14,15-Epoxycosatetraenoic acid (ETs) are cytochrome P450 metabolites of arachidonic acid. The vascular endothelium synthesizes and releases ETs in response to vasoactive agonists such as bradykinin and acetylcholine. In the coronary circulation, ETs activate smooth muscle membrane large-conductance, calcium-activated K+ (BKCa) channels to cause hyperpolarization and vascular relaxation. Therefore, they function as endothelium-derived hyperpolarizing factors (EDHFs). Besides ETs, hydrogen peroxide, K+, and endocannabinoids are potential EDHFs. The specific chemical mediator of EDHF activity varies, depending on vascular size, vascular bed, and species. Additionally, in small resistance arteries, electronic spread from the endothelium to the smooth muscle through gap junctions may mediate this activity.

The functional characterization of EDHF activity has depended on the use pharmacological inhibitors. To investigate the role of ETs in EDHF-dependent relaxation, inhibitors of cytochrome P450 enzymes are used. In some studies, inhibitors of cytochrome P450 blocked the relaxation to bradykinin and acetylcholine, whereas in other studies, these inhibitors were without effect. In this regard, cytochrome P450 inhibitors may have other nonspecific vascular effects. For example, relaxation to potassium channel activators such as cromakalim or pinacidil was inhibited by some cytochrome P450 inhibitors. Additionally, the P450 inhibitor clotrimazole directly inhibits Ca2+-activated K+ channels. Therefore, to establish the role of endogenous ETs in vascular relaxation, pharmacological tools are required that selectively inhibit only the action or synthesis of ETs without other nonspecific effects.

Chemical modification of the ET molecule alters ET vascular activity. For 14,15-ET, shortening the distance between the carboxyl and epoxy groups, converting the epoxy oxygen to a sulfur or nitrogen, and changing the carboxyl group at carbon-1 to an alcohol or elimination of the Δ8 double bond results in loss of agonist potency. Furthermore, the 14,15-ET analogue 14,15-epoxycosalenoic acid, with saturated double bonds between carbons 5 and 6, acts as an ET-specific antagonist. This analogue inhibits relaxation induced by 5,6-, 8,9-, 11,12-, 14,15-ET, and 14,15-DHET and inhibits the ET component of bradykinin-induced and acetylcholine-induced relaxation.

**Key Words:** vasodilation ■ arachidonic acids ■ endothelium-derived factors ■ acetylcholine ■ bradykinin

**Abstract**—Endothelium-dependent hyperpolarizations and relaxation of vascular smooth muscle induced by acetylcholine and bradykinin are mediated by endothelium-derived hyperpolarizing factors (EDHFs). In bovine coronary arteries, arachidonic acid metabolites, epoxycosatrienoic acids (ETs), function as EDHFs. The 14,15-ET analog 14,15-epoxycosatetraenoic acid (14,15-EEZE-mSI) was synthesized and tested for agonist and antagonist activity. In bovine coronary arterial rings, 14,15-, 11,12-, 8,9-, and 5,6-ET induced maximal concentration-related relaxation averaging 75% to 87% at 10 μmol/L, whereas, 14,15-EEZE-mSI induced maximal relaxation averaging only 7%. 14,15-EEZE-mSI (10 μmol/L) preincubation inhibited relaxation to 14,15- and 5,6-ET but not 11,12- or 8,9-ET. 14,15-EEZE-mSI also inhibited indomethacin-resistant relaxation to arachidonic acid and indomethacin-resistant and l-nitroarginine-resistant relaxation to bradykinin and methacholine. It did not alter the relaxation to sodium nitroprusside, iloprost, or the K+ channel opener bimakalim or NS1619. In cell-attached patches of isolated bovine coronary arterial smooth muscle cells, 14,15-EEZE-mSI (100 nmol/L) blocked the 14,15-ET-induced (100 nmol/L) activation of large-conductance, calcium-activated K+ channels. Mass spectrometric analysis of rat renal cortical microsomes incubated with arachidonic acid showed that 14,15-EEZE-mSI (10 μmol/L) increased ET concentrations while decreasing the concentrations of the corresponding dihydroxyicosatetraenoic acids. Therefore, 14,15-EEZE-mSI inhibits relaxation to 5,6- and 14,15-ET and the K+ channel activation by 14,15-ET. It also inhibits the EDHF component of bradykinin-induced, methacholine-induced, and arachidonic acid-induced relaxation. These results suggest that 14,15- or 5,6-ET act as an EDHF in bovine coronary arteries. (Hypertension. 2003;42:555-561.)
induced relaxation. This because these alterations resulted in antagonist activity, further modifications may increase the potency of the antagonist activity or enhance regioisomer-specific properties. Therefore, we synthesized 14,15-epoxyeicosa-5(Z)-enoic-methylsulfonylimide (14,15-EEZE-mSI). 14,15-EEZE-mSI differs from 14,15-EEZE in that the carbon-1 carboxyl has been substituted with a methylsulfonimide group. Sulfonimide groups are commonly used as a substitute for carboxyl groups because they have a similar pKa.21 The sulfonimide group could enhance biological availability by blocking metabolism by β-oxidation and by preventing esterification into membrane lipids.22-24 This study evaluated agonist and antagonist properties of 14,15-EEZE-mSI and has characterized this molecule as a regioisomer-specific EET antagonist.

Methods

Vascular Reactivity Studies

Fresh bovine hearts were purchased from a local slaughterhouse. Sections of the left anterior descending coronary artery were dissected, cleaned, and cut into 1.5 to 2.0 mm diameter rings (3 mm length). The arterial rings were suspended in a tissue bath containing a Krebs-bicarbonate buffer equilibrated with 95% O2–5% CO2 and maintained at 37°C as previously described.25 Tension was measured with a model FT-03C force transducer (Grass Instruments), ETH-400 bridge amplifier, and MacLab 8e A/D converter with MacLab software and a Macintosh computer. The arterial rings were slowly stretched to a basal tension of 3.5 g and equilibrated for 1.5 hours. KCl (40 to 60 mmol/L) was repeatedly added and rinsed until reproducible stable contractions were observed. The thromboxane mimetic U46619 (20 nmol/L) was added to increase basal tension to ~50% to 75% of maximal KCl contraction. Relaxation responses to cumulative additions of the EETs, 14,15-EET, sodium nitroprusside, bimakalim, NS1619, and iloprost were accepted at a value of P<0.05.

Statistical Analysis

Vascular reactivity, patch-clamp, and mass spectrometry data are expressed as mean±SEM. Significance of differences between mean values was evaluated by Student t test or ANOVA, followed by the Student-Newman-Keuls multiple comparison test. Significance was accepted at a value of P<0.05.

Results

Structure of 14,15-EEZe-mSI

14,15-EEZE-mSI was synthesized by methods previously described.19 Figure 1 compares the chemical structures of 14,15-EET and 14,15-EEZE-mSI. 14,15-EEZE-mSI differs from 14,15-EET in that the double bonds at carbons 8,9 and 11,12 are saturated and the carbon-1 carboxyl was altered to a methylsulfonimide group.

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

In U46619 precontracted arteries, 14,15-EEZE-mSI inhibited concentration-related relaxation, as previously described.20 In this study maximal relaxation to the EETs averaged 75% to 87% (Figure 2, A through D). 14,15-EEZE-mSI pretreatment caused a concentration-dependent inhibition of the 14,15-EET-induced relaxation. 14,15-EET maximal relaxation of 76±2% decreased to 67±6% and 33±5% after pretreatment with 1 μmol/L and 10 μmol/L 14,15-EEZE-mSI, respectively. 14,15-EEZE-mSI (10 μmol/L) pretreatment did not alter relaxation to 11,12- or 8,9-EET. However, 14,15-EEZE-mSI inhibited relaxation to 5,6-EET. 5,6-EET maximal relaxation of 75±3% decreased to 46±3% with pretreatment of this analogue. Relaxation to 14,15-DHET was also inhibited by 14,15-EEZE-mSI (Figure 2E). 14,15-DHET maximal relaxation of 88±3% decreased to 41±8% with 14,15-EEZE-mSI pretreatment. In contrast, 14,15-EEZE-mSI had little agonist activity. It maximally

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\text{14,15-Epoxycosatrienoic acid (14,15-EET)}
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\text{14,15-Epoxycosatrienoic acid (14,15-EET)}
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relaxed the precontracted arteries by 7±2% at 10 μmol/L (Figure 2F). Basal tension of the coronary arterial rings was not altered by 14,15-EEZE-mSI (1 nmol/L to 10 μmol/L, data not shown).

Next we evaluated the ability of this analogue to alter relaxation to other dilators. Sodium nitroprusside, an NO donor, iloprost, a prostacyclin analog, bimakalim, an ATP-sensitive K⁺ channel opener, and NS1619, a large-conductance, Ca²⁺-activated K⁺ channel (BKCa) opener, relaxed the coronary arteries (Figure 3). 14,15-EEZE-mSI (10 μmol/L) did not alter the concentration-dependent relaxation to iloprost or sodium nitroprusside (Figures 3A and 3B). Additionally, 14,15-EEZE-mSI did not alter the relaxation to the K⁺ channel activators bimakalim and NS1619 (Figures 3C and 3D). Therefore, 14,15-EEZE-mSI appears to specifically inhibit 14,15-induced and 5,6-EET-induced relaxation.

Additionally, contraction of arteries to increasing K⁺ concentrations (10 mmol/L to 80 mmol/L) was similar in arterial rings pretreated with either vehicle or 14,15-EEZE-mSI, with 14,15-EEZE-mSI causing a slight shift to the left of the concentration-response curve (EC₅₀ = 36.3±1.2 mmol/L, control versus 31.8±1.2 mmol/L, 14,15-EEZE-mSI, 10 μmol/L). Pre-incubation with 14,15-EEZE-mSI shifted the concentration-response curve of U46619 (10 pmol/L to 24 nmol/L) to the to the right, with EC₅₀s averaging 4.6±0.6 nmol/L and 13.2±1.1 nmol/L for control and 14,15-EEZE-mSI, respectively. This shift further indicates the slight agonist activity of this analog.

The ability of 14,15-EEZE-mSI to inhibit endothelium-dependent relaxation to bradykinin, methacholine, and arachidonic acid was evaluated (Figure 4). In the presence of indomethacin and L-NA, bradykinin-induced relaxation was shifted to the right 1000-fold by 14,15-EEZE-mSI (10 μmol/L) and maximal relaxation was reduced from 98±2% to 55±10% (Figure 4A). Similarly, indomethacin and L-NA-resistant relaxation to methacholine was reduced by 14,15-EEZE-mSI from a maximum of 75±4% to 48±8% (Figure 4B). The indomethacin-resistant relaxation to arachidonic acid was also reduced from a maximum of 87±7% to 54±4% by this inhibitor (Figure 4C). The supplementary addition of MSPPOH further decreased the arachidonic acid–induced maximal relaxation to 21±7%. Importantly, these data show that 14,15-EEZE-mSI inhibits the EDHF component of
methacholine- and bradykinin-induced relaxation. This suggests that EETs, specifically 14,15- or 5,6-EET, contribute to the non-NO and nonprostaglandin relaxation to these agonists in bovine coronary arteries.

Effect of 14,15-EEZE-mSI on 14,15-EET-Induced Activation of BKCa Channels
In cell-attached patches of isolated bovine coronary arterial smooth muscle cells, we have previously demonstrated that 14,15-EET activates BKCa channels. Consequently, we investigated the ability of 14,15-EEZE-mSI to block this activation. Recordings of BKCa channel activity in cell-attached patches are shown in Figure 5A. In the cell incubated with the vehicle, 14,15-EET (100 nmol/L) activated BKCa channel activity. In contrast, in the cell that was incubated with 14,15-EEZE-mSI (100 nmol/L), 14,15-EET failed to increase BKCa channel activity. Figure 5B summarizes the effect of 14,15-EEZE-mSI on channel mean open time (NPo). 14,15-EET produced a 4-fold activation of channel NPo. Incubation with 14,15-EEZE-mSI did not alter basal K+ channel activity but blocked the 14,15-EET-induced activation. Channel unitary conductance was similar in cells incubated with either vehicle or 14,5-EEZE-mSI and averaged 236±10 pS. Thus, 14,15-EEZE-mSI inhibits the 14,15-EET-induced activation of coronary smooth muscle BKCa channels.

Effect of 14,15-EEZE-mSI on Arachidonic Acid Metabolism
To determine if 14,15-EEZE-mSI alters the synthesis of EETs, we investigated the effect of this analogue on arachidonic acid metabolism of rat renal cortical microsomes. Renal microsomes were chosen for this study because they produce large quantities of the four EET regioisomers and their metabolites, the DHETs. Microsomes converted arachidonic acid to 14,15-, 11,12-, 8,9-, 5,6-EET, and 20-HETE (Table). Larger amounts of DHETs were also formed. The presence of 14,15-EEZE-mSI (10 μmol/L) decreased concentrations of the DHET regioisomers while increasing the concentrations of the EETs. It did not alter 20-HETE concentrations. Therefore, 14,15-EEZE-mSI does not interfere with the synthesis of EETs or 20-HETE but appears to decrease the metabolism of EETs to DHETs by epoxide hydrolase.

Discussion
EETs function as EDHFs in the coronary, renal, and cerebral circulation. The four EET regioisomers are equipotent in relaxing preconstricted bovine coronary arterial rings, with maximal relaxation averaging 80%. The four EET regioisomers are equipotent in relaxing preconstricted bovine coronary arterial rings, with maximal relaxation averaging 80%. In this vasculature, the specific position of the epoxide group does not appear critical for EET-induced relaxation. 14,15-DHET also induces relaxation of bovine coronary arterial rings, although relaxation is ≈5-fold less potent that the EETs. Similar to 14,15-EET, 14,15-DHET activates coronary smooth muscle cell BKCa channels through G-protein-dependent mechanisms. Thus, 14,15-EET and 14,15-DHET appear to induce smooth muscle hyperpolarization and relaxation through similar cellular mechanisms.

Previously, we characterized 14,15-EEZE as an EET-specific antagonist. This analogue blocked relaxation to all four EET regioisomers and 14,15-DHET but was most
effective in inhibiting 14,15-EET. It also blocked the EDHF component of methacholine and bradykinin-induced relaxation.\textsuperscript{20} 14,15-EEZE-mSI differs from 14,15-EEZE in that the carbon-1 carboxyl is replaced with a methylsulfonimide group. A similar substitution of carbon 1 of 14,15-EET with a methylsulfonimide group (14,15-EET-mSI) did not alter agonist properties.\textsuperscript{19} Additionally, 14,15-EET-mSI is not metabolized by smooth muscle cells or incorporated into cellular lipids.\textsuperscript{24} Similarly, by using LC/ESI–mass spectrometric analysis of 14,15-EEZE-mSI metabolism in bovine coronary smooth muscle cells, we did not see evidence of β-oxidation (data not shown). This substitution, therefore, could increase bioavailability by preventing membrane esterification and metabolism. However, it was not clear if this same substitution would alter agonist or antagonist properties of 14,15-EEZE. 14,15-EEZE-mSI showed little agonist activity. Conversely, preincubation with 14,15-EEZE-mSI blocked relaxation to 5,6-EET, 14,15-EET and 14,15-DHET. It did not alter relaxation to 8,9- and 11,12-EET. Therefore, this analogue appears to inhibit activity of eicosanoid agonists with an epoxide or hydroxyl group in either the 5,6 or 14,15 position. The reason for this specificity is not clear. 14,15-EEZE-mSI did not alter the ability of the bovine coronary arterial rings to relax to the NO-donor, sodium nitroprusside, the prostacyclin analog iloprost, or the K⁺ channel activators bimakalim or NS1619. Thus, 14,15-EEZE-mSI is not a nonselective inhibitor of vascular relaxation.

14,15-EEZE-mSI also inhibited the L-NA–resistant and indomethacin-resistant relaxation to bradykinin and methacholine and the indomethacin-resistant relaxation to arachidonic acid. Because this analogue only inhibited relaxation to 14,15- and 5,6-EET, this suggests that the EET regioisomer responsible for this EDHF activity is either 5,6- or 14,15-EET. Indeed, the EET regioisomer produced in the highest concentration by bovine coronary artery endothelial cells when stimulated by bradykinin or methacholine is 14,15-EET.\textsuperscript{33} Therefore, these results provide additional evidence that 14,15-EET is the principle EET regioisomer responsible for EDHF activity in bovine coronary arteries.

14,15-EEZE-mSI did not completely block arachidonic acid–induced relaxation and was less effective than 14,15-EEZE.\textsuperscript{20} The 14,15-EEZE-mSI–resistant relaxation to arachidonic acid was nearly eliminated by the epoxygenase inhibitor MSPPOH. Arachidonic acid is metabolized by the bovine coronary endothelium to all four EET regioisomers.\textsuperscript{25} 14,15-EEZE-mSI, unlike 14,15-EEZE, only inhibits relaxation to 14,15- and 5,6-EET.\textsuperscript{20} Thus, under these conditions, 14,15-EEZE-mSI could only inhibit the component of arachidonic acid–induced relaxation that was mediated by 14,15- and 5,6-EET. The remaining relaxation was inhibited by MSPPOH, an epoxygenase inhibitor that blocks the synthesis of all 4 EET regioisomers.

It is possible that 14,15-EEZE-mSI antagonism of EDHF activity occurs through the inhibition of EET synthesis rather than the blockade of EET activity. However, 14,15-EEZE-mSI increased the EET concentrations while decreasing DHET concentrations in the assays evaluating arachidonic acid metabolism. EETs are hydrolyzed to DHETs by epoxide hydrolases.\textsuperscript{34} Similarly, 14,15-EEZE-mSI may be a substrate

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**Figure 4.** Effect of 14,15-EEZEm-SI (10 μmol/L) on bradykinin-induced (A), methacholine-induced (B), or arachidonic acid–induced (C) relaxation of bovine coronary arteries. Arterial segments were pretreated with U46619 (20 nmol/L). For bradykinin and methacholine, segments were pretreated with indomethacin (10 μmol/L) and L-nitroarginine (30 μmol/L); for arachidonic acid, segments were pretreated with indomethacin (10 μmol/L) or indomethacin plus MSPPOH (10 μmol/L). Changes in isometric tension were measured. *Significantly different from control, $P<0.05$. All values are mean±SEM (n=6 to 16).
for epoxide hydrolases and compete with EETs for the epoxide hydrolase binding site. This competition would reduce the conversion of EETs to DHETs. However, this decreased conversion of EETs to DHETs would not explain the decreased EDHF-dependent relaxation induced by 14,15-EEZE-mSI.

In addition, we evaluated the ability of 14,15-EEZE-mSI to inhibit EET-induced activation of smooth muscle BKCa channels. In cell-attached patches, 14,15-EET activated BKCa channel open time by 4-fold. Incubation with 14,15-EEZE-mSI did not alter basal BKCa activity but blocked the 14,15-EET-induced activation. These results suggest that 14,15-EEZE-mSI antagonism of EDHF activity occurs through the blockade of EET action at the smooth muscle and not through the inhibition of EET synthesis in the endothelial cell or through the nonspecific inhibition of BKCa channels.

It is unclear if EET-induced vascular relaxation involves receptor-mediated mechanisms, even though EETs may stimulate BKCa channel activity through G-protein–dependent mechanisms. In this regard, EETs may act at multiple regioisomer-specific binding sites. This is supported by the evidence that 14,15-EEZE-mSI inhibited 14,15-induced and 5,6-EET-induced relaxation but not relaxation induced by 8,9- or 11,12-EET. Furthermore, arteries from different vascular beds vary in the EET regioisomers that cause relaxation. For example, 11,12-EET but not 14,15-EET relaxed the rat renal artery, only 5,6-EET induced relaxation in the rat tail artery, and 14,15-EET and 11,12-EET induced relaxation whereas 8,9- and 5,6-EET did not in porcine coronary arterial rings. Interestingly, 14,15-EEZE-mSI blocked 14,15-EET as well as 14,15-DHET–induced relaxation. The reasons for this interaction are not clear. However, this alludes to the possibility that 14,15-EET and 14,15-DHET act through the same receptor or binding site. Together, these results implicate a role for regioisomer, receptor-dependent mechanisms in EET-induced vascular activity and alteration of the EET molecule modifies these interactions.

**Perspectives**

The physiological importance of EDHF activity is becoming increasingly apparent as a major regulator of vascular tone. In the coronary circulation, the EETs function as EDHFs, and investigations of the role of endogenous EETs have used cytochrome P450 inhibitors. However, these inhibitors may have nonspecific vascular effects and also block other vascular cytochrome P450 enzymes. Therefore, specific inhibitors of EET synthesis or action are required to fully characterize their activity. The results from this study have shown that the 14,15-EET analogue 14,15-EEZE-mSI acts as a regioisomer-specific antagonist of EET activity. 14,15-EEZE-mSI and other EET analogue antagonists will provide useful tools for the future evaluation of the role of endogenous EETs. Most importantly, impaired EDHF function may contribute to decreased dilator activity in vascular pathologies such as diabetes or hypertension.

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14,15-Epoxyeicosa-5(Z)-Enoic-mSI: A 14,15- and 5,6-EET Antagonist in Bovine Coronary Arteries
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