction and enhancing NO-mediated relaxation.

AMP-ACTIVATED PROTEIN KINASE (AMPK) is emerging as a potential regulator of vascular function. Although recognized primarily as a modulator of cellular energy status and metabolism (28, 57), the identification of its existence in vascular cells and of its ability to be stimulated by numerous physical (8, 20, 59), energetic (17), hormonal (5, 8, 43), and chemical (8, 9, 11, 12, 29, 35, 40, 53) mediators of vasomotor function suggest a potential role for AMPK in the regulation of vascular control. Of particular importance to the modulation of vasomotor tone, cell culture and in vitro biochemical experiments have shown that activated endothelial AMPK is able to increase phosphorylation and activation of endothelial nitric oxide synthase (eNOS) at Ser^{1177} (5, 6, 8, 12, 29, 40, 53) and Ser^{633} (7) and to increase NO availability (5, 7, 12, 40). Although the potential for AMPK to generate endothelium-dependent, NO-mediated relaxation in vivo and in intact blood vessels is implied by these data, very few observations have been made to verify this hypothesis in isolated vessels, or in vivo. In addition, endothelium-independent relaxation has been documented in isolated porcine, mouse, and rat conduit arteries from healthy animals in response to AMPK activators such as hypoxia (48), 5-aminomimidazole-4-carboxamide 1-β-d-ribofuranoside (AICAR) (22), and metformin (37), suggesting that vasorelaxation can be generated directly by activation of vascular smooth muscle AMPK. Biochemical experiments have shown that AMPK can directly regulate myosin light-chain kinase (MLCK) by decreasing its sensitivity to intracellular calcium (31) and thus presumably reduce vascular tone. Collectively, these observations suggest that AMPK may modulate vasomotor function through both endothelium-dependent and -independent mechanisms.

Hypertension is associated with vasomotor dysfunction, which manifests in part as impaired endothelium-dependent vasorelaxation and also as altered vascular smooth muscle function (18, 24, 26, 27, 42, 54). Impaired endothelium-dependent vasomotor function in hypertensive models has been attributed to both reduced NO bioavailability and enhanced endothelium-dependent vasoconstriction (13, 18, 24, 26, 27, 39, 54). Endothelium-derived contracting factors (EDCFs) are products of cyclooxygenase (COX) and have been identified as prostaglandins that exert contraction through thromboxane-prostanoid (TP)-receptor mediated signaling in the vascular smooth muscle (13, 18, 54). Recent work suggests that activation of AMPK may be able to interact with the COX vasomotor signaling axis to suppress the EDCF-mediated vasoconstriction in dysfunctional vessels (38). Since AMPK may also play a role in facilitating the generation of NO and vasodilation of vascular smooth muscle, it is conceivable that exogenous activation of AMPK may be able to improve vasomotor function by stimulating processes that are otherwise impaired in diseased arteries. Interestingly, depressed AMPK activation, or AMPK dysregulation, has been observed in arteries of rodent models where vascular dysfunction exists, including in streptozotocin-induced diabetes (55), Zucker diabetic fatty rats (2), aged rats (47), and Otsuka Long Evans Tokushima Fatty (OLETF) rats (34). In pilot work, we determined that basal AMPK activation is also blunted in aorta of spontaneously hypertensive rats (SHR; a genetic model of essential hyperten-
tion and vasomotor dysfunction) compared with levels in normotensive control Wistar-Kyoto rats (WKY). However, it is unknown whether the ability to activate AMPK is impaired in arteries of hypertensive animals and whether pharmacological activation of AMPK in dysfunctional arteries is able to generate relaxation in a similar manner to that observed in healthy vessels.

The objectives of this study therefore are to characterize the endothelial dependency and independency of AMPK-mediated vasorelaxation and AMPK activation in aortic rings from normotensive (WKY), to determine whether these responses are altered in aortic rings from hypertensive (SHR) animals, and to elucidate potential mechanisms responsible for AMPK-mediated vasomotor outcomes. Our data illustrate that AMPK-mediated relaxation in response to exogenous AMPK activation occurs through both endothelium-dependent and -independent mechanisms in arteries from normotensive animals, demonstrate for the first time that these responses also occur and are actually enhanced in arteries from hypertensive animals despite depressed basal AMPK activation, and suggest that the mechanisms responsible for endothelium-dependent relaxation are exclusively NO mediated in WKY, but both NO- and COX-, EDCF-dependent in SHR aorta. Activation of AMPK suppresses the exaggerated EDCF-dependent contractions observed in SHR compared with WKY arteries, and this effect likely contributes to the enhanced endothelium-dependent, AICAR-mediated relaxation observed in SHR.

MATERIALS AND METHODS

Animal Care and Procedures

The University of Waterloo Animal Care Committee approved all animal-related procedures in this study. Experiments were performed using a total of 50 male WKY and 50 male SHR obtained from Harlan (Indianapolis, IN). Animals were group-housed at a constant air temperature (20–21°C) and humidity (~50%) in a 12-h:12-h reverse light-dark cycle. Rats had free access to standard 22/5 Rodent Diet (W) lab chow (Harlan) and tap water. Before all experiments, body mass was recorded and rats were anesthetized by pentobarbital sodium injection (50–65 mg/kg ip; Vetquinoil N-A., Lavaltrie, QC, Canada). To confirm blood pressure levels in SHR and WKY, a subset of anesthetized animals from each group were instrumented for mean arterial pressure and heart rate measurements by inserting a Mikro-Tip Pressure Transducer catheter (Millar Instruments, Houston, TX) into the left common carotid artery (14, 23). Data were collected as previously described (14, 23).

Vasomotor Responses in Isolated Vessels

Animals were euthanized by rapidly removing the heart, which was dissected and weighed. The thoracic aorta was excised and 2-mm aortic rings were prepared for vascular myography as previously described (21). In some experiments, the endothelium was removed by inserting a 256 μm diameter titanium wire through the vessel lumen and rolling it on Whatman blotting paper (Whatman, Maidstone, England) soaked with 4°C Krebs-bicarbonate buffer. Consistent removal of the endothelium by this method was verified functionally by nonresponsiveness to a maximal dose of the endothelium-dependent vasodilatory agent ACh in phenylephrine (PE) preconstricted aortic rings and biochemically by substantial removal of eNOS protein content (i.e., <10–15% residual eNOS compared with endothelium-intact aortic rings from the same animals) assessed by Western blotting (data not shown). Rings were mounted onto a vascular myography apparatus (Radnoti, Monrovia, CA) where they were immersed in 37°C Krebs-bicarbonate buffer continuously aerated with 95% O2-5% CO2, and data were collected as already described (21). Gradual stretching to a predetermined optimal resting tension of 7 g (24) was achieved by increasing the tension by 0.5 g increments from 1 g every 5 min, and rings were equilibrated at optimal resting tension for 30 min. Each vessel was then contracted by two consecutive exposures to 60 mM potassium chloride (KCl, with washouts in between) to ensure contractile integrity of all aortic rings.

Dose-dependent Vasorelaxation to AICAR

Following washout of KCl and return to baseline tension, rings were allowed to equilibrate for 30 min and then precontracted with PE (10–6.5 M). When a stable plateau in tension was achieved, each ring was exposed to increasing concentrations of the AMPK activator AICAR (10–6 to 10–2 M) to generate dose-dependent relaxation responses. In some experiments, some rings with and without endothelium were preincubated with the NOS inhibitor Nω-nitro-L-arginine methyl ester (l-NAME; 10–4 M), the COX inhibitor indomethacin (Indo; 10–5 M), or both l-NAME and Indo for 30 min before PE preconstriction. The dose range for AICAR was based on prior experiments examining rodent aorta (22) and on our own pilot work. The functional viability of aortic rings following exposure to this dose and duration of AICAR was also tested in pilot experiments by evaluating the ability of rings to generate tension to KCl and PE following the AICAR dose-response curve (DRC) protocol. Tension generated to KCl or PE post-AICAR DRC was not significantly different from values obtained before the AICAR DRC in rings both with and without endothelium (KCl: E–, 83 ± 7% and E+, 103 ± 9% of KCl exposure pre-AICAR; and PE: E–, 108 ± 2% and E+, 92 ± 1% of PE precontracted tension pre-AICAR in n = 3 rats) when rings underwent extensive wash out (i.e., 6 consecutive exchanges of buffer bath) and a 1-h recovery period post-AICAR DRC. This is consistent with previous reports that it is possible to wash out AICAR (10, 17) and that AICAR does not produce nonspecific toxic effects under these conditions.

Functional Index of NO Bioavailability

In other experiments, rings were preincubated with no drug, l-NAME (10–4 M), AICAR (2 mM), or AICAR + l-NAME for 30 min (following KCl exposures) and then contracted with PE (10–6.5 M). The difference in tension generated to PE was compared across groups to assess the interactive effects of l-NAME and AICAR on NO bioavailability.

Endothelium-dependent Contractions

Quiescent aortic rings (not precontracted) were preincubated with l-NAME (10–4 M, to block the NO-mediated component of the response to ACh) and with either no drug, AICAR (500 μM, 30 min), the AMPK inhibitor Compound C (CC; 20 μM, 45 min), or AICAR + CC. The lowest dosages of AICAR and CC capable of generating maximal functional effects were chosen based on pilot experiments (data not shown). Rings were then exposed to increasing concentrations of ACh to elicit endothelium-dependent, COX-EDCF-mediated contractions as previously described (13, 14).

ZMP and Adenine Nucleotide Content in Aortic Rings

With the use of other WKY and SHR, aortic rings were prepared either with or without endothelium, mounted on the vascular myography apparatus as described above, and subjected to the same set-up and equilibration protocol. Following incubation with or without AICAR (2 mM) for 30 min, rings were quickly removed from the apparatus, snap frozen in liquid nitrogen, and stored at –80°C. Samples were freeze-dried under vacuum, weighed, cut into small pieces, and extracted for analysis of ZMP [the intracellular metabolite of AICAR responsible for AMPK activation (10)] and adenine nucleotide content by high-performance liquid chromatography as previ-
ously described (25, 32). ZMP (Sigma) was included in the methodological calibration standards and was clearly resolved from other peaks in standards and samples.

**Immunoblot Analysis of Protein Content and Phosphorylation Levels**

Aortic rings from a different set of WKY and SHR were prepared with and without endothelium and mounted on the vascular myography apparatus as described above. Rings were exposed to the protocol used above to obtain the AICAR dose-response functional measures (with all rings being assigned to the no drug condition) and were removed before and during the AICAR DRC to be snap frozen for Western blotting. Some rings were removed immediately before administration of the first AICAR dose (following PE precontraction) for baseline measures and when stable responses were obtained at each consecutive AICAR dose. Some rings were also snap frozen as time controls (precontracted with PE and allowed to remain contracted for the duration of the AICAR DRC protocol but not exposed to AICAR) to ensure that AMPK activation did not occur as a function of time during the collection period of the DRC. Rings were rapidly removed from the apparatus at appropriate points (care was taken not to damage the endothelium), snap frozen in liquid nitrogen, and stored at −80°C. The time from removal of the rings from the buffer to immersion in liquid nitrogen was <5 s. One ring was removed for each control point and AICAR dose, and all rings (controls and AICAR doses) were obtained from a single rat for each E− or E+ experiment. Sample preparation and immunoblotting procedures were performed as described previously (21). Briefly, 30 μg of protein were loaded per well, and membranes were Ponceau stained to confirm consistent protein loading across lanes. All blots were normalized to a thoracic aorta standard (aorta from young male Sprague-Dawley rats) that was run on all gels.

**Drugs, Chemicals, and Antibodies**

All drugs and chemicals were purchased from either Sigma-Aldrich (St. Louis, MO) or Bioshop Canada (Burlington, ON, Canada), with the exception of AICAR, which was purchased from Toronto Research Chemicals (Toronto, ON, Canada). For immunoblotting experiments, primary antibodies specific for the α-subunit of AMPK (recognizing both α1- and α2-subunit isoforms, 1:500 dilution) and P(Thr172)-AMPK (1:1,000 dilution), acetyl-CoA carboxylase (ACC; recognizing both 2-subunit isoforms, 1:500 dilution) and P(Thr172)-eNOS (1:3,000) were obtained from Cell Signaling Technology (via New England Biolabs, St. Louis, MO) or Bioshop Canada (Burlington, ON, Canada), with Drakos (recognizing both substrates, 1:250), P(Ser79)-ACC (1:500), and P(Ser1177)-eNOS (1:3,000 dilution), acetyl-CoA carboxylase (ACC; top tracing). Precontracted tension to 10−6 M AICAR (Fig. 1C). Removal of the endothelium blunted relaxation to AICAR at all concentration points, with relaxation reaching only 60 ± 1% at 10−2 M (P < 0.0001 vs. WKY E+; Fig. 1D). Relaxation was not detectable in either E+ or E− WKY rings until 10−3 M AICAR (Fig. 1, C and D).

**RESULTS**

- **Animal Characteristics**

  Animals were 20–24 wk of age at the time of experiment (Table 1). Hypertension was confirmed in SHR by measuring mean arterial pressure in a subset of animals before removal of the aorta for in vitro experiments (Table 1). Left ventricular hypertrophy, apparent in the greater left ventricle-to-body mass and heart weight-to-body mass ratios, also confirms typical cardiovascular pathology in SHR versus WKY (Table 1).

<table>
<thead>
<tr>
<th>Table 1. Animal characteristics</th>
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<tr>
<td><strong>WKY</strong></td>
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<td>Age, wk</td>
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<td>Whole body mass, g</td>
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<td>Tissue masses</td>
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<td>Left ventricle, mg</td>
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<td>Left ventricle/body mass, mg/g body mass</td>
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<td>Right ventricle/body mass, mg/g body mass</td>
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<td>Heart, mg</td>
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<td>Heart/body mass, mg/g</td>
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<tr>
<td>Mean blood pressure, mmHg</td>
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<td>Heart rate, beats/min</td>
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Values are means ± SE; n, number of animals per group. Hemodynamic parameters were assessed in the left common carotid artery of anesthetized Wistar-Kyoto rats (WKY) and spontaneously hypertensive rats (SHR) using a Millar pressure probe (n = 3); n = 12 for all other measures. Due to differences in body weight between strains, values were expressed both in absolute terms and relative to body weight.

- **Endothelium-dependent and -independent Vasomotor Responses to AICAR**

  AICAR generates both endothelium-dependent and endothelium-independent relaxation in aortic rings from WKY. Figure 1A displays representative tracings of responses to AICAR for both WKY E+ (middle tracing) and WKY E− (bottom tracing) rings and for a PE-contracted time control (WKY E+ ring, no AICAR; top tracing). Precontracted tension to 10−6 M PE was not different between WKY rings with or without endothelium [E+: 2.39 ± 0.32 g and E−: 2.31 ± 0.12 g; P = not significant (NS)]. WKY rings with intact endothelium relaxed from PE preconstriction in an AICAR dose-dependent manner to a maximum of 99 ± 7% in response to 10−2 M AICAR (Fig. 1C). Removal of the endothelium blunted relaxation to AICAR at all concentration points, with relaxation reaching only 60 ± 1% at 10−2 M (P < 0.0001 vs. WKY E+; Fig. 1D). Relaxation was not detectable in either E+ or E− WKY rings until 10−3 M AICAR (Fig. 1, C and D).

  Endothelium-dependent and -independent relaxation to AICAR is enhanced in aortic rings from SHR compared with those from WKY. Representative tracings for SHR E+, E−, and time control are shown in Fig. 1B. Since SHR E+ rings unexpectedly relaxed ~50% of precontracted tension at the lowest AICAR concentration (10−6 M) used in our experimental protocol, we retrospectively performed a small number of experiments, in SHR E+ rings only, at lower concentrations of AICAR to demonstrate the full dose-response relationship in this group. A sample tracing over the expanded [AICAR] range (i.e., beginning 10−9 M AICAR) has been included in Fig. 1B for SHR E+ rings. Relaxation to AICAR in SHR rings with endothelium intact was elevated above that observed in WKY aorta at 10−6 M AICAR and remained higher at every concentration to 10−2 M AICAR (Fig. 1C). In rings with endothelium removed, SHR rings
relaxed more than rings from WKY beginning at $10^{-4}$ M AICAR and at each increasing concentration to $10^{-2}$ M (Fig. 1D). PE precontraction was 1.88 ± 0.25 g in SHR E+ rings and 1.84 ± 0.25 g in SHR E- rings, and these values were not different from each other or from those of the respective WKY E+ and E- rings.

### ZMP and Adenine Nucleotide Content Following Incubation with AICAR

ZMP and adenine nucleotide content is not different in SHR versus WKY aortic rings following AICAR treatment. ZMP was not detectable in control rings from either WKY or SHR (Table 2). Following exposure to AICAR, ZMP content was not different in WKY or SHR rings in either the presence or absence of the endothelium (Table 2). Similarly, ATP, AMP, and the AMP-to-ATP ratio were not different in WKY or SHR rings with or without endothelium following AICAR treatment, and AICAR-treated values for these parameters were not different from those of the nontreated controls (Table 2).

### AMPK Protein Content and Activation Status in SHR and WKY Aortic Rings Before and During the AICAR Dose-response Protocol

Basal AMPK activation is blunted in SHR versus WKY aortic rings with endothelium before AICAR exposure. Phosphorylation of AMPK at Thr$^{172}$, a mandatory modification required for enzyme activation (28), and phosphorylation of ACC at Ser$^{79}$, a downstream target and well-established marker of AMPK activity (28), were evaluated as surrogate markers of AMPK activation. AMPK phosphorylation was expressed as the phosphorylated-to-total protein ratio to account for slightly higher levels of AMPK total protein expression in SHR versus WKY rings with endothelium (total AMPK: 1.31 ± 0.04 vs. 1.00 ± 0.13 in SHR and WKY E+ rings, respectively; $P = 0.0317$). Before AICAR exposure, the P(Thr$^{172}$)-AMPK/AMPK ratio in SHR rings with endothelium was only ~39% of that observed in WKY rings (Fig. 2A; 0.39 ± 0.07 vs. 1.00 ± 0.11, respectively, at baseline; $P = 0.0008$). P(Ser$^{79}$)-ACC was similarly depressed in E+ rings from SHR versus WKY rings before AICAR exposure (Fig. 3A; 0.33 ±

Fig. 1. Vasorelaxation to 5-aminoimidazole-4-carboxamide 1-β-D-ribofuranoside (AICAR) is both endothelium dependent and -independent and is enhanced in aorta from spontaneously hypertensive rats (SHR). Representative tracings demonstrate the relaxation responses to increasing concentrations of AICAR in aortic rings from Wistar-Kyoto rats (WKY; A) and SHR (B) with (E+; middle tracings) and without (E-; bottom tracings) endothelium following phenylephrine (PE; $10^{-6.5}$ M) precontraction. Time control tracings (top; rings precontracted with PE but not exposed to AICAR doses) demonstrate the ability of rings to maintain contraction over the duration of the AICAR dose-response protocol. Relaxation of WKY and SHR aortic rings with (C) and without (D) endothelium to increasing concentrations of AICAR is shown. Responses are expressed as mean percent relaxation from PE precontraction (PE $10^{-6.5}$ M). #$P < 0.05$, *$P < 0.01$ vs. WKY; n = 4–6 rings per group from different animals. Con, no drug.
0.10 vs. 1.00 ± 0.20, respectively, at baseline; \( P = 0.0185 \), although total ACC protein content was not different in SHR versus WKY rings with endothelium (1.00 ± 0.26 vs. 0.93 ± 0.16, respectively; \( P = 0.8304 \)). In contrast, in rings lacking endothelium, neither P(Thr\( ^{172} \))-AMPK and P(Ser\( ^{79} \))-ACC (Figs. 2B and 3B) nor total AMPK and ACC protein levels were different in SHR versus WKY (AMPK: 1.05 ± 0.17 vs. 1.00 ± 0.24 in SHR and WKY E+ rings, \( P = 0.8583 \); ACC: 1.09 ± 0.37 vs. 1.00 ± 0.26 in SHR and WKY E+ rings, respectively, \( P = 0.8601 \)). Thus the depressed AMPK activation in SHR versus WKY aorta appears to be localized to the endothelium rather than the smooth muscle.

### Table 2. ZMP and adenine nucleotide content of SHR and WKY aortic rings

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<thead>
<tr>
<th></th>
<th>WKY</th>
<th>SHR</th>
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<tbody>
<tr>
<td>ZMP</td>
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<tr>
<td>No drug</td>
<td>Not detectable</td>
<td>Not detectable</td>
<td>Not detectable</td>
<td>Not detectable</td>
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<tr>
<td>AICAR</td>
<td>4.0 ± 0.9</td>
<td>4.0 ± 0.3</td>
<td>3.4 ± 0.2</td>
<td>3.7 ± 0.3</td>
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<tr>
<td>ATP</td>
<td></td>
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<tr>
<td>No drug</td>
<td>0.89 ± 0.15</td>
<td>1.32 ± 0.26</td>
<td>0.82 ± 0.12</td>
<td>0.78 ± 0.17</td>
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<td>AICAR</td>
<td>0.79 ± 0.13</td>
<td>1.12 ± 0.19</td>
<td>0.62 ± 0.13</td>
<td>0.69 ± 0.17</td>
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<tr>
<td>AMP</td>
<td></td>
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<tr>
<td>No drug</td>
<td>0.67 ± 0.06</td>
<td>0.46 ± 0.10</td>
<td>0.53 ± 0.08</td>
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<td>AICAR</td>
<td>0.72 ± 0.04</td>
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<td>AMP/ATP</td>
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<tr>
<td>No drug</td>
<td>0.87 ± 0.15</td>
<td>0.56 ± 0.24</td>
<td>0.77 ± 0.18</td>
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<td>AICAR</td>
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<td>0.55 ± 0.19</td>
<td>1.00 ± 0.27</td>
<td>0.99 ± 0.31</td>
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Values are means ± SE; \( n = 5 \) to 6 rings per group from different animals. All values are expressed as micromoles per gram dry weight except the AMP-to-ATP ratio, which is unitless. Aortic rings were loaded on the myography apparatus, subjected to the same start and equilibration protocol as used in the collection of functional measures, and then incubated with 2 mM 5-aminoimidazole-4-carboxamide 1-β-D-ribofuranose (AICAR) or no drug for 30 min. Rings were snap frozen and then extracted for analysis by high-performance liquid chromatography. No significant differences were present between groups following AICAR incubation or compared with control rings.

### Fig. 2. Phosphorylation of AMP-activated protein kinase (AMPK) activation site Thr\( ^{172} \) during AICAR dose-response curves in SHR and WKY aortic rings. P(Thr\( ^{172} \))-AMPK and total protein content of the AMPK-α subunit were assessed by Western blotting in homogenates of aortic rings that were removed during the AICAR dose-response curve protocol. The ratio of phosphorylated to total AMPK protein is expressed in rings with (E+; A) and without (E−; B) endothelium immediately before AICAR exposure [baseline (B), PE precontraction only], at each AICAR concentration during the dose-response curve (expressed in log M), and in time controls (TC; PE precontraction for the duration of the dose-response curve with no AICAR exposure). Representative blots are shown for phosphorylation of AMPK at Thr\( ^{172} \) (p-AMPK) and AMPK in SHR and WKY aortic rings with (C) and without (D) endothelium. The bottom 2 panels show the same data expressed as fold increase normalized to the respective WKY or SHR baseline in E+ (E) and E− (F) rings to account for initial baseline differences between WKY and SHR. Legend conventions are conserved across panels. Results represent data collected from 3 independent experiments where aortic rings from a single animal were used to generate all treatment conditions for each experiment. Blots were run in duplicate for each set of samples. Data are expressed as means ± SE of densitometry analyses. \#\( P < 0.05 \), *\( P < 0.01 \) vs. WKY.
AMPK activation is enhanced in SHR versus WKY aortic rings with endothelium at low AICAR doses but is similar in SHR and WKY aortic rings without endothelium. The levels of P(Thr\(^{172}\))-AMPK (Fig. 2) and P(Ser\(^{79}\))-ACC (Fig. 3) were increased significantly from baseline with increasing concentrations of AICAR in E\(^{+}\) and E\(^{-}\) rings from WKY and SHR. Time controls, precontracted with PE, but not exposed to any AICAR doses (designated as TC in Figs. 2, A and B, and 3, A and B), showed no changes in P(Thr\(^{172}\))-AMPK/AMPK or P(Ser\(^{79}\))-ACC from baseline in SHR or WKY rings with (Figs. 2A and 3A) or without (Figs. 2B and 3B) endothelium, demonstrating that no changes in AMPK activation occurred as a function of time over the duration of the protocol in the absence of AICAR. When data were expressed relative to WKY baseline values (Fig. 2, A and B), P(Thr\(^{172}\))-AMPK/AMPK in SHR E\(^{+}\) was either not different or was lower (at 10\(^{-4}\) P = 0.0041 and 10\(^{-3}\) M P = 0.0029) than in WKY E\(^{+}\) rings in response to AICAR (Fig. 2A) and was not different at any concentration in SHR versus WKY E\(^{-}\) rings (Fig. 2B). Similarly, P(Ser\(^{79}\))-ACC was not significantly different at any AICAR concentrations in SHR versus WKY rings either with (Fig. 3A) or without (Fig. 3B) endothelium. However, when values were expressed as fold increase from their respective baseline values, the increase in P(Thr\(^{172}\))-AMPK/AMPK over baseline was significantly greater in SHR than in WKY E\(^{+}\) rings at 10\(^{-6}\) M (P = 0.0430) and 10\(^{-5}\) M (P = 0.0065; Fig. 2E), and P(Ser\(^{79}\))-ACC was increased in SHR over WKY at 10\(^{-5}\) M (P = 0.0137) and 10\(^{-4}\) M (P = 0.0250) in rings with endothelium (Fig. 3E). In contrast, no differences were observed between P(Thr\(^{172}\))-AMPK/AMPK and P(Ser\(^{79}\))-ACC fold increases from baseline between SHR and WKY in E\(^{-}\) rings at any AICAR dose (Figs. 2F and 3F).

**Pharmacological Dissection of Mechanisms Responsible for Endothelium-dependent Relaxation to AICAR**

Endothelium-dependent relaxation to AICAR in WKY aortic rings is NO mediated. Aortic rings were incubated with L-NAME, Indo, or L-NAME + Indo to determine the involvement of NO- and COX-dependent signaling in the vasodilatory responses to AICAR. Relaxation was blunted in WKY E\(^{+}\) aortic rings treated with either L-NAME or L-NAME + Indo compared with the untreated controls (Fig. 4A), to levels resembling those of E\(^{-}\) rings (P = NS vs. E\(^{-}\) control; Fig. 4C). Indo alone, however, did not alter relaxation compared with the E\(^{-}\) control condition at any AICAR concentration (Fig. 4A). In WKY E\(^{-}\) rings, relaxation to AICAR was not affected by L-NAME, Indo, or L-NAME + Indo (Fig. 4C). Precontraction to PE was not affected by drug incubation and was not significantly different across groups (WKY E\(^{+}\) control: 2.39 ± 0.32 g, E\(^{+}\) L-NAME: 2.72 ± 0.27 g, E\(^{+}\) Indo: 2.13 ± 0.24 g, E\(^{+}\) L-NAME Indo: 2.82 ± 0.20 g; and E\(^{-}\) control: 2.31 ± 0.12 g).
Fig. 4. Mechanisms of AICAR-mediated endothelium-dependent relaxation in SHR and WKY aortic rings. Aortic rings from SHR and WKY were incubated with either nitric oxide (NO) synthase (NOS) inhibitor L-NAME or LN, cyclooxygenase (COX) inhibitor indomethacin (Indo), or L-NAME + Indo (LN Indo) to determine the NO and endoperoxide dependency of relaxation responses to AICAR. Percent relaxation from phenylephrine preconstriction is displayed in WKY and SHR rings with (A and B) and without (C and D) endothelium. Legend conventions are conserved across E+ and E− panels. SHR and WKY E+ no drug (Con) and E− Con data have been repeated in the figure above (from previous figures) for comparison. Each treatment was performed in aortic rings from each animal. *P < 0.01, †P < 0.05 vs. E− Con; ‡P < 0.01 vs. E+ LN Indo; n = 3–6 rings per group from different animals.

g, E− l-NAME: 2.42 ± 0.28 g, E− Indo: 2.62 ± 0.36 g, E− l-NAME Indo: 2.55 ± 0.10 g).

Endothelium-dependent relaxation to AICAR in SHR aortic rings is NO and endoperoxide mediated. Across the lowest three concentrations of the AICAR dose response curve (10^{-6}, 10^{-5}, and 10^{-4} M), aortic rings with endothelium intact and preincubated with Indo relaxed only ∼20–40% from precontraction, whereas no drug controls relaxed 57–71% (P < 0.0001; E− Indo vs. control at each of the 3 lowest concentrations; Fig. 4B). E+ rings treated with l-NAME relaxed only ∼15–25% (P < 0.0001 vs. E+ control at 10^{-6}, 10^{-5}, and 10^{-4} M; Fig. 4B). Relaxation was absent across the lowest three AICAR concentrations in E+ rings preincubated with both l-NAME + Indo (P < 0.0001 vs. E+ Con at 10^{-6}, 10^{-5}, and 10^{-4} M; Fig. 4B) and was not different from responses observed in SHR E+ control rings (P = NS vs. E− control; Fig. 4D). l-NAME and Indo groups were also each significantly different from the l-NAME + Indo group across the first three doses of AICAR (P < 0.0001 for both l-NAME and Indo vs. l-NAME + Indo at 10^{-6}, 10^{-5}, and 10^{-4} M; Fig. 4B). In contrast, preincubation with l-NAME, Indo, or l-NAME + Indo did not alter relaxation responses to AICAR in any of in E− rings compared with E− control (Fig. 4D). In general, tension generated to PE was not affected by drug preincubation and was not different from the respective WKY treatment groups, except that SHR E+ l-NAME preconstricted tension was greater than SHR E+ control (P = 0.0321), and preconstricted tension in SHR E− Indo was lower than that of WKY E+ Indo (P = 0.0043) and of SHR E+ control (P = 0.0163). All other preconstricted tension values were not significantly different from each other (SHR E+ control: 1.88 ± 0.25 g, E+ l-NAME: 2.98 ± 0.15 g, E+ Indo: 0.95 ± 0.16 g, E+ l-NAME Indo: 1.91 ± 0.15 g; and E− control: 1.84 ± 0.25 g, E− l-NAME: 1.77 ± 0.14 g, E− Indo: 2.17 ± 0.20 g, E− l-NAME Indo: 2.05 ± 0.23 g). Vasorelaxation responses to the endothelium-independent vasodilator sodium nitroprusside in rings precontracted with PE were not altered between drug conditions where precontraction values were different (as determined by comparison of EC50 and maximal response in each group; data not shown), confirming that drug-dependent variations in precontraction values per se did not significantly influence relaxation responses.

Phosphorylation of eNOS at activation site Ser^{1177} was not increased in aortic rings over the AICAR DRC. To determine whether AMPK-mediated phosphorylation of eNOS activation site Ser^{1177} could account for the NO-dependent vasomotor responses to AICAR (a potential mechanism suggested by previous work of others), P(Ser^{1177})-eNOS was assessed in the homogenates of aortic rings collected over the functional AICAR DRC. P(Ser^{1177})-eNOS was not significantly different between SHR and WKY aortic rings at baseline and was not significantly affected by AICAR over the AICAR DRC protocol (Fig. 5A). Total eNOS protein content also did not change over the AICAR DRC in either SHR or WKY (data not shown), although total eNOS content was greater in SHR versus WKY (SHR, 1.56 ± 0.07 vs. WKY, 1.00 ± 0.05; P < 0.0001).

NO bioactivity is increased with AICAR incubation in SHR and WKY aortic rings as determined by the influence of AICAR on PE contraction in the presence or absence of l-NAME. The additional tension generated to PE in the presence versus the absence of NOS inhibition (l-NAME) is an established functional indicator of NO bioavailability in in vitro pharmacological studies (15, 26). Greater NO bioavailability is revealed by a larger increment in PE-generated tension (l-NAME vs. no l-NAME) in one condition compared with that in another condition. Since the functional data in Fig. 4 indicate a role for NO in AICAR-induced relaxation generated in WKY and SHR aortic rings despite a lack of increase in eNOS Ser^{1177} phosphorylation with AICAR treatment (Fig. 5), we evaluated the influence of AICAR on PE contraction in the presence versus absence of l-NAME as an additional functional index of the...
Fig. 5. Phosphorylation of endothelial NOS (eNOS) at Ser1177 was not increased in response to AICAR in SHR or WKY aortic rings. P(Ser1177)-eNOS content was assessed by Western blotting in aortic rings removed during the AICAR dose-response curve protocol. A: P(Ser1177)-eNOS is expressed in rings with endothelium either immediately before AICAR exposure [baseline (B), PE precontraction only], at each AICAR concentration during the dose-response curve (expressed using log M nomenclature), and in time controls (TC; PE precontraction for the duration of the dose-response curve with no AICAR exposure). Representative blots depict phosphorylation of eNOS at Ser1177 (p-eNOS; B) for all conditions in the figures above. Results represent data collected from 3 independent experiments where aortic rings from a single animal were used to generate all treatment conditions for each experiment. Blots were run in duplicate for each set of samples. Legend conventions are conserved across panels. Data represent means ± SE. No significant differences were observed with AICAR treatment.

The effect of AICAR on NO bioavailability (15, 26; Fig. 6). As expected, preincubation of aortic rings with l-NAME alone resulted in greater PE-generated tension compared with the no-l-NAME control group in both WKY and SHR, demonstrating the influence of NO on net PE contraction. Preincubation with AICAR significantly decreased the contraction generated to PE compared with the no-drug condition in both SHR and WKY aortic rings. This effect was fully prevented in WKY and partially prevented in SHR rings that had been pretreated with AICAR and l-NAME (Fig. 6, A and B). The resulting differential in PE generated tension comparing l-NAME with no l-NAME groups is thus much greater in rings treated with AICAR than in the respective control rings, demonstrating that AICAR enhances NO bioavailability in both WKY and SHR groups.

Endothelium-dependent, EDCF-mediated contraction is blunted in the presence of AICAR but recovered with AMPK inhibitor CC in both SHR and WKY aortic rings. In addition to producing NO-dependent relaxation, ACh also generates COX-, EDCF-dependent contraction that is augmented in conduit arteries of SHR versus WKY and is considered to be a major contributor to the vascular dysfunction in this and other models of cardiovascular disease (13, 18, 54). As expected based on previous work in SHR and WKY conduit arteries (13, 18, 54), endothelium-dependent contractions generated to increasing concentrations of ACh in quiescent vessel preparations were robust in aortic rings from SHR (Fig. 7B) but were of a much lower magnitude in those from WKY (Fig. 7A). Both removal of the endothelium and preincubation of rings with the COX inhibitor Indo abolished contractions in WKY and SHR aortic rings (Fig. 7, A and B), confirming the endothelium- and COX dependency of these responses, respectively, as has been previously reported (13, 54). Preincubation with AICAR similarly blunted the COX-EDCF-mediated contraction to ACh in both WKY and SHR compared with the E+ controls (Fig. 7, C and D). CC almost completely reversed the effect of AICAR in WKY (Fig. 7C) and SHR (Fig. 7D) rings, although contractions were not completely restored in SHR at lower ACh concentrations. Responses in rings preincubated with CC alone were not different from those treated with AICAR + CC in either SHR or WKY (data not shown), confirming the AMPK specificity of the AICAR effect. Collectively, these data imply that AMPK suppressed the COX-EDCF-mediated contractile response that is more robust in SHR than in WKY aortic rings.

DISCUSSION

The main findings of our study are that AICAR generates both endothelium-dependent and -independent relaxation in arteries from normotensive rats, that these responses also occur and are actually enhanced in arteries from hypertensive animals despite depressed basal AMPK activation, and that the mechanisms responsible for endothelium-dependent relaxation are exclusively NO mediated in WKY but NO- and COX dependent in SHR aorta. Further investigation into the mechanisms responsible for the enhanced endothelium-dependent relaxation to AICAR in SHR revealed that AMPK-mediated suppression of downstream COX-EDCF-dependent vasoconstrictory signaling pathways and enhanced AMPK activation at low AICAR doses likely account for a significant portion of the augmented sensitivity of SHR versus WKY E+ aortic rings. These results are novel and add significantly to our understanding of AMPK-mediated vasomotor function by being the first to investigate endothelium-dependent and -independent func-

Fig. 6. AICAR preincubation blunts contraction to PE in an NO-dependent manner demonstrating an AICAR-mediated increase in NO bioavailability. Aortic rings from WKY (A) and SHR (B) were preincubated with no drug (Con), AICAR (2 mM), l-NAME (10−4 M), or AICAR + l-NAME for 30 min and then contracted with PE (10−6.5 M). Values are expressed as the mean difference in developed tension from the no-drug control group (E+ Con). *P < 0.05, †P < 0.001 vs. E+ Con or vs. an adjacent group where indicated; n = 5–15 rings per group from different animals.
Ser79 on ACC) responded in a dose-dependent manner over the Ser1177 (5, 6, 8, 12, 29, 40) and that AMPK activation has been demonstrated by AICAR in SHR and WKY aortic rings. A number of functional responses to AICAR from WKY and SHR.

Endothelium-independent relaxation to AICAR and other AMPK activators (i.e., deoxy-glucose/hypoxia and metformin) has been observed in arteries of hypertensive animals and by elucidating mechanisms responsible for the endothelium-dependent functional response to AICAR in a conduit artery from WKY and SHR. Preincubation with L-NAME and in the absence of precontraction (quiescent rings). The endothelium- and COX dependency of these responses was verified in WKY (A) and SHR (B) by removal of the endothelium (E−) and by preincubation with the COX inhibitor Indo, respectively. In some experiments rings from WKY (C) or SHR (D) were preincubated with either no drug (Con), AICAR (500 μM), or AICAR + AMPK inhibitor Compound C (CC; 20 μM) to determine the influence of AMPK on this response. Some rings were also preincubated with CC alone; these responses were not different from the AICAR + CC response in SHR or WKY and have therefore not been illustrated in the figure for clarity. Values are expressed as mean percent of tension developed to 60 mM potassium chloride (KCl). Each treatment was performed in aortic rings from each animal.

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Preincubation with L-NAME completely removed the endothelial component of AICAR-mediated relaxation in WKY and partially blocked the endothelium-dependent component in SHR aortic rings (Fig. 4), suggesting that AICAR-mediated endothelium-dependent relaxation is completely NO dependent in WKY and only partially NO dependent in SHR. Our observations indicate no AICAR-dependent increases in phosphorylation of eNOS activation site Ser1177 in SHR or WKY aortic rings (Fig. 5), but further experiments verified that functional NO bioavailability is increased with AICAR nonetheless by demonstrating that preincubation with AICAR blunts contraction to PE in rings from WKY and SHR in an NO-dependent manner (Fig. 6). Therefore we conclude that AMPK must be facilitating the eNOS-mediated production of NO through mechanisms other than Ser1177 phosphorylation such as phosphorylation of eNOS Ser633 (8), assisting in eNOS association with heat shock protein 90 (12, 51), promoting the deacetylation of endothelial cells (5, 8, 12, 40), establishing NO as a potential mediator of endothelium-dependent effects of AMPK. However, AMPK activation does not result in eNOS phosphorylation under some conditions (19, 41), suggesting that AMPK activity and eNOS phosphorylation can be dissociated and that the influence of AMPK on NO-mediated vasomotor function may also involve other mechanisms in addition to the Ser1177 phosphorylation-mediated activation of eNOS. Although some studies infer that NO-dependent relaxation stimulated by AMPK-mediated activation of eNOS may be possible, there is limited evidence from previous studies that acute activation of AMPK is capable of generating functional vasodilatory outcomes by this mechanism in an intact blood vessel or in the context of an in vivo vascular system (3, 4). Our data, along with the recently published work of Bradley et al. (4), are some of the first to our knowledge to demonstrate an association between endothelium, NO-dependent relaxation and the activation of AMPK in isolated intact vessels. Preincubation with L-NAME completely removed the endothelial component of AICAR-mediated relaxation in WKY and partially blocked the endothelium-dependent component in SHR aortic rings (Fig. 4), suggesting that AICAR-mediated endothelium-dependent relaxation is completely NO dependent in WKY and only partially NO dependent in SHR. Our observations indicate no AICAR-dependent increases in phosphorylation of eNOS activation site Ser1177 in SHR or WKY aortic rings (Fig. 5), but further experiments verified that functional NO bioavailability is increased with AICAR nonetheless by demonstrating that preincubation with AICAR blunts contraction to PE in rings from WKY and SHR in an NO-dependent manner (Fig. 6). Therefore we conclude that AMPK must be facilitating the eNOS-mediated production of NO through mechanisms other than Ser1177 phosphorylation such as phosphorylation of eNOS Ser633 (8), assisting in eNOS association with heat shock protein 90 (12, 51), promoting the deacetylation of eNOS (7) to assemble active eNOS complexes, etc. Alternatively, AMPK may increase NO bioavailability independently of NO

We were interested in investigating mechanisms responsible for the endothelium-dependent component of the relaxation generated by AICAR in SHR and WKY aortic rings. A number of cell culture and in vitro biochemical experiments have demonstrated that AMPK can phosphorylate eNOS at activation site Ser1177 (5, 6, 8, 12, 29, 40) and that AMPK activation has been associated with increased NO release from endothelial cells (5, 8, 12, 40), establishing NO as a potential mediator of endothelium-dependent effects of AMPK. However, AMPK activation does not result in eNOS phosphorylation under some conditions (19, 41), suggesting that AMPK activity and eNOS phosphorylation can be dissociated and that the influence of AMPK on NO-mediated vasomotor function may also involve other mechanisms in addition to the Ser1177 phosphorylation-mediated activation of eNOS. Although some studies infer that NO-dependent relaxation stimulated by AMPK-mediated activation of eNOS may be possible, there is limited evidence from previous studies that acute activation of AMPK is capable of generating functional vasodilatory outcomes by this mechanism in an intact blood vessel or in the context of an in vivo vascular system (3, 4). Our data, along with the recently published work of Bradley et al. (4), are some of the first to our knowledge to demonstrate an association between endothelium, NO-dependent relaxation and the activation of AMPK in isolated intact vessels. Preincubation with L-NAME completely removed the endothelial component of AICAR-mediated relaxation in WKY and partially blocked the endothelium-dependent component in SHR aortic rings (Fig. 4), suggesting that AICAR-mediated endothelium-dependent relaxation is completely NO dependent in WKY and only partially NO dependent in SHR. Our observations indicate no AICAR-dependent increases in phosphorylation of eNOS activation site Ser1177 in SHR or WKY aortic rings (Fig. 5), but further experiments verified that functional NO bioavailability is increased with AICAR nonetheless by demonstrating that preincubation with AICAR blunts contraction to PE in rings from WKY and SHR in an NO-dependent manner (Fig. 6). Therefore we conclude that AMPK must be facilitating the eNOS-mediated production of NO through mechanisms other than Ser1177 phosphorylation such as phosphorylation of eNOS Ser633 (8), assisting in eNOS association with heat shock protein 90 (12, 51), promoting the deacetylation of eNOS (7) to assemble active eNOS complexes, etc. Alternatively, AMPK may increase NO bioavailability independently of NO
production per se via possible effects on NO destruction or the sensitivity of vascular smooth muscle to NO (i.e., the other determinants of NO bioavailability). For example, activation of AMPK has been associated with decreased oxidative stress in human aortic endothelial cells and mouse aorta (36, 56) and could thus potentially increase NO bioavailability by reducing the interaction of NO with reactive oxygen species. However, the mechanisms attributed to the AMPK-mediated reduction in oxidative stress in these studies involved AMPK-mediated modifications in protein transcription, translation, etc., processes which require a time frame of hours to days to produce effects, and therefore such mechanisms do not likely contribute to the acute functional responses observed here. Further studies will be required to determine whether oxidative stress is involved in mediating acute effects of AMPK activation on vasomotor function.

Interestingly, a portion of the endothelium-dependent component of the relaxation generated by AICAR was COX dependent in SHR but not WKY aortic rings, since the COX 1 and 2 inhibitor Indo partially blocked endothelium-dependent relaxation in SHR but not in WKY vessels (Fig. 4). Since a portion of the tension generated by PE is EDCF mediated in SHR aorta from our experiments [see PE precontracted tension values in RESULTS, as also observed previously by our laboratory (23) and others (1, 39)], it appeared that AICAR was causing endothelium-dependent relaxation of precontracted SHR aortic rings by inhibiting the enhanced COX-EDCF-mediated contraction that is present in SHR [an effect that would not be expected to occur in WKY since the contribution of EDCFs to PE-mediated contraction in WKY rings is minimal (1, 23, 39)]. To test this hypothesis, we assessed ACh-induced contraction in quiescent rings in the presence of 1-NAME according to an established protocol that has previously shown these contractions to be endothelium-, COX-, and TP-receptor dependent, and to be greatly enhanced in SHR versus WKY (13, 18, 54). Indeed, our results herein confirm the endothelium- and COX dependency of the ACh-induced contractions and that the magnitude of these contractions is greater in SHR versus WKY aorta. We found that these responses were robustly blunted with AICAR and that the effect of AICAR was largely prevented by preincubation of rings with the AMPK inhibitor CC, confirming AMPK specificity of this observation (Fig. 7).

Therefore, these novel results demonstrate that AMPK is suppressing COX-EDCF-mediated contractions in SHR aortic rings, through inhibition of either COX-EDCF production/release or TP receptor signaling in the vascular smooth muscle. These results are consistent with related work in other models from other investigations that have reported that AICAR and metformin both blunt endothelium-dependent contraction in mesenteric arteries from OLETF rats (another model of enhanced endothelium-derived contraction) likely by suppressing the release of both prostacyclin and thromboxane A2 (38). These findings also support the hypothesis that AICAR-mediated dilation of precontracted SHR aortic rings was achieved in part through suppression of COX-EDCF-mediated contraction.

The relaxation responses to AICAR in SHR and WKY aortic rings were also partially endothelium independent (Fig. 1). Although we did not investigate intracellular signaling mechanisms within the vascular smooth muscle that could contribute to AICAR-mediated endothelium-independent relaxation in the present study, previous in vitro biochemical experiments demonstrate that AMPK is able to phosphorylate and desensitize MLCK and thus could presumably induce relaxation in the context of an intact vascular system (31). Endothelium-independent relaxation to AICAR may also occur due to effects of AMPK on HMG-CoA reductase (10, 16, 33, 44), which could involve modulation of Rho-kinase and L-type channel extracellular calcium entry (45), thus potentially exerting influence over vascular smooth muscle mechanisms of calcium sensitization and calcium-mediated contraction. As indicated, however, we did not undertake to study these mechanisms in the current study, and additional cellular and physiological studies will be required to elucidate mechanisms of AMPK-mediated relaxation in vascular smooth muscle.

Both the endothelium-dependent and -independent components of the relaxation response to AICAR were enhanced in SHR aortic rings compared with those of WKY (Fig. 1). Initially, it was tempting to speculate that functional responses in SHR vessels would be less responsive to AICAR than those of WKY, since our own pilot work indicated depressed activation of AMPK in SHR aortas before AICAR treatment [P(Thr172)-AMPK and P(Ser79)-ACC in SHR vs. WKY E+ rings; Figs. 2A and 3A]. However, studies performed in tissues from diseased or aging models have shown that the ability to activate AMPK pharmacologically is not necessarily compromised when AMPK activation is depressed in the basal state (46, 49, 58), and thus there does not appear to be dysfunction in the capacity to activate the AMPK protein per se under these conditions. To help explain the different functional sensitivities of SHR and WKY rings to AICAR, we assessed the intracellular stimulus for AMPK activation and the activation of AMPK over the AICAR DRC protocol in SHR and WKY vessels. The stimulus for AMPK activation was not different between SHR and WKY rings, since all rings were similarly capable of uptake and conversion of AICAR to ZMP (Table 2). ZMP is the intracellular metabolite of AICAR and acts as an AMP analog to activate AMPK both allosterically and by rendering it a poorer substrate to upstream phosphatases (50, 52). However, both P(Thr172)-AMPK and P(Ser79)-ACC fold increases from baseline were approximately twofold greater in SHR versus WKY E+ rings at the lower concentrations of AICAR (Figs. 2E and 3E), but P(Thr172)-AMPK and P(Ser79)-ACC were similar in SHR versus WKY E- rings over all AICAR doses (Figs. 2F and 3F). It is unknown whether expression and/or activity of upstream kinases (i.e., LKB1, CaMKK-β, TAK-1) and phosphatases (i.e., PP2C), which phosphorylate and dephosphorylate AMPK at Thr172, respectively, are altered in SHR versus WKY vascular tissue. Thus it is difficult to speculate on how these might contribute to the SHR/WKY differences observed here. Although slightly elevated AMPK activation may have contributed to greater relaxation in SHR E+ rings than those from WKY (although this does not appear to be the case for SHR and WKY E- rings), it is likely that enhanced sensitivity of downstream signaling targets, rather than differences in the activation of AMPK per se, is the main contributor to the differential responses of SHR versus WKY E+ and E- rings to AICAR. Indeed, as already discussed, dissection of the mechanisms responsible for the endothelium-dependent component of the relaxation response to AICAR provided insight into the interpretation of differences in tissue sensitivity by revealing that a COX-dependent component of the vasodilatory response to AICAR in SHR but not WKY (Fig. 7), contributing to the responsiveness of SHR E+ rings at lower AICAR concentrations. Furthermore, conduit vessels from SHR exhibit higher levels of eNOS protein content expression than those from WKY (see RESULTS) (14, 24), presenting a more
abundant target for AMPK stimulation and NO generation and thus possibly contributing to greater sensitivity and enhanced NO-mediated relaxation observed in SHR aortic rings. In the vascular smooth muscle, signaling through mechanisms such as the Ca²⁺ sensitization that are responsible for regulating contraction is upregulated in aorta from SHR versus WKY (30, 42). Any or all of these mechanisms may contribute to the SHR versus WKY differences observed in relaxation to AICAR, and further investigation will be necessary to elucidate the contribution of these and other mechanisms to vascular smooth muscle responses in SHR and WKY aortic rings.

A limitation of the present study is that we cannot rule out the potential for nonspecific activity of AICAR (10, 57), and caution should be exercised in interpreting results generated using only pharmacological tools. However, two features of the current study help mitigate against this limitation: first, the consistency between the functional responses to AICAR and the biochemical indexes of AMPK activation under control and pharmacological dissection conditions (L-NAME, Indo) across a wide range of AICAR concentrations, and second, the reversibility of the AICAR-mediated effects demonstrated by that AMPK inhibitor CC. Although there has been some concern regarding toxicity of AICAR in cell and tissue preparations at high doses, we determined in pilot work that the contractile viability of vessels was not compromised following the AICAR DRC, since tension generated in response to KCl and PE was not different from that obtained before AICAR exposure (see discussion of pilot work in MATERIALS AND METHODS). Thus relaxation to AICAR in our study was not due to a decreased viability of our tissue preparations or an artefact of AICAR toxicity in our aortic rings.

In summary, AICAR-mediated relaxation is composed of both endothelium-dependent and -independent components in aortic rings from WKY and SHR. Endothelium-dependent relaxation is NO mediated in WKY but NO- and COX-mediated in SHR. Greater activation of AMPK in vessels with intact endothelium as well as the suppression of COX-, EDCF-mediated vasoconstriction that is enhanced in SHR versus WKY are likely the main contributors to the enhanced endothelium-dependent relaxation, respectively, of SHR versus WKY aortic rings to AICAR. These results suggest AMPK may be a useful target for improving vasorelaxation in arteries from hypertensive rats and demonstrate that these vessels maintain their ability to respond despite lower basal AMPK activation. Activation of AMPK in the vasculature may be a potential strategy for improving the vascular dysfunction that exists in cardiovascular disease states such as hypertension or other disease states exhibiting vasomotor dysfunction due to impaired NO-dependent relaxation and/or enhanced COX-mediated contraction.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

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