Epoxycosatrienoic acid-induced relaxation is impaired in insulin resistance

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INSULIN RESISTANCE AND HYPERINSULINEMIA are common in patients with essential hypertension and are implicated in the pathogenesis of this disease as well as in its complications (8, 13, 20). Although the mechanisms that link insulin resistance and vascular dysfunction remain unclear, impairment of endothelial function has been proposed as one potential mechanism. This hypothesis is supported by studies in both insulin-resistant human subjects (27) as well as animals (16, 21, 28) that demonstrated impaired endothelium-dependent relaxation.

Previous studies (21, 22, 28) using the fructose-fed rat model of insulin resistance have documented an impaired endothelium-dependent relaxation, as defined by a decreased response to acetylcholine and/or bradykinin, in small mesenteric and coronary arteries. Furthermore, this impaired endothelium-dependent relaxation is related to a defect in a nitric oxide/prostacyclin-independent relaxing factor that induces vasodilation through activation of Ca2+-activated K+ channels (KCa) (17). On the basis of the current literature (23, 25), this relaxation is likely due to endothelium-derived hyperpolarizing factor (EDHF).

To date, the identity of EDHF is unclear. On the basis of the current literature, it is likely that there is more than one definitive EDHF depending on the animal species and vascular bed studied. However, consistent throughout the majority of data is the suggestion that EDHF is a metabolite of arachidonic acid (3, 9, 12). Although this area remains controversial, considerable evidence (19, 24, 26, 33) has clearly shown that arachidonic acid metabolites of cytochrome P-450 monoxygenase enzyme system, such as epoxycosatrienoic acids (EET) and their dihydroxyeicosatrienoic acid metabolites, exhibit EDHF-like activity in coronary, cerebral, renal, and mesenteric arteries of various species.

A recent study (18) by our laboratory has demonstrated that endothelium-dependent and EDHF-mediated relaxation can be restored in small mesenteric arteries by induction of the cytochrome P-450 monoxygenase enzyme system. These data suggest that decreased EDHF production is the mechanism for impaired endothelium-mediated relaxation; however, other issues could be involved, such as an enhanced breakdown of EDHF or impaired K+ channel function on vascular smooth muscle (VSM) or the endothelium. The current study was designed to assess the ability of the EETs, a putative EDHF, to induce relaxation in small mesenteric arteries of control and insulin-resistant rats.

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METHODS

The animal care committees at Wake Forest University School of Medicine and the Medical College of Georgia approved the current protocol. Male Sprague-Dawley rats were obtained at 6 wk of age and randomized into one of the following two groups: 1) insulin-resistant (n = 24) and 2) control (n = 40) rats. Animals in the insulin-resistant group were fed a fructose-rich diet containing 66% fructose, 22% casein, and 12% lard, plus essential vitamins and minerals (Teklad Labs; Madison, WI), whereas control animals received standard rat chow.

After a 4-wk diet treatment, the rats (in a fasting state) were anesthetized with pentobarbital sodium (50 mg/kg ip) and anticoagulated with heparin sodium (500 units ip). A midline incision was made and the abdominal and chest cavities were opened. Approximately 1 ml of blood was removed for evaluation of insulin and glucose concentrations. Hyperinsulinemia was used as a marker of insulin resistance in this model (31). Subsequently, a section of the small intestine was clamped, removed, and placed in a chilled oxygenated modified Krebs-Ringer bicarbonate solution concentration composed of (in mM) 118.3 NaCl, 4.7 KCl, 2.5 CaCl₂, 1.2 MgSO₄, 1.2 KH₂PO₄, 25 NaHCO₃, and 11.1 dextrose. Fourth-order branches of the superior mesenteric artery (≈210 μm in diameter) were isolated from surrounding perivascular tissue and removed from the mesenteric vascular bed for either functional studies or VSM patch-clamp experiments.

Determination of vascular reactivity. Small mesenteric arteries (~2 mm in length) isolated from the mesenteric vascular bed were transferred to a vessel chamber and mounted and secured between two glass micropipettes with a 10-μm ophthalmic suture. The vessel chamber was transferred to an inverted light microscope stage coupled to a video dimension analyzer (Living Systems Instrumentation; Burlington, VT). The video dimension analyzer was connected to both a video monitor (for visualization of the vessel) and to a strip-chart recorder (Kipp and Zonen) for constant recording of the lumen of the vessel. Oxygenated (20% O₂-5% CO₂) Krebs solution, maintained at 37°C, was continuously circulated through the vessel bath. In addition, the lumen of the vessel was filled with Krebs solution through the micropipettes and maintained at a constant pressure of 40 mmHg. Only one concentration-response experiment was performed per artery; however, several arteries were taken from each rat.

Mesenteric arteries were allowed to equilibrate for 30 min and subsequently preconstricted to ~40% of their resting diameter with phenylephrine, an α₁-receptor agonist. Concentration-response experiments were performed with two of the EET regioisomers: 11,12-or 14,15-EET (1 × 10⁻¹⁰ to 3 × 10⁻⁶ M) or vehicle (ethanol) in arteries from both control and insulin-resistant rats. These two regioisomers were chosen based on preliminary experiments where their effects were not altered by inhibition of cyclooxygenase products with indomethacin (data not shown). To determine the mechanism of EET-induced relaxation in this vascular bed, additional studies were performed with 14,15-EET. Just one regioisomer was chosen as a prototype EET because both EETs studied induced similar responses in control arteries and the substantial costs of performing all of the mechanistic studies with both agents. To determine the role of the endothelium in EET-induced relaxation, arteries were denuded of endothelium before the concentration response experiment with 14,15-EET. Endothelial denudation was performed by perfusing air through the lumen of the artery. Endothelial disruption was verified by the absence of a dilator response to acetylcholine and viability was tested by vasodilator response to nitroprusside. In addition, these pharmacological tests were verified by electron microscopy (Fig. 1). To determine the role of Ca²⁺-dependent potassium channels (K₉Ca) arteries were pretreated with iberiotoxin (IBTX) (0.1 μM), IBTX (0.1 μM) + apamin (0.5 μM) or charybdotoxin (CTX) (0.1 μM) + apamin (0.5 μM). IBTX was used to specifically inhibit large conductance K₉Ca, whereas the combination of IBTX + apamin was used to assess the role of large and small conductance K₉Ca. CTX is a nonspecific antagonist of K₉Ca, whereas apamin is an antagonist of the small-conductance K₉Ca. The combination of CTX + apamin was used because it has been shown to inhibit EDHF, likely via its effects on the intermediate and small-conductance K₉Ca (1, 5). Finally, to determine whether EETs induce relaxation via the release of nitric oxide, arteries were pretreated with Nω-nitro-L-arginine (L-NNA, 100 μM).

VSM patch-clamp experiments. Myocytes were isolated from identical vessels used in the functional studies as previously described (4). Briefly, the endothelium was removed and the adventitia of the arteries was carefully teased away under a microscope. The remaining smooth muscle-rich media layer was enzymatic digested. The muscles were incubated at 37°C in a solution consisting of (in mg) 6 papain, 4 dithiothreitol, 2 collagenase, and 0.02% bovine serum albumin. After 30 min of gentle shaking, the muscle strips were lightly triturated, and the enzyme solution was diluted by the addition of excess enzyme-free solutions. The solution was removed and centrifuged at 500 rpm for 15 min. The remaining muscle-rich layer was transferred to a micropipette and maintained in a bath containing oxygenated Krebs solution. Myocytes were isolated by carefully teasing apart the muscle layer. The isolated myocytes were then transferred to a micropipette and expanded at 5 x 10⁻⁶ M phenylephrine to maintain resting membrane potential. VSM patch-clamp experiments were performed using an Axopatch 200B amplifier (Axon Instruments, Union City, CA) and data were analyzed using Clampfit (Axon Instruments) and Origin (OriginLab, Northampton, MA).
pellet was resuspended in fresh medium composed of (in mM) 110 NaCl, 5 KCl, 2 CaCl2, 2 MgCl2, 10 HEPES, 0.94 NaH2PO4, 10 glucose, 0.49 EDTA, and 10 taurine. The pellet was kept at 4°C. The recordings were performed within 6–8 h after cell dissociation.

Small aliquots of cell suspension were placed in a recording chamber (Warner Instruments). The external recording bath solution was composed of the following (in mM): 140 KCl, 10 MgCl2, 0.1 CaCl2, 10 HEPES, and 30 glucose (pH 7.2, 25°C). A gigaseal between the cell and the pipette was formed and a pCLAMP 7 (Axopatch 200B Amp, Axon Instruments) amplifier was used to record the current. Current and voltage traces were digitized with Digidata 1200 series (Axon Instruments) and stored for analysis. Capacitative and leakage currents were subtracted digitally. Single-K⁺ channels were measured in cell-attached or inside-out patches. In cell-attached configuration, the patch pipette (2–5 MΩ) was filled with a Ringer solution containing (in mM) 140 NaCl, 2 MgCl2, 10 HEPES, 20 glucose, and 10 calcium (pH 7.2, 25°C) and a gigaseal seal was made on an intact cell to measure channel activity at a voltage of +50 mV. The effect of 11,12-EET and 14,15-EET was determined at a concentration of 1 μM.

In the experiments measuring K⁺ channel-activity in cell-free inside-out patches, the bathing solution exposed to the cytoplasmic surface of the membrane composed of (in mM) 60 K2SO4, 30 KCl, 2 MgCl2, 0.16 CaCl2, 1 1,2-bis(2-aminoephosphonyl)ethane-N,N,N',N'-tetraacetic acid (pCa 7), 10 HEPES, 5 ATP, and 10 glucose (pH 7.4, 22–25°C). The solution in contact with the external surface was the Ringer solution described above. Average channel activity in patches with stably attached arteries, 11,12-EET and 14,15-EET was determined as described previously (29). For the inside-out experiments, the effect of 11,12-EET and 14,15-EET was determined at a concentration of 1 μM.

Biochemical measurements. Plasma insulin was assayed by using a dextran-coated charcoal immunoassay with rat antibody. Glucose concentrations were measured using a Glucose Trinder Kit (Sigma; St. Louis, MO).

Chemicals. The EETs were obtained from Cayman Chemicals. For all experiments, the EETs were kept in the dark and on ice to minimize metabolism. All other chemicals were obtained from Sigma. All agents were dissolved in deionized water and diluted with Krebs buffer. L-NNA was dissolved in water and titrated to a pH of ~2 with hydrochloric acid for dissolution. The pH was then titrated to physiological level (7.4) with sodium hydroxide.

Data analysis. Data from vascular reactivity studies are expressed as a percentage of relaxation after preconstriction. All data are expressed as means ± SE. All concentration response curves were evaluated for changes in maximal response and differences at each concentration using analysis of variance with repeated measures, followed by Fisher’s pairwise least-significant difference test for multiple comparisons. Statistical comparison between groups for patch-clamp experiments was evaluated by Student’s t-test. The criteria for significance were P < 0.05.

RESULTS

Vascular reactivity experiments. Resting intraluminal diameter of small mesenteric arteries did not differ between groups (207 ± 6 μm for control and 212 ± 4 μm for insulin-resistant arteries). Moreover, the percentage of arterial constriction after phenylephrine was similar between groups with 42 ± 2% for control and 41 ± 2% for the insulin-resistant group. Neither endothelial denudation nor pharmacological inhibition significantly altered the resting diameter compared with the arteries in the control group. The percentage of constriction in experiments with endothelial denudation or pharmacological inhibition also did not differ compared with normal control arteries; however, the concentration of phenylephrine was reduced by one-half (from 200 to 100 μM) to produce the same degree of vasoconstriction.

In arteries from control animals, 11,12-EET and 14,15-EET induced a concentration-dependent relaxation (Fig. 2). By contrast, neither of the EET regioisomers induced relaxation in arteries from the insulin-resistant rats. In fact, a small but significant vasoconstriction was induced with both EET regioisomers (Fig. 3). Because the EETs that do not induce relaxation in arteries from the insulin-resistant rats, no further functional experiments were performed with these arteries. It should be noted that the vehicle (ethanol) induced a small vascular relaxation in both control and insulin-resistant arteries (Figs. 2 and 3) that was significant versus time control (data not shown).

In control arteries, further experiments were performed with 14,15-EET to determine the mechanism of EET-induced relaxation. Endothelium denudation significantly reduced relaxation to 14,15-EET (Fig. 4). Maximal relaxation to 14,15-EET in endothelium-intact arteries was 82 ± 7% compared with 62 ± 4% in endothelium-denuded arteries (P < 0.05). Pretreatment of control arteries with L-NNA (100 μM) also significantly reduced relaxation to 14,15-EET (maximal relaxation = 54 ± 5%) (Fig. 4). Pretreatment of arteries with IBTX (0.1 μM) markedly inhibited relaxation to 14,15-EET; however, a significant vasodilation was elicited for the final two concentrations studied with a maximal relaxation of 52 ± 6% (Fig. 5). In

![Fig. 2. Cumulative concentration response to two epoxyeicosatrienoic acid (EET) regioisomers and vehicle (ethanol) in phenylephrine-preconstricted small mesenteric arteries from control rats (n = 3–5 rats). *P < 0.05, statistical significance between vehicle and EET responses. Both EET regioisomers induced a significant vasodilation compared with vehicle for each concentration indicated.](http://ajpheart.physiology.org/)
contrast, pretreatment of control arteries with the combination of IBTX (0.1 μM) + apamin (0.5 μM) or CTX (0.1 μM) + apamin (0.5 μM) almost completely abolished relaxation to 14,15-EET (Fig. 5). It should be noted that the relaxation induced in the presence of IBTX + apamin or CTX + apamin was not different from that induced by ethanol.

Patch-clamp experiments. In cell-attached patches of smooth muscle cells from control and insulin-resistant mesenteric microvessels, the BKCa channel exhibited a single-channel conductance of 142 ± 6 and 134 ± 3 pS, respectively. These values did not differ from one another and were similar to what has previously been described (4). The effects 11,12- and 14,15-EET on BKCa channel opening probability were measured in myocytes from control and insulin-resistant rats. In control cells, both of the EET compounds significantly increased the BKCa channel opening probability (Fig. 6). The addition of 11,12-EET (n = 3) and 14,15-EET (n = 3) caused an increase in BKCa channel opening probability by 60- and 79-fold, respectively. In contrast, these compounds had no effect on channel opening probability in myocytes from insulin-resistant rats recorded in the cell-attached configuration (Fig. 6).

We also examined the direct effect of both 11,12-EET and 14,15-EET on the BKCa channel opening probability using excised inside-out patches. BKCa channel activity in excised patches of myocytes from control and insulin-resistant rats was enhanced by both EET regioisomers. In control cells, the 11,12-EET increased the opening probability of the BKCa channels from 0.002 ± 0.004 to 0.049 ± 0.08 (n = 4) (P < 0.05), whereas 14,15-EET increased the opening probability from 0.002 ± 0.006 to 0.055 ± 0.02 (n = 4), P < 0.05 (Fig. 7). In cells from insulin-resistant rats, 11,12-EET increased the opening probability of the BKCa channels from 0.0035 ± 0.003 to 0.123 ± 0.08 (n = 4) (P < 0.05), whereas application of 14,15-EET enhanced the open probability of the BKCa channel from 0.005 ± 0.005 to 0.097 ± 0.04 (n = 4), P < 0.05 (Fig. 7). The degree by which the open probability was enhanced by the EETs did not differ between the two groups.

Biochemical measurements. Mean body wt (303 ± 8 g for control and 310 ± 6 g for insulin resistant) and fasting glucose (149 ± 11 mg/dl for control and 142 ± 8 mg/dl for insulin resistant) were similar among control and insulin-resistant rats. In contrast, fasting plasma insulin (97 ± 27 pmol/l for control and 234 ± 37 pmol/l...
Fig. 6. Continuous recordings of Ca$^{2+}$-activated K$^+$ channel (BK$_{Ca}$) openings in cell-attached patch configuration before and after 1 μM of 11,12- or 14,15-EET. Channel openings are upward deflections from baseline (dashed line) or closed state. BK$_{Ca}$ channel opening in mesenteric myocytes from control (A) and insulin-resistant (B) rats.

Fig. 7. Continuous recordings of BK$_{Ca}$ channel openings in inside-out patch configuration (+50 mV) before and after 1 μM of 11,12- or 14,15-EET. Channel openings are upward deflections from baseline (dashed line) or closed state. BK$_{Ca}$ channel opening in mesenteric myocytes from control (A) and insulin-resistant (B) rats.
for insulin resistant, $P < 0.05$) was significantly elevated in insulin-resistant rats compared with control.

**DISCUSSION**

The current study assessed the effect of EETs on vascular relaxation and VSM BK$_{Ca}$ activation in mesenteric arteries from control and insulin-resistant rats. There are several important findings herein. First, in control mesenteric arteries both of the EET regioisomers tested induced a similar concentration-dependent relaxation. Second, in myocytes from control mesenteric arteries the EETs enhanced the open probability of the BK$_{Ca}$ channel both in the cell-attached and inside-out patch-clamp configurations. Third, although EET-induced relaxation in control arteries appears to be mostly due to their effect on VSM, the endothelium is also involved because endothelial denudation and inhibition of nitric oxide synthase reduced relaxation to 14,15-EET. Fourth, the BK$_{Ca}$ channel is not the only K$_{Ca}$ channel activated by the EETs because IBTX, a specific inhibitor of BK$_{Ca}$ channels, reduced, but did not abolish, relaxation to 14,15-EET. In contrast, the combination of IBTX + amapin or CTX + amapin eliminated 14,15-EET-induced relaxation. Fifth, neither of the EET regioisomers induced relaxation in arteries from insulin-resistant rats. Likewise, in the cell-attached mode, EETs did not affect the open probability of BK$_{Ca}$ channels in myocytes from insulin-resistant rats. Finally, both EETs increased BK$_{Ca}$ channel open probability in inside-out patches of myocytes from insulin-resistant animals similar to that observed in control myocytes.

The present study provides direct evidence in intact microvessels and single myocytes from mesenteric arteries of control rats that EETs induce relaxation through K$_{Ca}$ channels. This relaxation is primarily mediated through BK$_{Ca}$ channels because a significant portion of EET-induced relaxation was inhibited by IBTX, a specific inhibitor of the BK$_{Ca}$ channel. Moreover, we noted a marked increase in the open probability of the BK$_{Ca}$ channel in the presence of the EETs. The relaxation that is resistant to IBTX appears to be due to activation of small conductance K$_{Ca}$ channels because it was completely abolished by the combination of IBTX + amapin or CTX + amapin. The majority of studies (10, 12, 24) support these findings where EET-induced relaxation was inhibited by nonspecific antagonists of K$_{Ca}$ (tetroethylammonium, tetrabutylammonium, and CTX) in porcine coronary, canine coronary, and cat cerebral arteries.

Regarding the effect of EETs on BK$_{Ca}$ channels of control myocytes, we have shown that the EETs increase the open probability of the BK$_{Ca}$ channel using both the cell-attached and inside-out VSM patch-clamp configurations. These data suggest that the EETs directly activate the channel without the need of G proteins or other second-messenger systems. Previous studies (14, 19) using the patch-clamp technique have also shown that the EETs activate VSM BK$_{Ca}$ channels in control myocytes. In addition, they have been shown to activate BK$_{Ca}$ in other tissues including porcine coronary endothelial cells (2) and pituitary GH3 cells (30). There have been two previous studies that have described the effect of EETs on VSM BK$_{Ca}$ in the cell attached and inside-out patch-clamp configurations; however, their results differ from our own. Hu and Kim (14) assessed the effect of EETs on VSM cells from rabbit portal vein, rat caudal artery, guinea pig aorta, and porcine coronary artery. These investigators found that all EET isomers potentiated BK$_{Ca}$ channel activity in the cell-attached mode. In contrast, these investigators found that the EETs had no effect in the inside-out configuration. Likewise, Li and Campbell (19) assessed EET-induced BK$_{Ca}$ channel activity in bovine coronary VSM cells and found that BK$_{Ca}$ channel activity was increased in the cell attached mode, but not in the inside-out configuration in the absence of guanosine 5’-triphosphate (GTP). However, in the presence of GTP, BK$_{Ca}$ activity was enhanced in the inside-out configuration, leading these authors to conclude that EET-induced activation of BK$_{Ca}$ involves GTP binding proteins. In the current study, we demonstrated that EETs could activate BK$_{Ca}$ in the inside-out configuration in the absence of GTP. We are not sure why our findings differ from those of Li and Campbell; however, it may be explained by differences in animal model, vascular bed, artery size, or free Ca$^{2+}$ content. In contrast to these studies, it has been shown in porcine coronary artery endothelial cells that the EETs enhance BK$_{Ca}$ activation in the inside-out configuration without the addition of GTP (2). Similar findings are also reported in pituitary GH3 cells (30).

The current study also assessed the role of the endothelium in EET-induced relaxation of control arteries. We demonstrate that removal of the endothelium or pretreatment with L-NNA in control arteries diminishes EET-induced relaxation to a similar degree, suggesting that these two interventions eliminate the same mechanism of vasodilation. In addition, it is likely that endothelium-dependent EET-induced relaxation is mediated through activation of K$_{Ca}$ channels because pretreatment with IBTX + amapin or CTX + amapin completely abolished vasodilation. In other words, it appears from the current data that EET stimulation results in the activation of endothelial cell K$_{Ca}$ channels and the production of nitric oxide. These data are supported by a previous study (11) in cultured endothelial cells from bovine coronary arteries and human umbilical cord, where 5,6-EET increased intracellular Ca$^{2+}$ to a similar degree as what is observed with bradykinin. In addition, this study showed that inhibiting the production of the EETs resulted in a decreased formation of nitric oxide (11). In contrast to these data and to our own, endothelial denudation of porcine and canine coronary arteries does not alter the vasodilatory response to the EETs (12, 24).

In contrast to experiments with control arteries, arteries from insulin-resistant rats did not relax to any of the EET regioisomers. In fact, a small but significant concentration-dependent vasoconstriction was induced with each of the three EET regioisomers. Recent data...
insulin resistance promotes hypertension and vascular dysfunction.

In summary, the EETs induce a concentration-dependent relaxation of small mesenteric arteries from control rats. In addition, the EETs enhance the open probability of the VSM BKCa channel in both the cell-attached and inside-out configurations in control myocytes from rat mesenteric arteries. In contrast, the EETs induce a small vasoconstriction in small mesenteric arteries from insulin-resistant rats and they do not affect the VSM BKCa channel as assessed in the cell-attached patch-clamp configuration. Interestingly, the activation of the BKCa channel in the inside-out patch-clamp configuration is not different from control. Thus, the impaired response to EETs in insulin-resistant arteries is not due to a direct BKCa channel dysfunction, but is more likely due to an alteration of the signal transduction pathways regulating the BKCa channel opening.

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