Effects of the selective EET antagonist, 14,15-EEZE, on cardioprotection produced by exogenous or endogenous EETs in the canine heart

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Submitted 21 February 2008; accepted in final form 22 April 2008

Gross GJ, Gauthier KM, Moore J, Falck JR, Hammock BD, Campbell WB, Nithipatikom K. Effects of the selective EET antagonist, 14,15-EEZE, on cardioprotection produced by exogenous or endogenous EETs in the canine heart. Am J Physiol Heart Circ Physiol 294: H2838–H2844, 2008. First published April 25, 2008; doi:10.1152/ajpheart.00186.2008.—Previously, we demonstrated (17) that 11,12- and 14,15-epoxyeicosatrienoic acids (EETs) produce marked reductions in myocardial infarct size. Although it is assumed that this cardioprotective effect of the EETs is due to a specific interaction with a membrane-bound receptor, no evidence has indicated that novel EET antagonists selectively block the EET actions in dogs. Our goals were to investigate the effects of 11,12- and 14,15-EET, the soluble epoxy hydrolase inhibitor, 12-(3-adamantan-1-yl-ureido)-dodecanoic acid (AUDA), and the putative selective EET antagonist, 14,15-epoxyeicos-5(Z)-enoic acid (14,15-EEZE), on infarct size of barbital anesthetized dogs subjected to 60 min of coronary artery occlusion and 3 h of reperfusion. Furthermore, the effect of 14,15-EEZE on the cardioprotective actions of the selective mitochondrial ATP-sensitive potassium channel opener diazoxide was investigated. Both 11,12- and 14,15-EET markedly reduced infarct size [expressed as a percentage of the area at risk (IS/AAR)] from 21.8 ± 1.6% (vehicle) to 8.7 ± 2.2 and 9.4 ± 1.3%, respectively. Similarly, AUDA significantly reduced IS/AAR from 21.8 ± 1.6 to 14.4 ± 1.2% (low dose) and 9.4 ± 1.8% (high dose), respectively. Interestingly, the combination of the low dose of AUDA with 14,15-EET reduced IS/AAR to 5.8 ± 1.6% (P < 0.05), further than either drug alone. Diazoxide also reduced IS/AAR significantly (10.2 ± 1.9%). In contrast, 14,15-EEZE had no effect on IS/AAR by itself (21.0 ± 3.6%), but completely abolished the effect of 11,12-EET (17.8 ± 1.4%) and 14,15-EET (19.2 ± 2.4%) and AUDA (19.3 ± 1.6%), but not that of diazoxide (10.4 ± 1.4%). These results suggest that activation of the EET pathway, acting on a putative receptor, by exogenous EETs or indirectly by blocking EET metabolism, produced marked cardioprotection, and the combination of these two approaches resulted in a synergistic effect. These data also suggest that 14,15-EEZE is not blocking the mitochondrial ATP-sensitive potassium channel as a mechanism for antagonizing the cardioprotective effects of the EETs.

1Cytochrome P-450 epoxyenase; epoxyeicosatrienoic acids; soluble epoxy hydrolase; ATP-sensitive potassium channel opener

ARACHIDONIC ACID IS METABOLIZED to various bioactive eicosanoids by cyclooxygenase, lipooxygenase, and the cytochrome P-450 (CYP) monooxygenase pathways (21). We and others have recently found that inhibition of the CYP ω-hydroxylase pathway (6, 7, 15) and activation of the CYP epoxyenase pathway by overexpressing CYP2J2 (26, 27) or by administering exogenous epoxyeicosatrienoic acids (EETs) result in an improvement in the recovery of contractile function in isolated, perfused mouse hearts (19, 27), subjected to global ischemia and reperfusion, and a reduction in infarct size (IS) in intact canine or rat hearts (7, 17), subjected to regional ischemia and reperfusion. These protective effects were mediated by activation of the sarcolemmal (sarc) or the mitochondrial (mito) ATP-sensitive potassium (KATP) channel (7, 12, 17, 19). Similarly, exogenous EETs protect endothelial cells from apoptosis produced by TNF-α via a MAPK and phosphatidylinositol 3-kinase/Akt signaling pathway (25, 28), and similar protective effects have been reported by Dhanasekaran et al. (2) in human endothelial cells obtained from the pulmonary and coronary vasculature and cardiac myocytes exposed to anoxia (3). Although it is assumed that the beneficial effects of CYP ω-hydroxylase inhibition and CYP2J2 overexpression are the result of an increase in endogenous EETs, there is no direct evidence that the protective actions of these two interventions are actually the result of the actions of EETs on a putative membrane receptor.

In this regard, Gauthier et al. (4, 5) recently identified and characterized a unique EET antagonist, 14,15-epoxyeicosa-5(Z)-enoic acid (EEZE). 14,15-EEZE behaves as a direct EET antagonist at a receptor binding site on the cell membrane or intracellularly (30) and antagonizes selectively many of the vascular actions of the EETs (4, 5, 29), although one study in mesenteric artery demonstrated that 14,15-EEZE had intrinsic vasodilator activity (8). However, the effects of 14,15-EEZE on the cardioprotective actions of the EETs have not been thoroughly addressed and are a major focus of the present study.

Another method that has been shown to be cardioprotective in mice involves inhibition of the major enzymatic hydrolysis pathway of the EETs, soluble epoxy hydrolase (EPHX2) (9, 10, 18, 20). This tactic has been used effectively to increase endogenous EET concentrations under basal conditions and particularly during ischemia-reperfusion. Recently, Seubert et al. (20) studied the functional role of deleting the EPHX2 gene, Ephpx2, on cardioprotection in isolated wild-type (C57BL6) and Ephpx2 null mouse hearts subjected to 20 min of ischemia and 40 min of reperfusion. The Ephpx2 null hearts had a better recovery of contractile function and a modest reduction in IS compared with the wild-type hearts. These results suggest...
that enhancing cellular concentrations of EETs by blocking their hydrolysis may be a potential new therapeutic way to produce cardioprotection. We have further addressed the beneficial effects of inhibiting this pathway with a pharmacological blocker of EPHX2, 12-(3-adamantan-1-yl-ureido)-dodecanoic acid (AUDA) (10), to determine the cardioprotective potential of inhibiting EPHX2 (18), in a large animal model of ischemia-reperfusion injury, and also to determine the possibility that EPHX2 inhibition, in combination with exogenous EETs, might have additive or synergistic effects on irreversible injury.

MATERIALS AND METHODS

This study was approved by performed in accordance with the guidelines of the Animal Care Committee of the Medical College of Wisconsin, which is accredited by the American Association of Laboratory Animal Care.

Materials. 11,12-EET, 14,15-EET, and 14,15-EEZE were synthesized in the laboratory of Dr. J. R. Falck. AUDA was synthesized as described (13). All other chemicals were of the highest analytic or purity grades. Distilled, deionized water was used in all experiments.

General preparation of dogs. The protocol used has been thoroughly described in detail in previous publications from our laboratory (15, 17). Briefly, adult mongrel dogs of either sex, weighing 15–25 kg, were fasted overnight, anesthetized with the combination of barbitual sodium (200 mg/kg) and pentobarbital sodium (15 mg/kg), and ventilated with room air supplemented with 100% oxygen. Body temperature was carefully controlled at 38 ± 1°C with a heating pad. Atelectasis was prevented by maintaining an end-expiratory pressure of 5–7 cm with a trap. Arterial blood pH, PCO2, and PO2 were monitored at selected intervals by an AVL blood-gas analyzer and maintained within normal physiological limits (pH = 7.35–7.45, PCO2 30–40 Torr, and PO2 85–130 Torr) by adjusting the respiratory rate and the oxygen flow rate or by adding 1.5% sodium bicarbonate intravenously if necessary. An electromagnetic flowmeter (Statham 2202) was used to measure left anterior descending (LAD) blood flow. A mechanical occluder was placed distal to the flow probe and the occluder, such that there were no branches between the flow probe and the occluder. A double-tipped Millar (model 770) catheter was placed into the carotid artery and left ventricle (LV) to measure aortic and LV pressures and to determine LV change in pressure over time (positive and negative). The left atrium was cannulated via the appendage for radioactive microsphere injections.

Experimental protocol. Dogs were sequentially assigned to 12 groups for different treatments (Fig. 1). In all groups, including the AUDA-treated dogs, eight dogs were included for statistical analysis. At 15 min before the 60-min LAD occlusion period, 11,12-EET (0.128 mg/kg), 14,15-EET (0.128 mg/kg), or 14,15-EEZE (0.128 mg/kg), vehicle was administered by 10.220.32.246 on July 3, 2017 http://ajpheart.physiology.org/ Downloaded from
by the radioactive microsphere technique, as previously reported (17).
Microspheres were administered 30-min into the 60-min occlusion period
and at the end of 3 h of reperfusion. Transmural blood flow was
calculated as the weighted average of the subepicardium, midmyo-
cardium, and subendocardium of each region.

Statistical analysis. All values are expressed as means ± SE (N = 8/group).
Differences between groups in hemodynamics and blood
gases were compared by using a two-way ANOVA. Differences
between groups in tissue blood flows, AAR, and IS/AAR were
compared by a one-way ANOVA followed by Tukey’s post hoc test.
Differences between groups were considered significant if P < 0.05.
Linear regression analysis was performed to determine the correlation
between transmural collateral blood flow in the ischemic area and
myocardial IS (IS/AAR). Analysis of covariance, with collateral
blood flow as the covariate, was used to determine whether differ-
ences in this relationship were observed among the treatment groups
analyzed.

RESULTS

Hemodynamics. Heart rate and mean arterial blood pressure at
baseline and at 30 min of ischemia or at 3 h of reperfusion
were not different between all of the experimental groups
(Table 1). These data suggest that changes in hemodynamics
were not responsible for the differences in IS/AAR observed
between control dogs and those treated with the various EET mimetics, diazoxide, or the EET antagonist EEZE. Blood gases
and pH were also monitored throughout the experiments and
were not different between groups at any time points measured
(data not shown).

Regional myocardial blood flow. Transmural blood flow in
the nonischemic (left circumflex coronary bed) and the ischemic
(LAD bed) were measured at 30 min of occlusion and at 3 h of reperfusion with radioactive microspheres. There were
no significant differences in nonischemic transmural blood flow
between groups or transmural collateral blood flow
(ischemic bed) at the measured points. Most importantly, there
were no differences in flow in the ischemic reperfused area
during occlusion, which suggested that all groups were subject-
ted to similar degrees of ischemia (Table 2). There were also
no differences in AAR between groups (data not shown). Since the two major determinants of the ultimate IS/AAR in dogs are
the AAR and collateral blood flow, these data suggest that
changes in these two parameters were not responsible for any
differences in IS/AAR observed between control and drug-
treated dogs.

Effects of 11,12-EET and 14,15-EET on IS/AAR in the
absence and presence of the EET antagonist 14,15-EEZE. Both
11,12-EET and 14,15-EET produced a marked reduction in
IS/AAR compared with the vehicle-treated dogs (Fig. 2).
Control IS/AAR was 21.8 ± 1.6%, and 11,12- and 14,15-EET
reduced IS/AAR to 8.7 ± 2.2 and 9.4 ± 1.3%, respectively
(P < 0.001). These results are in agreement with previously
published results (17) with this same dose of EETs (0.128
mg/kg) in dogs. However, in the presence of the selective EET
antagonist (4, 5) 14,15-EEZE, the cardioprotective effects
of 11,12- and 14,15-EET were abolished (IS/AAR = 19.2 ± 2.4

Table 1. Hemodynamic values

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Occlusion (30 min)</th>
<th>Reperfusion (3 h)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Heart rate, beats/min</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>156±4</td>
<td>155±4</td>
<td>155±6</td>
</tr>
<tr>
<td>11,12-EET</td>
<td>154±4</td>
<td>156±4</td>
<td>154±2</td>
</tr>
<tr>
<td>14,15-EET</td>
<td>153±3</td>
<td>158±4</td>
<td>148±3</td>
</tr>
<tr>
<td>AUDA (LD)</td>
<td>154±8</td>
<td>153±3</td>
<td>153±7</td>
</tr>
<tr>
<td>AUDA (HD)</td>
<td>156±2</td>
<td>151±6</td>
<td>153±4</td>
</tr>
<tr>
<td>AUDA (LD) + 14,15-EET</td>
<td>146±2</td>
<td>146±3</td>
<td>149±2</td>
</tr>
<tr>
<td>14,15-EETE</td>
<td>152±2</td>
<td>152±2</td>
<td>153±4</td>
</tr>
<tr>
<td>14,15-EZEE + 14,15-EET</td>
<td>160±6</td>
<td>160±8</td>
<td>154±4</td>
</tr>
<tr>
<td>14,15-EZEE + 11,12-EET</td>
<td>152±5</td>
<td>151±3</td>
<td>154±4</td>
</tr>
<tr>
<td>Diazoxide</td>
<td>151±5</td>
<td>154±7</td>
<td>154±4</td>
</tr>
<tr>
<td>14,15-EZEE + Diazoxide</td>
<td>152±3</td>
<td>158±7</td>
<td>153±4</td>
</tr>
<tr>
<td>14,15-EZEE + AUDA (HD)</td>
<td>155±4</td>
<td>152±1</td>
<td>144±7</td>
</tr>
</tbody>
</table>

**Mean arterial pressure, mmHg**

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Occlusion (30 min)</th>
<th>Reperfusion (3 h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>100±4</td>
<td>104±6</td>
<td>110±4</td>
</tr>
<tr>
<td>11,12-EET</td>
<td>97±3</td>
<td>100±5</td>
<td>104±5</td>
</tr>
<tr>
<td>14,15-EET</td>
<td>92±6</td>
<td>90±7</td>
<td>99±5</td>
</tr>
<tr>
<td>AUDA (LD)</td>
<td>97±7</td>
<td>95±8</td>
<td>101±7</td>
</tr>
<tr>
<td>AUDA (HD)</td>
<td>94±8</td>
<td>88±8</td>
<td>99±6</td>
</tr>
<tr>
<td>AUDA (LD) + 14,15-EET</td>
<td>108±5</td>
<td>104±4</td>
<td>110±4</td>
</tr>
<tr>
<td>14,15-EETE</td>
<td>115±8</td>
<td>110±7</td>
<td>117±4</td>
</tr>
<tr>
<td>14,15-EZEE + 14,15-EET</td>
<td>130±5</td>
<td>120±8</td>
<td>123±4</td>
</tr>
<tr>
<td>14,15-EZEE + 11,12-EET</td>
<td>115±2</td>
<td>108±3</td>
<td>114±4</td>
</tr>
<tr>
<td>Diazoxide</td>
<td>99±3</td>
<td>96±4</td>
<td>107±5</td>
</tr>
<tr>
<td>14,15-EZEE + Diazoxide</td>
<td>105±5</td>
<td>96±7</td>
<td>100±4</td>
</tr>
<tr>
<td>14,15-EZEE + AUDA (HD)</td>
<td>102±6</td>
<td>98±5</td>
<td>101±8</td>
</tr>
</tbody>
</table>

All values are means ± SE; N = 8 dogs. There were no significant
differences between groups by ANOVA followed by Newman-Keuls post hoc
analysis.

Table 2. Transmural blood flow values

<table>
<thead>
<tr>
<th>Group</th>
<th>Nonischemic Region</th>
<th>Ischemic Region</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>30-min Occlusion</td>
<td>3-h Reperfusion</td>
</tr>
<tr>
<td></td>
<td>30-min Occlusion</td>
<td>3-h Reperfusion</td>
</tr>
<tr>
<td>Control</td>
<td>0.85±0.16</td>
<td>0.98±0.16</td>
</tr>
<tr>
<td>11,12-EET</td>
<td>0.65±0.11</td>
<td>0.85±0.10</td>
</tr>
<tr>
<td>14,15-EET</td>
<td>0.78±0.06</td>
<td>1.09±0.08</td>
</tr>
<tr>
<td>AUDA (LD)</td>
<td>1.17±0.11</td>
<td>1.29±0.19</td>
</tr>
<tr>
<td>AUDA (HD)</td>
<td>1.03±0.08</td>
<td>1.31±0.20</td>
</tr>
<tr>
<td>AUDA(LD) + 14,15-EET</td>
<td>1.10±0.08</td>
<td>1.46±0.11</td>
</tr>
<tr>
<td>14,15-EZEE</td>
<td>1.16±0.08</td>
<td>1.04±0.08</td>
</tr>
<tr>
<td>14,15-EZEE + 14,15-EET</td>
<td>1.19±0.17</td>
<td>1.21±0.11</td>
</tr>
<tr>
<td>14,15-EZEE + 11,12-EET</td>
<td>1.14±0.16</td>
<td>1.20±0.08</td>
</tr>
<tr>
<td>Diazoxide</td>
<td>1.62±0.18</td>
<td>1.67±0.19</td>
</tr>
<tr>
<td>14,15-EZEE + Diazoxide</td>
<td>1.60±0.17</td>
<td>1.42±0.12</td>
</tr>
<tr>
<td>14,15-EZEE + AUDA (HD)</td>
<td>1.14±0.11</td>
<td>0.92±0.08</td>
</tr>
</tbody>
</table>

Values are means ± SE in ml·min⁻¹·g⁻¹; N = 8 dogs.
Effect of the EPHX2 inhibitor AUDA on IS/AAR alone and in combination with 14,15-EET. AUDA produced a dose-dependent reduction in IS/AAR (control: 21.8 ± 1.6%; AUDA: 0.157 mg/kg, 14.4 ± 1.2%; AUDA: 0.314 mg/kg, 9.4 ± 1.8%; Fig. 4). When the low dose of AUDA was combined with 0.128 mg/kg of 14,15-EET, IS/AAR was further reduced to 5.8 ± 1.3%, which was significantly less than the small dose of AUDA given alone (P < 0.05). This combination was also smaller than the high dose of AUDA or 14,15-EET alone, although this effect did not reach statistical significance. Analysis of 14,15-EET in plasma samples at 5- and 30-min reperfusion by liquid chromatography-electrospray ionization-mass spectrometry (16) indicates that 14,15-EET concentrations increased with AUDA administrations, as shown in Fig. 5. At 5-min reperfusion, AUDA at 0.157 and 0.314 mg/kg increased 14,15-EET concentrations from 9.2 ± 7.7 pg/ml (control) to 147.2 ± 39.0 and 211.0 ± 54.7 pg/ml, respectively. At 30-min reperfusion, AUDA increased 14,15-EET concentrations from 9.6 ± 6.4 pg/ml (control) to 153.3 ± 41.0 and 226.0 ± 54.7 pg/ml, respectively.

**DISCUSSION**

The present study confirms the potent cardioprotective efficacy of administering exogenous EETs to the ischemic heart (17). This study demonstrates that an analog of 14,15-EET, 14,15-EEZE (4, 5), is a potent antagonist of 11,12- and 14,15-EET to reduce IS in the canine heart at a dose that had no effect on IS alone. These data suggest that the effect of 14,15-EET to reduce IS is independent of its effect on coronary collateral blood flow (Fig. 3).
from our laboratory and those of others suggest that 14,15-EEZE selectively blocks the effects of EETs in various species and organs, most importantly in the vasculature and heart (4, 5, 20, 21). Furthermore, our results indicate that inhibition of the EPHX2 by AUDA produced a reduction in IS when administered alone and was able to potentiate the effect of 14,15-EET when these two agents were given together. These results also suggest that one may be able to combine lower doses of EETs and EPHX2 inhibitors to reduce ischemia-reperfusion injury, which would lessen the worry about adverse side effects that might possibly occur when higher doses of either drug are given alone. Finally, the effect of 14,15-EEZE appeared to be selective for a direct EET-mediated action at a receptor, since it did not block the downstream cardioprotection produced via activation of the mito K<sub>ATP</sub> channel.

The 14,15-EET analog 14,15-EEZE was synthesized in 2002 (4) and was tested in a number of in vitro systems to determine its activity as an EET antagonist. This compound blocks EET-induced vascular relaxations produced by all four EET regioisomers in bovine coronary artery rings (4). Furthermore, it also inhibited indomethacin-resistant relaxations to methacholine and arachidonic acid and indomethacin-resistant and l-nitroarginine-resistant relaxations to bradykinin. However, this compound did not block relaxations to sodium nitroprusside, iloprost, or the potassium channel openers, bimakalim or NS1619. The results obtained with diazoxide support the previous ones obtained in the presence of the above-mentioned K<sup>+</sup> channel openers, bimakalim and NS1619. In rat renal microsomes, 14,15-EEZE did not affect EET synthesis or 20-HETE synthesis (4, 5, 9). A more recent study, in which the 14,15-EEZE selectively blocks the effects of EETs in various species and organs, most importantly in the vasculature and heart (4, 5, 20, 21)

Another possibility that we considered is that 14,15-EEZE is a K<sub>ATP</sub> channel antagonist, since it has been well established that the EETs are mito and sarc K<sub>ATP</sub> channel openers (11, 12, 17, 19). In this regard, the cardioprotective effects of the EETs were blocked by glibenclamide, a nonselective K<sub>ATP</sub> antago-

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**Fig. 5.** 14,15-EET concentrations (pg/ml) in coronary venous plasma at 5 and 30 min of reperfusion (R) following treatment with either the LD of AUDA (0.157 mg/kg) or the HD of AUDA (0.314 mg/kg) compared with predrug control values. *P < 0.01 vs. control.

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**Fig. 6.** Effects of AUDA (HD) and the mitochondrial ATP-sensitive potassium channel opener diazoxide (DZ) on myocardial IS/AAR alone or in the presence of 14,15-EEZE in dogs subjected to 60 min of coronary artery occlusion and 3 h of reperfusion. 14,15-EEZE completely abolished the effect of AUDA, but had no effect on the cardioprotective effect of DZ. *P < 0.01 vs. control.
Ephx2 knockout and may provide more definitive conclusions. EPHX2 is a bifunctional enzyme with both epoxide hydrolase activity and lipid phosphatase activity (1, 14). Targeted deletion of the enzyme eliminates both activities and cannot be ascribed to inhibition of epoxide hydrolysis only. The lipid phosphatase activity may have an unidentified role(s) in cardioprotection. This possibility needs further investigation.

Interestingly, when combining the EPHX2 inhibitor with 14,15-EET, a greater protective effect was observed than that observed with the EET administered alone. This is not surprising, since preventing the metabolism and inactivation of 14,15-EET would effectively increase the concentration of EETs in the heart and produce a greater effect on reducing IS/AAR. Since EPHX2 inhibitors are antihyperensive models of hypertension and protect the kidney from end-organ damage, the present results and those of Seubert et al. (20) demonstrate an efficacious effect to reduce reversible and irreversible cardiac damage, resulting from ischemia and/or reperfusion. It also suggests that EPHX2 inhibitors or the administration of more long-acting epoxygenase products (EET derivatives) or the combination of the two may have potential benefit in patients with coronary artery disease. In this regard, several studies have shown that the risk of coronary atherosclerosis is increased in patients with polymorphisms of the CYP2J2, which further supports the importance and protective potential of this system at several points in the heart and coronary vasculature (23, 24).

REFERENCES


