14,15-Epoxyeicosa-5(Z)-enoic Acid

A Selective Epoxyeicosatrienoic Acid Antagonist That Inhibits Endothelium-Dependent Hyperpolarization and Relaxation in Coronary Arteries


Abstract—Endothelium-dependent hyperpolarization and relaxation of vascular smooth muscle are mediated by endothelium-derived hyperpolarizing factors (EDHFs). EDHF candidates include cytochrome P-450 metabolites of arachidonic acid, K+ hydrogen peroxide, or electrical coupling through gap junctions. In bovine coronary arteries, epoxyeicosatrienoic acids (EETs) appear to function as EDHFs. A 14,15-EET analogue, 14,15-epoxyeicosa-5(Z)-enoic acid (14,15-EEZE) was synthesized and identified as an EET-specific antagonist. In bovine coronary arterial rings preconstricted with U46619, 14,15-EET, 11,12-EET, 8,9-EET, and 5,6-EET induced concentration-related relaxations. Preincubation of the arterial rings with 14,15-EEZE (10 μmol/L) inhibited the relaxations to 14,15-EET, 11,12-EET, 8,9-EET, and 5,6-EET but was most effective in inhibiting 14,15-EET–induced relaxations. 14,15-EEZE also inhibited indomethacin-resistant relaxations to methacholine and arachidonic acid and indomethacin-resistant and L-nitroarginine-resistant relaxations to bradykinin. It did not alter relaxation responses to sodium nitroprusside, iloprost, or the K+ channel activators (NS1619 and bimakalim). Additionally, in small bovine coronary arteries pretreated with indomethacin and L-nitroarginine and preconstricted with U46619, 14,15-EEZE (3 μmol/L) inhibited bradykinin (10 nmol/L)–induced smooth muscle hyperpolarizations and relaxations. In rat renal microsomes, 14,15-EEZE (10 μmol/L) did not decrease EET synthesis and did not alter 20-hydroxyeicosatetraenoic acid synthesis. This analogue acts as an EET antagonist by inhibiting the following: (1) EET-induced relaxations, (2) the EDHF component of methacholine-induced, bradykinin-induced, and arachidonic acid–induced relaxations, and (3) the smooth muscle hyperpolarization response to bradykinin. Thus, a distinct molecular structure is required for EET activity, and alteration of this structure modifies agonist and antagonist activity. These findings support a role of EETs as EDHFs. (Circ Res. 2002;90:1028-1036.)

Key Words: epoxyeicosatrienoic acids • arachidonic acid • endothelium-derived hyperpolarizing factors • endothelium • 20-hydroxyeicosatetraenoic acid

Epoxyeicosatrienoic acids (EETs) are cytochrome P-450 metabolites of arachidonic acid. They are synthesized by the vascular endothelium and released in response to vasoactive agonists, such as bradykinin and acetylcholine, and are stimulated by cyclic stretch.1–6 Additionally, in the coronary circulation, they cause vascular smooth muscle hyperpolarization and relaxation and, therefore, function as endothelium-derived hyperpolarizing factors (EDHFs).2 To investigate the role of endogenous EETs in the relaxations induced by endothelium-dependent vasoactive substances, inhibitors of cytochrome P-450 are used, but these investigations have given variable results. In some studies, inhibitors of cytochrome P-450 block the relaxations to bradykinin and acetylcholine, whereas in other studies, these inhibitors are without effect or also inhibit relaxations to K+ channel activators, such as cromakalim or pinacidil.1,2,4,5,7–11 This latter finding suggests possible nonspecific actions of these inhibitors, including the direct inhibition of K+ channels. In this respect, several reports have indicated that the cytochrome P-450 inhibitor, clotrimazole, directly inhibits Ca2+-activated K+ (BKCa) channels; however, this property does not seem to be shared by other imidazole-containing inhibitors of cytochrome P-450.12–14

The vascular endothelium metabolizes arachidonic acid to the vasodilatory EETs via the cytochrome P-450 2C or 2J epoxygenases.3,15–17 Alternatively, the vascular smooth muscle metabolizes arachidonic acid to 20-hydroxyeicosatetraenoic acid (20-HETE) by cytochrome P-450 4A ω-hydroxy-
lase.\textsuperscript{18–20} Specifically, in the renal, mesenteric, and cerebral arterial circulation, 20-HETE causes vascular constriction and contributes to myogenic tone.\textsuperscript{18–21} Thus, the activity of cytochrome P-450 inhibitors on vascular tone will depend on which arachidonic acid metabolite predominates.\textsuperscript{22} Variability in arachidonic acid metabolism between vascular beds may account for the observed inconsistent effects of cytochrome P-450 inhibitors. Consequently, to determine the role of endogenous EETs in vascular relaxation, a pharmacological tool is required that selectively inhibits the action or synthesis of EETs but not the action or synthesis of 20-HETE. Therefore, we synthesized a series of 14,15-EET analogues and tested for their ability to antagonize the vasodilator activity of the EETs. The present study describes the characterization of one of the 14,15-EET analogues, 14,15-epoxyeicosa-5(Z)-enoic acid (14,15-EEZE), which acts as an EET antagonist.

**Materials and Methods**

**Vascular Reactivity Studies**

Bovine hearts were obtained from a local slaughterhouse. Sections of the left anterior descending coronary artery were dissected, cleaned of adhering fat and connective tissue, and cut into 1.5- to 2.0-mm-diameter rings (3-mm length), with care taken not to damage the endothelium. The arterial rings were suspended in a tissue bath containing a Krebs-bicarbonate buffer containing the following (in mmol/L): NaCl 118, NaHCO\textsubscript{3} 24, KCl 4.8, CaCl\textsubscript{2} 3.2, KH\textsubscript{2}PO\textsubscript{4} 1.2, MgSO\textsubscript{4} 1.2, glucose 11, and EDTA 0.03. The Krebs buffer was equilibrated with 95% O\textsubscript{2}/5% CO\textsubscript{2} and maintained at 37°C. Isometric tension was measured as previously described.\textsuperscript{2} Briefly, arterial rings were slowly stretched at 0.5-g increments to a basal tension of 3.5 g. After equilibration, KCl (40 to 60 mmol/L) was repeatedly added and rinsed until reproducible stable contractions were observed. The thromboxane mimetic, U46619 (20 mmol/L), was added to increase basal tension to 50% of the maximal KCl contraction. Cumulative concentrations of EETs, 14,15-dihydroxyeicosatrienoic acid (14,15-DHET), sodium nitroprusside, NS1619, bimakalin, or iloprost were added. The vessels were rinsed and then pretreated with 14,15-EEZE, and the concentration response was repeated. Similar studies were performed in rings pretreated with indomethacin (10 mmol/L) by using methacholine and arachidonic acid as agonists or in rings pretreated with indomethacin (10 mmol/L) and l-nitroarginine (30 mmol/L) by using bradykinin as the agonist. Basal tension represents tension before the addition of U46619. When appropriate, the endothelium was removed by gentle rubbing. Results are expressed as percent relaxation of the U46619-treated rings, with 100% relaxation representing basal tension.

**E\textsubscript{m} and Relaxations of Small Cannulated Bovine Coronary Arteries**

Small coronary arteries were dissected from the apical section of the left ventricle and cannulated on tapered glass micropipettes in a heated (37°C) Lucite perfusion and superfusion chamber as previously described.\textsuperscript{23} Arteries were maintained at a constant perfusion pressure of 60 mm Hg in perfusion and superfusion solutions of the following composition (in mmol/L): NaCl 119, KCl 4.7, CaCl\textsubscript{2} 1.6, MgSO\textsubscript{4} 1.17, glucose 5.5, NaHCO\textsubscript{3} 24, NaH\textsubscript{2}PO\textsubscript{4} 1.18, and EDTA 0.026. All solutions were equilibrated with 95% O\textsubscript{2}/5% CO\textsubscript{2} and N\textsubscript{2} to maintain a pH of 7.4 and PO\textsubscript{2} of 140 mm Hg. Membrane potential (E\textsubscript{m}) recordings were obtained by using procedures previously described.\textsuperscript{23} Briefly, the cannulated arteries were impaled from the external adventitia with glass microelectrodes filled with 3 mol/L KCl connected to a high-impedance amplifier. Impalements were obtained and averaged for each experimental condition. Diameters were obtained by using a Nikon SMZ-800 inverted microscope/Spot RT camera (Diagnostic Instruments, Inc), with images captured and analyzed with the use of Spot/Metaview acquisition/analysis/graphics software. All measurements were performed with U46619 (20 mmol/L) in the superfusate solution. Vehicle or 14,15-EEZE (3 mmol/L), indomethacin (10 mmol/L), and l-nitroarginine (30 mmol/L) were present in perfusion and superfusion solutions. Bradykinin (10 mmol/L) was added to the superfusion solution. Diameters and E\textsubscript{m} were measured either as a control experiment with vehicle (0.03%), vehicle with bradykinin (10 mmol/L), 14,15-EEZE (3 mmol/L), or 14,15-EEZE with bradykinin.

**20-HETE Vasostimulation**

Rat middle cerebral arteries obtained from 10- to 12-week-old male Sprague-Dawley rats (Harlan Sprague Dawley, Inc) were cannulated and equilibrated for 30 minutes. Arteries were pretreated with 17-octadecynoic acid (10 mmol/L) in the perfusion and superfusion solutions for 20 minutes to block endogenous 20-HETE production. Diameter responses to the addition of 20-HETE (1 mmol/L) were measured as described above. The arteries were rinsed and incubated with 14,15-EEZE (10 mmol/L) in the perfusion and superfusion solutions for 20 minutes, and the diameter responses to 20-HETE (1 mmol/L) were repeated.

**Metabolism of [\textsuperscript{14}C]Arachidonic Acid**

Kidneys were dissected from 15-week-old male Sprague-Dawley rats. The renal medulla was removed, and the renal cortical sections were minced and homogenized by using a hand-hold glass tissue homogenizer. Microsomal proteins were prepared by differential centrifugation as previously described and quantified by using the Bio-Rad Bradford assay.\textsuperscript{24} Microsomes were incubated in assay buffer of the following composition (in mmol/L): KH\textsubscript{2}PO\textsubscript{4} 80, K\textsubscript{2}HPO\textsubscript{4} 20, EDTA 1, and MgCl\textsubscript{2} 10. The microsomes (2 mg protein) were incubated for 30 minutes at 37°C with [\textsuperscript{14}C]arachidonic acid (10 mmol/L), NAPD (1 mmol/L), and a NADPH-regenerating system containing isocitrate (10 mmol/L) and isocitrate dehydrogenase (0.1 U/mL) (total volume 0.4 mL). Incubations were performed with either vehicle (0.095% ethanol) or 14,15-EEZE (10 mmol/L). Reactions were terminated by the addition of 5 μL glacial acetic acid with 1.5 mL distilled H\textsubscript{2}O. The solutions were extracted with ethyl acetate and dried under a stream of nitrogen gas. The extracts were redissolved in acetonitrile and chromatographed by reverse-phase high-performance liquid chromatography (HPLC) with the use of methods previously described.\textsuperscript{25} Column effluent was collected in 0.2-mL fractions and analyzed for radioactivity by liquid scintillation spectrometry. Similar incubations were performed with 9 mg microsomal protein for 15 minutes at 37°C with arachidonic acid (10 mmol/L) (total volume 2 mL) and either vehicle (0.095% ethanol) or 14,15-EEZE (10 mmol/L). After incubation, 0.65 mL of 95% ethanol was added, and the samples were subjected to solid-phase extraction by using C\textsubscript{18} Bond Elut columns and dried under a stream of nitrogen. These samples were re-dissolved in acetonitrile and analyzed by liquid chromatography/electrospray ionization–mass spectrometry (LC/ESI-MS, Agilent 1100 LC/MSD, SL model) as previously described.\textsuperscript{25}

**Statistical Analysis**

Vascular reactivity, E\textsubscript{m}, and mass spectrometry data are expressed as mean±SEM. Significance of differences between mean values was evaluated by the Student t test or ANOVA followed by the Student-Newman-Keuls multiple comparison test. Significance was accepted at P<0.05.

**Drugs and Chemicals**

Bradykinin, sodium nitroprusside, l-nitroarginine, arachidonic acid, NS1619, and indomethacin were purchased from Sigma Chemical Co. Methacholine was purchased from ICN Biochemicals. Bradykinin, sodium nitroprusside, l-nitroarginine, and methacholine were mixed to their appropriate stock concentrations in water. Bimakalin, indomethacin, arachidonic acid, EET regioisomers, and 14,15-EEZE were prepared as 10 mmol/L stock, and NS1619 was prepared as a 30 mmol/L stock in 95% ethanol. EET regioisomers were synthe-
sized by the method of Corey et al., and 14,15-EET analogues were synthesized as previously described.

**Results**

**Analogue of 14,15-EET With Antagonist Activity**

Figure 1 shows the chemical structures of the 14,15-EET analogues that were tested for antagonist activity. Only two analogues inhibited relaxations to 14,15-EET. These analogues were 14,15-EEZE and 14,15-epoxyeicosanoic acid (14,15-EEA). 14,15-EEZE differs from 14,15-EET in that the double bonds at carbons 8,9 and 11,12 are saturated, and 14,15-EEA differs from 14,15-EET in that all the double bonds are saturated. 14,15-EEZE was chosen for further study.

**Effect of 14,15-EEZE on Agonist-Induced Relaxations of Coronary Arterial Rings**

In U46619-precontracted arteries, 14,15-EET, 11,12-EET, 8,9-EET, and 5,6-EET caused a concentration-related relaxation as previously described (Figures 2A through 2D). The four EET regioisomers were equipotent, with maximal relaxations averaging 80% to 90%. 14,15-EEZE pretreatment caused a concentration-dependent inhibition of the 14,15-EET–induced relaxations. In the presence of 14,15-EEZE (10 μmol/L), 14,15-EET–induced maximal relaxations averaged only 18%. 14,15-EEZE also inhibited the concentration-related relaxations to 11,12-EET, 8,9-EET, and 5,6-EET, although it was most effective in inhibiting the 14,15-EET–induced relaxations. Similarly, 14,15-EEZE (10 μmol/L) pretreatment inhibited the relaxations to 14,15-DHET (Figure 2E), 14,15-EEZE alone maximally relaxed U46619-precontracted arteries by 21% at 10 μmol/L (Figure 2F).

In precontracted vessels, sodium nitroprusside (an NO donor), iloprost (a prostacyclin analogue), bimakalim (an ATP-sensitive K+ channel opener), and NS1619 (a large-conductance BK Ca opener) relaxed coronary arteries (Figure 3). 14,15-EEZE (10 μmol/L) did not alter the concentration-dependent relaxations to sodium nitroprusside or iloprost (Figures 3A and 3B). Additionally, pretreatment of the vessels with 14,15-EEZE did not alter the concentration-relaxation curves to bimakalim or NS1619 (Figures 3C and 3D). 14,15-EEZE pretreatment also did not alter concentration-relaxation curves to NS1619 in bovine coronary arterial rings in which the endothelium was removed (data not shown). Therefore, 14,15-EEZE appears to specifically inhibit EET-induced relaxations.

We evaluated the ability of 14,15-EEZE to block endothelium-dependent relaxations to methacholine, bradykinin, and arachidonic acid (Figure 4). In the presence of indomethacin, arachidonic acid and methacholine maximally relaxed the preconstricted arteries by 90% and 70%, respectively. Pretreatment of the arteries with 14,15-EEZE (10 μmol/L) nearly eliminated these relaxations. Additionally, in the presence of...
indomethacin and L-nitroarginine, bradykinin caused a concentration-related relaxation. The bradykinin concentration-response curve was shifted to the right nearly 1000-fold by 14,15-EEZE, and maximal relaxation was reduced from 95% to 55%. Thus, the EDHF component of methacholine- and bradykinin-induced relaxations was inhibited by 14,15-EEZE, suggesting that EETs mediate the non-NO and non-prostaglandin (non-PG) relaxations to these agonists in bovine coronary arteries.

Effect of 14,15-EEZE on Bradykinin-Induced Vascular Smooth Muscle Hyperpolarizations and Dilations of Small Bovine Coronary Arteries

Small cannulated bovine coronary arteries pretreated with indomethacin and L-nitroarginine were stimulated with U46619 (20 nmol/L) to cause smooth muscle depolarization and vasoconstriction. $E_m$ of the U46619-treated vessels averaged $-33\pm2$ mV, and internal diameter averaged $144\pm27$ µm (Figures 5A through 5C). Under these conditions, the addition of bradykinin (10 nmol/L) induced an average 8-mV hyperpolarization to $-41\pm1$ mV and an average 43-µm relaxation to $187\pm34$ µm (Figure 5C). Preincubation of the U46619-treated vessels with 14,15-EEZE (3 µmol/L) did not alter $E_m$ or diameter, and the addition of bradykinin to the 14,15-EEZE–treated artery resulted in a significantly blunted hyperpolarization (5 mV) and dilation (14 µm). Thus, 14,15-EEZE inhibits bradykinin-induced vascular smooth muscle hyperpolarizations and dilations of small bovine coronary arteries.

Effect of 14,15-EEZE on Synthesis of EETs and 20-HETE

To determine whether 14,15-EEZE alters arachidonic acid metabolism, we investigated the effect of 14,15-EEZE on arachidonic acid metabolism of rat renal cortical microsomes. Rat renal cortical microsomes were used because they produce large quantities of 20-HETE and EETs.37

Figure 2. Effect of 14,15-EEZE on EET-induced (A through D) and 14,15-DHET–induced (E) relaxations of bovine coronary arteries and 14,15-EEZE–induced relaxations (F). Arterial segments were pretreated with U46619 (20 nmol/L). Changes in isometric tension were measured. 14,15-EEZE significantly inhibited EET-induced and 14,15-DHET–induced relaxations at concentrations of 1 and 10 µmol/L ($P\leq0.01$). All values are mean±SEM ($n=6$ to 16).
cortical microsomes were incubated with [14C]arachidonic acid in the presence of either vehicle or 14,15-EEZE (10 μmol/L), and the metabolites were analyzed by reverse-phase HPLC. Figure 6 shows that the [14C]metabolites comigrated with 20-HETE and EET standards. 14,15-EEZE did not alter the production of 20-HETE but appeared to increase the amount of EETs and decrease the EET metabolites, the DHETs. Further analyses of microsomal metabolites were performed by LC/ESI-MS (Table). The presence of 14,15-EEZE decreased the concentrations of all DHET regioisomers and significantly increased the concentrations of the EET regioisomers. The synthesis of 20-HETE was not altered. These results show that 14,15-EEZE does not alter the synthesis of 20-HETE but appears to decrease the metabolism of EETs to DHETs.
Effect of 14,15-EEZE on 20-HETE–Induced Constriction

In isolated rat middle cerebral arteries pretreated with 10 μmol/L 17-octadecynoic acid (86±9 μm), 14,15-EEZE (10 μmol/L) did not alter the diameter (86±9 μm) or the constriction to 20-HETE. 20-HETE (1 μmol/L) decreased the luminal diameter by 14±3% in the presence of 14,15-EEZE and by 17±5% in its absence (n=5, P=NS).

Discussion

Previous studies from a number of laboratories have shown that in the coronary, renal, and cerebral circulation, EETs function as EDHFs. EETs are diffusable paracrine factors that are synthesized by the endothelium and that activate vascular smooth muscle BKCa channels. The results of the present study characterized a structural analogue of 14,15-EET (14,15-EEZE) as an EET-specific antagonist. Compared with 14,15-EET, 14,15-EEZE has little agonist activity. In U46619-precontracted bovine coronary rings, 14,15-EET induced relaxations in a concentration-dependent manner, with maximal relaxations of 80%, whereas 14,15-EEZE induced maximal relaxations of only 21%. In contrast, preincubation with this EET analogue blocked relaxations induced by 5,6-EET, 8,9-EET, 11,12-

Effect of 14,15-EEZE on 20-HETE–Induced Constriction

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>Control, pg/mg Protein</th>
<th>14,15-EEZE, pg/mg Protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>14,15-DHET</td>
<td>1470±19</td>
<td>529±34*</td>
</tr>
<tr>
<td>11,12-DHET</td>
<td>3770±148</td>
<td>2874±119*</td>
</tr>
<tr>
<td>8,9-DHET</td>
<td>2074±87</td>
<td>1146±52*</td>
</tr>
<tr>
<td>5,6-DHET</td>
<td>59±9</td>
<td>37±2*</td>
</tr>
<tr>
<td>20-HETE</td>
<td>1033±3</td>
<td>1020±38</td>
</tr>
<tr>
<td>14,15-EET</td>
<td>526±10</td>
<td>1625±52*</td>
</tr>
<tr>
<td>11,12-EET</td>
<td>436±5</td>
<td>1499±40*</td>
</tr>
<tr>
<td>8,9-EET</td>
<td>274±6</td>
<td>1356±32*</td>
</tr>
<tr>
<td>5,6-EET</td>
<td>413±17</td>
<td>427±25</td>
</tr>
</tbody>
</table>

Each value represents the mean±SEM (n=4).

*Significantly different from control.
EET, and 14,15-EET, with the greatest blockade occurring with 14,15-EET. Thus, it appears that the 8,9 and 11,12 double bonds are required for full agonist activity of 14,15-EET and that alterations of these bonds confers antagonistic properties. The 14,15-EET analogue in the present study inhibited relaxations induced by all four EET regioisomers and 14,15-DHET. The EET regioisomers differ structurally in the position of the epoxy group. This suggests that the position of the epoxy group in the EET molecule may not be critical for agonist or antagonist activity.

Inhibitors of cytochrome P-450 enzymes, including miconazole, SKF525A, and 17-octadecynoic acid, block the endothelial cell cytochrome P-450 epoxygenase that produces EETs as well as the smooth muscle cytochrome P-450 \( \omega \)-hydroxylase, which produces 20-HETE. In the vascular smooth muscle, 20-HETE is activated by stretch or increased intravascular pressure and acts as an intracellular signaling molecule to inhibit the opening of BK\(_{Ca}\) channels, causing depolarization and vasoconstriction. Consequently, the vascular effect of cytochrome P-450 inhibitors will depend on whether the constrictor or dilator cytochrome P-450 pathway predominates. To evaluate the contributions of EETs to vascular tone, an inhibitor that selectively blocks the epoxygenase pathway is needed. In the bovine coronary arteries from the present study, 14,15-EEZE blocked the relaxations induced by EETs, 14,15-DHET, and the EDHF component of bradykinin and methacholine relaxations. This inhibition is not due to the inhibition of other endogenous dilators because 14,15-EEZE did not alter the ability of the bovine coronary arterial rings to relax to the NO donor (sodium nitroprusside), the prostacyclin analogue (iloprost), or the ATP-sensitive K\(^+\) channel opener (bimakalim). Thus, 14,15-EEZE is not a nonspecific inhibitor of vasodilation. We also evaluated the effect of 14,15-EEZE on 20-HETE–induced constrictions of rat cerebral arteries. We used this artery because 20-HETE has been shown to play a role in the autoregulation of rat cerebral blood flow. 14,15-EEZE did not alter 20-HETE constriction in this artery. Therefore, it appears that 14,15-EEZE specifically antagonizes EET-induced vascular effects and does not alter the constrictor effect of 20-HETE. Thus, it is a good pharmacological tool to study the role of endogenous EETs.

Several studies have shown that cytochrome P-450 inhibitors have direct effects on BK\(_{Ca}\) channels. The inhibitors, clotrimazole and ketoconazole, have been shown to directly inhibit BK\(_{Ca}\) channels in whole-cell, inside-out, and outside-out patch-clamp evaluations. Alternatively, in the bovine coronary arteries from the present study, the vascular effects of 14,15-EEZE were not due to the inhibition of BK\(_{Ca}\) channels. This is evident because 14,15-EEZE did not alter the relaxation response to the BK\(_{Ca}\) channel opener, NS1619, and because in the perfused coronary arteries, 14,15-EEZE did not alter \( E_{\text{re}} \).

Importantly, 14,15-EEZE blocked the non-PG relaxations to arachidonic acid and methacholine and the non-PG non-NO relaxations and hyperpolarizations to bradykinin. It is possible that antagonism of these responses occurs through the inhibition of EET synthesis and/or stimulation of 20-HETE synthesis rather than through the blockade of EET activity. However, 14,15-EEZE did not alter 20-HETE production by rat renal cortical microsomes. 14,15-EEZE increased EET concentrations while decreasing DHET concentrations. EETs are hydrolyzed to DHETs by epoxide hydrolases. We have previously shown that 14,15-DHET also relaxes bovine coronary arteries, although it is 5-fold less potent. 14,15-EEZE may similarly be a substrate for the epoxide hydrolases and could consequently compete for the epoxide hydrolase binding site and reduce the conversion of EETs to DHETs. Therefore, it is feasible that 14,15-EEZE could increase the ratio of EETs/DHETs, but this alteration would not explain the impaired vascular relaxations induced by 14,15-EEZE. These results suggest that the antagonism of the vascular effects occurs through the blockade of EET activity and not through the alteration of EET or 20-HETE synthesis. Additionally, this evidence that an EET-specific antagonist blocks the vascular responses to bradykinin and methacholine further substantiates the role of EETs as EDHFs.

It has been suggested that the vascular actions of EETs may occur through endothelium-dependent mechanisms. For example, in porcine coronary arterial strips, smooth muscle hyperpolarization responses to 11,12-EET are greater in the presence of an intact endothelium and are sensitive to blockade by charybdoxin and apamin. However, in bovine coronary arteries, we have previously shown that 11,12-EET–induced relaxations are similar in arterial rings with and without an intact endothelium. Therefore, 11,12-EET induces relaxation through smooth muscle–dependent mechanisms, and 14,15-EEZE impairs these actions. Although not addressed by the present study, 14,15-EEZE may provide a useful tool for the investigation of endothelium-dependent effects of EETs.

At this time, an EET receptor(s) has not been identified in smooth muscle even though EETs stimulate BK\(_{Ca}\) channel activity through a G-protein–dependent mechanism. The present study provides evidence that EETs interact with a precise binding site or receptor because a specific structure is required for their vascular actions. It is not clear whether EETs act at a single or multiple binding sites or receptors, inasmuch as the 14,15-EET analogue inhibited 14,15-EET–, 11,12-EET–, 8,9-EET–, and 5,6-EET–induced relaxations. High-affinity protease-sensitive binding of 14,15-EET has been demonstrated in guinea pig mononuclear cells, and in endothelial cells, high-affinity binding has been characterized for 12\((R)\)-hydroxyeicosatrienoic acid. Similar to our results, structural modification of 20-HETE alters agonist and antagonist activity, presumably by altering interaction with a putative binding site. Thus, EETs may induce their vascular effects through a receptor-dependent mechanism, and alteration of the EET molecule modifies this interaction.

The EETs are important mediators of numerous biological mechanisms, including vascular and bronchial smooth muscle tone, cellular proliferation, hormone secretion, fluid and electrolyte transport, and inflammation. Because of the unique and complex cellular effects of EETs, which are often masked by other cytochrome P-450 metabolites, specific inhibitors of EETs are required to fully characterize their actions. We have shown that the alteration of the EET
molecule makes it possible to prepare a specific EET antagonist. This EET analogue and future analogues will be useful for the further evaluation of the biological and pharmacological effects of endogenous EETs and may provide a basis for the development of therapeutic agents designed to modify the actions of EETs.

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