14,15-Epoxideicosatrienoic Acid Represents a Transferable Endothelium-Dependent Relaxing Factor in Bovine Coronary Arteries

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Abstract—Bradykinin causes arterial relaxation and hyperpolarization, which is mediated by a transferable endothelium-derived hyperpolarizing factor (EDHF). In coronary arteries, epoxyeicosatrienoic acids (EETs) are involved in the EDHF response. However, the role of EETs as transferable mediators of EDHF-dependent relaxation remains poorly defined. Two small bovine coronary arteries were cannulated and perfused in tandem in the presence of the nitric oxide synthase inhibitor, nitro-L-arginine (30 μmol/L), and the cyclooxygenase inhibitor, indomethacin (10 μmol/L). Luminal perfusate from donor arteries with intact endothelium perfused endothelium-denuded detector arteries. Detector arteries were constricted with U46619 and diameters were monitored. Bradykinin (10 nmol/L) added to detector arteries did not induce dilation (5±2%), whereas bradykinin addition to donor arteries dilated detector arteries by 26.5±7% (P<0.05). These dilations were blocked by donor artery endothelium removal and detector artery treatment with the EET-selective antagonist, 14,15-epoxyeicosa-5(Z)-monoenic acid (14,15-EEZE; 10 μmol/L, ~5±6%) but not 14,15-EEZE treatment of donor arteries (20±5%). 14,15-EET (0.1 to 10 μmol/L) added to detector arteries induced maximal dilations of 82±5% that were inhibited 50% by detector artery treatment with 14,15-EEZE (32±12%) but not donor artery treatment with 14,15-EEZE. Liquid chromatography–electrospray ionization mass spectrometry analysis verified the presence of 14,15-EET in the perfusate from an endothelium-intact but not denuded artery. These results show that bradykinin stimulates donor artery 14,15-EET release that dilates detector arteries. 14,15-EEZE blocked the donor artery, endothelium-dependent, bradykinin-induced relaxations, and attenuated relaxations to 14,15-EET. These results suggest that EETs are transferable EDHFs in coronary arteries. (Hypertension. 2005;45:1-6.)

Key Words: bradykinin ■ endothelium-derived factors ■ vasodilation

The vascular endothelium releases numerous factors in response to neurohormonal agonists or physical stimuli that mediate relaxation. These include prostacyclin, nitric oxide (NO), and a group of factors collectively called endothelium-derived hyperpolarizing factors (EDHFs).1–3 As reviewed in detail by Campbell and Harder, EDHFs cause vascular relaxation by hyperpolarizing smooth muscle.4 The chemical identity of EDHF varies between species, vascular size, and vascular bed. Cytochrome P450 (CYP450) metabolites of arachidonic acid, the epoxyeicosatrienoic acids (EETs), 15-lipoxygenase metabolites of arachidonic acid, the trihydroxieicosatetraenoic acids, hydrogen peroxide, potassium ion, and C-type natriuretic peptide all function as EDHFs.5–10 Additionally, transmission of endothelial cell hyperpolarization through myoendothelial-endothelial cell gap junctions may also contribute to the EDHF response.11 In the coronary circulation, EETs function as EDHFs.5,12 They relax bovine, porcine, canine, and guinea pig coronary arteries.5,6,12–15 Bovine coronary endothelial cells synthesize EETs, and this synthesis is stimulated by acetylcholine and bradykinin.5,16 In this vasculature, the primary EET regiosomer is 14,15-EET. Direct application of 14,15-EET to bovine coronary smooth muscle results in the activation of large-conductance, calcium-dependent K+ (BKCa) channels through a G-protein–dependent signaling cascade to cause membrane hyperpolarization.5,17,18

In the presence of cyclooxygenase and NO synthase inhibitors with indomethacin and nitro-L-arginine (L-NA), bradykinin stimulates endothelium-dependent relaxation of bovine coronary arteries that is nearly eliminated by CYP450 inhibitors.19 Previously, using isolated coronary artery smooth muscle patch clamp bioassay, we demonstrated that bradykinin stimulates the release of an endothelium-derived factor that activates BKCa channels and causes hyperpolarization of detector smooth muscle cells.20 The bradykinin-induced BKCa channel activation of detector smooth muscle cells was blocked by treatment of donor arteries with CYP450 inhibitors or donor artery endothelium removal.
Similarly, bradykinin stimulated the release of a CYP450 metabolite from donor porcine coronary arteries that induced membrane hyperpolarization or activated calcium-activated K⁺ channels in cell-attached patches of detector cultured smooth muscle cells. These studies established the role of a transferable endothelial cell CYP450 metabolite in mediating bradykinin-induced EDHF activity in coronary arteries.

However, the exact role of EETs in mediating the EDHF phenomenon remains controversial. EETs may act as transferable factors released by the endothelium that diffuses to the smooth muscle to cause relaxation directly. Alternatively, as suggested by Busse et al., EETs could act within the endothelial cell to activate K⁺ channels. This activation could stimulate K⁺ efflux and the K⁺ ion could act as an EDHF or K⁺ efflux, and the subsequent endothelial cell hyperpolarization could stimulate the release of an alternative EDHF. The goal of this study was to identify the cellular site of EET action in the EDHF-dependent EDHF activity of bovine coronary arteries by using a tandemly cannulated artery bioassay preparation.

Using cannulated and perfused coronary arteries, we evaluated the effect of the EET antagonist, 14,15-EEZE, on 14,15-EET dilations. Detector arteries were constricted with U46619 and treated with ethanol vehicle. The preconstricted arteries showed a concentration-related dilation to intraluminal 14,15-EET (maximal dilations = 81 ± 3%; Figure 2A). Pretreatment of the arteries with 14,15-EEZE (10 μmol/L) added to the bath significantly reduced the dilation response (maximal dilation = 32 ± 12%). Similarly, 14,15-EET caused concentration-related dilations when added to the detector artery bath solution (maximal dilations = 82 ± 5%; Figure 2B).

**Methods**

**Bioassay of Bovine Coronary Arteries**

Fresh bovine hearts were purchased from a local slaughterhouse. Small coronary arteries (100- to 600-μm diameter) were dissected from the left ventricle and cleaned of adhering fat and connective tissue in HEPES buffer of the following composition (in mmol/L): 155 NaCl, 5 KCl, 1.8 CaCl₂, 1 MgCl₂, 5.5 glucose, and 10 HEPES (pH = 7.4). The arteries were randomly cannulated in the direction of flow in a heated (37°C) bioassay dual-chamber perfusion apparatus with flow as diagrammed in Figure 1. Arterial segments were secured to tapered glass pipettes with 10-0 nylon suture and side branches were tied with a single strand teased from 2-0 silk suture. Arteries were perfused and superfused with physiological salt solution of the following composition (in mmol/L): 119 NaCl, 4.7 KCl, 1.6 CaCl₂, 1.17 MgSO₄, 5.5 glucose, 24 NaHCO₃, 1.18 NaH₂PO₄, and 0.026 EDTA equilibrated with 21% O₂, 5% CO₂-balance N₂ gas, and also contained indomethacin (10 μmol/L) and L-NA (30 μmol/L). Donor arteries (350 to 500 μm internal diameter [ID]) with an intact endothelium were followed-up by detector arteries (180 to 300 μm, ID) that had endothelium removed by air bolus. Inflow pipettes were connected to a gravity-feed reservoir that maintained perfusion pressure at 80 mm Hg. Digital images of detector arteries were captured and analyzed using a Nikon SMZ 100 zoom stereomicroscope, Spot Insight camera (Spot Diagnostic Instruments), Dell Pentium computer, and MetaVue software (Universal Imaging Corp.). After cannulation, the arteries were equilibrated for 30 to 45 minutes. Detector arteries were constricted with U46619 (50 to 100 μmol/L) added to the bath. Bradykinin (10 nmol/L), 14,15-EET (0.1 to 10 μmol/L), and 14,15-EEZE (10 μmol/L) were added to bath solutions. In one experimental protocol, 14,15-EET (0.01 to 10 μmol/L) was added to the luminal perfusate. At the end of each experiment, arteries were perfused and superfused with Ca²⁺-free buffer to estimate maximal passive diameter. Diameter changes are graphed as percent relaxation, with passive diameter representing 100% relaxation.

**Liquid Chromatography, Electrospray Ionization Mass Spectrometry**

Perfusates were collected for 10 minutes (0.19 mL/min to 0.3 mL/min) before and during the application of bradykinin (10 nmol/L). Perfusate metabolites were isolated using solid-phase C-18 Bond Elut SPE columns as previously described, and the samples were dried under a stream of nitrogen gas. Samples were redissolved in 20 μL of acetonitrile and analyzed by liquid chromatography–electrospray ionization mass spectrometry (Agilent 1100 LC/MSD, Sl model). Detection was made in the negative ion mode, 14,15-EET eluted from the column at 29.7 minutes, and a major ion of 319 m/z (M⁻) was produced.

**Materials**

Indomethacin, L-NA, and bradykinin were purchased from Sigma (St. Louis, Mo). U46199 was purchased from Biomol. 14,15-EEZE and 14,15-EET were provided by J.R.F. [14C](U)-Arachidonic acid (920 mCi/mmol) was purchased from NEN Life Science Products (Boston, Mass). All solvents were high-performance liquid chromatography grade and purchased from Burdick Jackson. 14,15-EEZE and 14,15-EET were prepared as 10 μmol/L stock in ethanol. L-NA was prepared as a 100 μmol/L stock in 0.1 N HCl, indomethacin was prepared as a 10 μmol/L stock in 0.2 mol/L Na₂CO₃, and bradykinin was prepared as a 1 μmol/L stock in water.

**Statistics**

Statistical analysis was performed using analysis of variance to determine the significance of differences within groups with subsequent Student Neuman Keul post hoc analysis used to determine the significance between groups. Data are expressed as mean±SEM.
Under these conditions, the addition of 14,15-EEZE to the donor artery bath did not alter the dilations to 14,15-EET added to the detector arteries (maximal dilations = 73 ± 8%). These results demonstrate that 14,15-EEZE inhibits 14,15-EET–induced dilations only when present at the site of smooth muscle dilator activity.

Our next goal was to demonstrate donor artery dependent dilations of detector arteries to bradykinin using the cumulated tandem bioassay. Preconstricted detector arteries with the endothelium removed did not dilate in response to the direct addition of bradykinin (10 nmol/L, maximal dilation = 5 ± 2%). Figure 3). Bradykinin addition to the bath of endothelium intact donor arteries resulted in the significant dilation of detector arteries (maximal dilation = 26.5 ± 7%). Removal of donor artery endothelium eliminated the donor-dependent bradykinin-induced dilation of detector arteries (maximum dilation = −5 ± 7.5%). Therefore, donor artery-dependent, bradykinin-induced dilation of detector arteries requires that donor arteries have an intact endothelium.

To determine the mediator of the donor-dependent relaxations to bradykinin, we repeated the bradykinin dilations with the EET antagonist, 14,15-EEZE. The addition of 14,15-EEZE to the donor artery bath did not alter the donor-dependent bradykinin dilation of detector arteries (Figure 4A). In contrast, 14,15-EEZE added to the detector bath blocked the donor-dependent, bradykinin dilations of the detector arteries (Figure 4B). These results implicate EETs as mediators of the bradykinin dilations of detector arteries and indicate that EETs act on detector artery smooth muscle and not the donor artery endothelium.

To determine the EETs released with bradykinin stimulation, we analyzed perfusates of endothelium-intact donor arteries using liquid chromatography-electrospray ionization mass spectrometry. Per fusates were collected under control conditions and when the arteries were stimulated with bradykinin (10 nmol/L). In perfusates collected from one artery under control and bradykinin-stimulated conditions, 14,15-EET eluted from the column at 29.7 minutes and produced a major ion of 319 m/z (M-1) (Figure 5). 11,12-EET eluted from the column at 32.5 minutes and was produced in much smaller amounts than 14,15-EET. Bradykinin added to the superfusate bath increased 14,15-EET and 11,12-EET as indicated by greater peak height (Figures 5A and 5B). These data verify the presence of EETs in the perfusate. EETs were not detected under basal or bradykinin-stimulated conditions in perfusates from an endothelium-denuded artery (data not shown). Thus, under these conditions, bradykinin stimulates the release of 14,15-EET from endothelium-intact coronary arteries. As in previous studies,16 14,15-EET was the major EET regioisomer released with bradykinin stimulation.

It is possible that EETs produced by the donor arteries act in an autocrine fashion in the endothelium to stimulate the release of an alternate factor that diffuses to detector arteries to cause dilation. To examine this possibility, we measured dilations of detector arteries when donor arteries were treated with 14,15-EET. 14,15-EET added to the bath of endothelium-intact donor arteries did not dilate detector arteries (Figure 6). The subsequent addition of 14,15-EET to the detector artery bath caused significant dilation (maximum dilation = 78 ± 8%). Therefore, 14,15-EET does not appear to cause the release of other relaxing factors from donor arteries.

Discussion

Bradykinin stimulates the release of NO, prostacyclin, and EDHF.20–22,27,28 It also stimulates the release of EETs.16 In the present study, bradykinin added to the donor artery stimulated dilation of a detector artery perfused in tandem.
Bradykinin was without effect if the endothelium was removed from the donor artery. This indicates that bradykinin stimulates the release of an endothelial cell relaxing factor that is transferable. This is in agreement with our previous findings that bradykinin stimulated the release of a transferable EDHF that activated BKCa channels and hyperpolarized isolated smooth muscle cells. Similarly, removal of donor artery endothelium inhibited the activity of the hyperpolarizing factor. Inhibition of CYP450 in the donor artery also blocked the BKCa activation by bradykinin, suggesting that a metabolite acting on the endothelium to release an alterna-
tive transferable hyperpolarizing factor.

The dual-cannulated perfused artery bioassay configuration separates the site of relaxing factor synthesis (donor artery with intact endothelium) from the site of relaxing factor action (detector artery with the endothelium removed). With this separation, site-specific pharmacological blockade with the EET-antagonist, 14,15-EEZE, was used to characterize the site of EET vascular activity. Previously, we had demonstrated that 14,15-EET causes concentration-related relaxations and hyperpolarizations of bovine coronary arterial rings. Similarly, in the perfused small bovine coronary arteries of this study, 14,15-EET caused concentration-related dilations with similar potency. In bovine coronary arteries, 14,15-EEZE inhibits relaxations to all EET regioisomers and 14,15-DHET with the greatest inhibition occurring with 14,15-EET. It also inhibited NO-independent and cyclooxygenase-independent hyperpolarizations and dilations to bradykinin. 14,15-EEZE application to detector arteries blocked the donor-dependent dilations to bradykinin. This antagonist did not alter the dilations to bradykinin when applied to the donor arteries. This confirms that 14,15-EEZE blocks the actions of a transferable relaxing factor at the site of the detector artery smooth muscle but does not alter its release from donor artery endothelium. The inhibition of the effect of the transferable dilator factor by 14,15-EEZE indicates that the factor is an EET.

We did not observe dilation of detector arteries when 14,15-EET was added to the donor artery bath. This suggests that extraluminal 14,15-EET does not diffuse through the donor artery vascular wall and flow to the detector arteries in concentrations required for dilation, and that 14,15-EET does not stimulate donor artery endothelium to release additional relaxing factors. Direct extraluminal application of 14,15-EET potently dilated detector arteries. Therefore, concentrations of 14,15-EET used in this study were sufficient to cause dilation directly but did not stimulate the production and/or release of other relaxing factors in concentrations required for the dilation of detector arteries. Because all experiments were performed in the presence of indomethacin and L-NA to inhibit prostacyclin and NO production, possible endothelial cell effects of EETs on the release of these relaxing factors was not considered.

In bovine coronary arterial smooth muscle, EETs activate BKCa channels through a G-protein–dependent pathway to cause hyperpolarization and vascular relaxation. Because the relaxation response is mediated through smooth muscle hyperpolarization, the EETs are considered EDHFs. Baron et al have documented that EETs activate BKCa channels in cultured porcine coronary endothelial cells. However, we have shown that freshly isolated bovine coronary endothelial cells do not express BKCa channels. Therefore, EET activation of endothelial cell BKCa channels should not contribute to the release of a transferable relaxing factor in bovine coronary arteries. Alternatively, the effect of EETs on other endothelial K+ channels has not been examined and this possibility should not be discounted. EETs could alter other signaling mechanisms in the endothelial cell to stimulate the release of other relaxing factors. In cultured endothelial cells, 11,12-EET potentiated Ca2+ mobilization during receptor-dependent stimulation with histamine and receptor-independent stimulation with thapsigargin. Nevertheless, our results did not provide evidence of EET autocrine activity.
in donor artery endothelium that contributed to the bradykinin-stimulated dilations of detector arteries.

With the mass spectrometry analysis of arterial perfusate, we have provided evidence that 14,15- and 11,12-EETs are present in the perfusate of donor arteries. 14,15-EET was the major EET regioisomer in the perfusate. The perfusate 14,15-EET appeared to increase with bradykinin stimulation. The epoxide hydrolase metabolite of 14,15-EET, 14,15-dihydroxyeicosatrienoic acid (14,15-DHET), also causes relaxation of bovine coronary arteries that is inhibited by 14,15-EEZE. Although not identified in this study, some contribution of DHETs to the donor-dependent dilations to bradykinin remains a possibility. Taken together, our observations of the pharmacological blockade of the dilatory factor activity with 14,15-EEZE and the mass spectrometry verification of perfusate EET provide compelling evidence that 14,15-EET is a transferable EDHF.

Perspectives

The contribution of EDHFs to the regulation of arterial diameter is altered by vascular disease. In salt-induced hypertension, EDHF may compensate for the loss of NO in acetylcholine dilations of rat mesenteric arteries. In insulin-resistant rats, normal mesenteric arterial dilations to 11,12-EET and 14,15-EET were not observed. In human coronary arteries, flow-induced dilation is mediated in part by NO; however, in arteries with coronary artery disease, flow-induced dilation is mediated solely by CYP450 metabolites. Clearly, EDHF-dependent regulation of vascular diameter is altered by vascular disease. In some pathologies, the EDHF contribution is increased to provide compensatory dilations, whereas in other pathologies, specific EDHF mechanisms are severely diminished. Understanding the mechanisms of EDHF-dependent dilation and alterations during vascular diseases will aid the development of therapies designed to enhance and maintain the function of the endothelium.

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