Epoxideicosatrienoic Acids Function as Selective, Endogenous Antagonists of Native Thromboxane Receptors: Identification of a Novel Mechanism of Vasodilation

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Received August 20, 2008; accepted September 30, 2008

ABSTRACT

Epoxy- and dihydroxy-eicosatrienoic acids (EETs and DHETs) are vasoreactive cytochrome P450 metabolites of arachidonic acid. Interestingly, however, the mechanism(s) by which EETs/DHETs mediate smooth muscle relaxation remains unclear. In contrast to previous reports, where dilation was purportedly large-conductance Ca\(^2\+\)-activated K\(^+\) (BK\(_{\text{ca}}\)) and/or transient receptor potential cation channel, subfamily V, member 4 (TRPV4) channel-mediated, 14,15-EET-induced vasodilation [reversal of contractile tone established with the thromboxane receptor (TP) agonist 15-hydroxy-11,9\(\alpha\)-(epoxymethano)prosta-5,13-dienoic acid (U-46619)] was unaltered in BK\(_{\text{ca}}\) and TRPV4 knockout mouse isolated aortae compared with wild-type conditions. The absence of a vasodilator effect for U-46619 is consistent with a role for EETs/DHETs as competitive TP antagonists. Competitive TP antagonism was also observed in nonvascular tissue, including rat fundus and tertiary bronchus, indicating that the effect is not specific to blood vessels. Such effects were TP-selective because 14,15-EET failed to inhibit “non-TP” prostanoic receptor-mediated function in multiple cell/tissue-based assays (K\(_i\) > 10 \(\mu\)M). In accordance, 14,15-EET inhibited specific [\(^3\)H]7-(3-((2-((phenylamino)carbonyl)hydrazino)methyl)-7-oxabicyclo(2,2,1)hept-2-yl)-5-heptanoic acid (SQ-29548) binding to human recombinant TP receptor, with a K\(_i\) value of 3.2 \(\mu\)M, and it showed weaker affinity for non-TP prostanoic receptors, including DP, FP, EP\(_{1-4}\), and IP receptors (K\(_i\) values of 6.1, 5.3, 42.6, 19.7, 13.2, 20.2, and >25 \(\mu\)M, respectively) and no appreciable affinity (K\(_i\) values >10 \(\mu\)M) for a diverse array of pharmacologically distinct receptors, including the leukotriene receptors Cys-LT\(_{1,2}\) and BLT\(_2\). As such, EETs/DHETs represent a unique class of “endogenous” G protein-coupled receptor competitive antagonists, inducing vasodilation via direct TP inhibition. Thus, EETs/DHETs represent novel autoregulatory agents, directly modulating the actions of cyclooxygenase-derived eicosanoids following arachidonic acid mobilization.

Upon release from cell membranes, arachidonic acid can be converted to a range of eicosanoids by three principal classes of enzymes: cyclooxygenases (COX), lipoxygenases, and cytochrome P450 monoxygenases. The P450 epoxygenases can introduce an epoxide to any of the four double bonds (5,6, 8,9, 11,12, and 14,15) of arachidonic acid, resulting in the generation of four distinct EET regioisomers (Capdevila et al., 1990). Each EET can be further metabolized to dihydroxyeicosatrienoic acids (DHETs) by soluble epoxide hydrolase (sEH), resulting in the generation of four corresponding DHET regioisomers. Since their discovery more than 25 years ago (Capdevila et...
al., 1981), there has been a recent resurgence in EET biology following their classification as "endothelium-derived hyperpolarization factors" in the mid-1990s (Campbell et al., 1996). EETs are primarily recognized for eliciting vasodilatory, anti-inflammatory, and thrombolytic effects and increasing their generation or reducing catabolism (e.g., increased CYP2J2, sEH inhibition) might be of potential utility in treating cardiovascular, pulmonary, and inflammatory diseases (Larsen et al., 2007; Spector and Norris, 2007). Indeed, human CYP2J2, CYP2C8, and sEH polymorphisms have been associated with increased risk for coronary heart disease, myocardial infarction, and stroke (Yasar et al., 2003; Fornage et al., 2005; Lee et al., 2006, 2007; Monti et al., 2008). Notably, both CYP2J2 transgenic mice (Seubert et al., 2004) and sEH null mice (Seubert et al., 2006) exhibit improved cardiac function following ischemia-reperfusion and small molecule sEH inhibitors lower blood pressure and attenuate end-organ damage in several animal models of hypertension (Yu et al., 2000; Zhao et al., 2004; Irim et al., 2005).

Despite increased interest in EET biology, the precise mechanism(s) of action of EET remains ambiguous. One proposed mechanism is that EETs function as endothelium-derived hyperpolarizing factors (EDHFs) (Campbell et al., 1996), modulating ion channel function, including large-conductance Ca\(^{2+}\)-activated K\(^{+}\) (BK\(_{Ca}\)) and transient receptor potential cation channel, subfamily V, member 4 (TRPV4) channels. For example, EET-mediated smooth muscle hyperpolarization and vasodilation is purportedly inhibited by either BK\(_{Ca}\) blockade withiberiotoxin (Zou et al., 1996) or antisense-mediated TRPV4 suppression (Earley et al., 2005).

An emerging mechanism for EET function is through direct binding to a putative plasma membrane "receptor," resulting in secondary activation of signal transduction pathways, and, ultimately, in downstream effects on ion channel function(s) and gene regulation. Membrane-impermeable 14,15-EET analogs retain biological activity (Snyder et al., 2002), and radiolabeled EET derivatives exhibit high-affinity, saturable binding in intact guinea pig monocytes and U937 cells (Wong et al., 2000; Yang et al., 2008). The proposed binding site is likely a G protein-coupled receptor (GPCR) since EET function was G\(_{o}\)-dependent in bovine endothelial and smooth muscle cells (Li and Campbell, 1997; Node et al., 2001). However, a specific molecular or pharmacological entity (i.e., binding site/membrane-bound receptor) that mediates such an effect has yet to be identified.

To further elucidate the mechanism(s) of EET function, the vasodilatory properties of 14,15-EET were investigated in the present study using both BK\(_{Ca}\) and TRPV4 knockout mice. Detailed characterization has led to the observation that EETs function as a unique class of "endoogenous" G protein-coupled receptor antagonists, inducing vasodilation via direct, competitive TP receptor inhibition. This specific interaction was confirmed in multiple species (mouse and rat) and tissues (vascular and nonvascular) and documented in direct radioligand binding studies using human recombinant prostanoid-leukotriene receptors.

**Materials and Methods**

All studies were conducted in Association for Assessment and Accreditation of Laboratory Animal Care-accredited facilities in accordance with institutional guidelines.

**Isolated Tissue Preparation.** After anesthesia with 5% isoflu-rane, adult male Sprague-Dawley rats (400 g; Charles River, Raleigh, NC), BK\(_{Ca}\) knockout mice (25 g; Stanford University, Palo Alto, CA; Meredith et al., 2004), EP\(_{3}\) knockout mice (25 g; University of North Carolina, Chapel Hill, NC) (Fleming et al., 1998), adult female guinea pigs (375 g; Charles River Canada, Montreal, QC, Canada), and TRPV4 knockout mice (25 g; GlaxoSmithKline, Collegeville, PA) (Thorneoe et al., 2008) were euthanized by cervical dislocation followed by exsanguination. Age-matched littersmates were used as wild-type controls for each mouse genotype.

Conduit smooth muscle rings (mouse and rat endothelium-intact thoracic aorta; guinea pig trachea) and nonvascular smooth muscle strips (mouse and rat fundus; rat tertiary bronchus and myome-trium) were suspended in 10-mL tissue baths containing Krebs-Henseleit solution aerated with 95% O\(_2\), 5% CO\(_2\), pH 7.4, at 37°C (Douglas et al., 2005). Endothelium-intact mesenteric resistance arteries (125 μm internal diameter) were mounted on a wire myograph (Danish Myotechnology, Aarhus, Denmark). Isometric force responses were recorded (Chart 5.0; ADInstruments, Colorado Springs, CO) under 0.5 g optimal resting tension unless noted otherwise (1.0 g in rat aorta, fundus, and myometrium and in guinea pig trachea).

After 1-h equilibration, tissues were treated with 60 mM KCl as a calibration standard. Vascular tissues were treated subsequently with 1 μM phenylephrine, and endothelial loss was confirmed using 10 μM carbachol.

**Isolated Tissue Relaxation and Contraction.** After precontraction with EC\(_{50}\) concentrations of contractile agent (1 μM U-46619, 300 nM phenylephrine, 30 nM endothelin-1, 40 nM KCl, and 1 μM carbachol), cumulative concentrations of vehicle or test relaxing reagents were added. When studying IP- and EP\(_{3}\)-mediated function, rat aorta and guinea pig trachea were precontracted with phenylephrine and carbachol, respectively. Before precontraction, selected tissues were treated for 30 min with 100 nM ibeteroxin or 10 μM indomethacin. For contractile studies, after pretreatment (30 min) with vehicle or test reagent, cumulative concentration-response curves to contractile agents were constructed.

**Platelet Aggregation.** Blood was collected from healthy volunteers through an established employee donor program within GlaxoSmithKline. Consent was obtained from each donor, and donors were de-identified. Human washed platelets were prepared as described previously (Wilson et al., 2006). Experiments were performed in the presence of the selective TP receptor antagonist SQ-29548 (1 μM) and indomethacin (1 μM). To evaluate DP agonism, platelets were incubated with 14,15-EET (0.1–10 μM) or the DP agonist BW245C (0.1 nM–10 μM) before the addition of 100 μM ADP. To evaluate DP antagonism, washed platelets were preincubated with 10 μM 14,15-EET or 11 μM of the DP antagonist BWA868C and then incubated with 100 μM BW245C before stimulation with ADP. All incubations were 15 min.

**Competition Binding.** Competition binding assays were performed using membranes prepared from Chinese hamster ovary (CysLT\(_1\), EP\(_{1/2/3}\), and TP), human embryonic kidney 293 (CysLT\(_2\), EP\(_{2}\), and FP), or U2OS (BLT\(_1\) and DP\(_{1}\)) cells expressing human recombinant receptor or human platelets (native IP) as described previously (Abramovitz et al., 2000). After 1-h incubation at room temperature, competition reactions were terminated and washed, and radioactivity was measured via scintillation counting.

**In Vitro Selectivity Profile.** The selectivity of 14,15-EET and 14,15-DHET (10 μM) binding at human TP was assessed further by examining binding interactions with a diverse range of 50 distinct "nonprostanoid" GPCRs, ion channels, and transporters (Cerep, Paris, France): adenosine (A\(_{1a}\)) and adrenergic (\(\alpha_{1a}\) and \(\beta_1\)), angio-tensin-II (AT\(_1\)), benzodiazepine (BZD; central), bradykinin (B\(_1\)), cholecystokinin (CCK\(_{A}\)), dopamine (D\(_{1a}\)), endothelin-1 (ET\(_{A}\)), GABA (nonselective), galanin (GAL\(_2\)), interleukin-8 (CXCR2), histamine (H\(_{1}\)), melancortin (MC\(_{3}\)), melatonin (MT\(_1\)), muscarinic (M\(_{1/2/4}\)), neurokinin (NK\(_{2}\)), neuropeptide Y (Y\(_{1}\)), neurotensin (NT\(_1\)), opioid
Results

EET-Induced Vasodilation Is Unaltered in BKCa and TRPV4 Knockout Mice. Relative to wild-type littermate controls, the vasodilatory effects of 14,15-EET were not significantly altered in aortae isolated from BKCa knockout mice (IC50 values of 5.4 ± 1.8 and 4.0 ± 0.0 μM; Emax values of 94 ± 16 and 106 ± 6% reversal U-46619 contractile tone, respectively; n = 3–4; Fig. 1). Likewise, compared with wild-type mice, responses in mesenteric resistance arteries were not significantly altered in BKCa knockout mice (IC50 values of 0.9 ± 0.3 and 2.4 ± 0.7 μM; Emax values 79 ± 8 and 68 ± 9% reversal U-46619 contractile tone, respectively; n = 5–6; Fig. 1). Relative to wild-type littermates, deletion of the TRPV4 channel also failed to alter the vasodilatory effects of 14,15-EET in isolated aortae (IC50 values of 1.3 ± 0.3 and 0.9 ± 0.1 μM; Emax values 92 ± 3 and 92 ± 1% reversal U-46619 contractile tone, respectively; n = 3–4; Fig. 1). Pharmacological BKCa channel inhibition with 100 nM iberiotoxin did not alter the vasodilatory effects of 14,15-EET in either wild-type (IC50 value of 1.6 ± 0.5 μM; Emax of 82 ± 3% reversal U-46619 contractile tone; n = 3–4; Fig. 1) or TRPV4 knockout (IC50 value of 2.0 ± 0.3 μM; Emax of 76 ± 3% reversal U-46619 contractile tone; n = 3–4; Fig. 1) mouse aortae.

EETs and DHETs Selectively Reverse U-46619 Contractile Tone. All 5,6-, 8,9-, 11,12-, and 14,15-EET and DHET regioisomers elicited a vasodilatory response when tissues were preconstricted with U-46619. Responses to EETs and DHETs were similar within arteries, with the exception of the 5,6- and 14,15-regiosomers in the mouse mesenteric artery in which the DHET isomers were 64% less efficacious and 2-fold less potent, respectively (Table 1; n = 3–7; Fig. 2). In contrast to U-46619, 14,15-EET (≤10 μM, used as representative ligand) failed to reverse contractile tone pre-established with phenylephrine, endothelin-1, or KCl (n = 3–6; Fig. 2).

The majority of EET and DHET regioisomers failed to reverse contraction established with phenylephrine (n = 4–7; Fig. 2). Indeed, only 5,6-EET reversed tone in aortae (63 ± 4% at 10 μM) and only 5,6-EET, 5,6-DHET, and 11,12-DHET reversed tone in mesenteric resistance artery (59 ± 22, 58 ± 10, and 50 ± 13% at 10 μM, respectively). Reversal of phenylephrine tone (but not U-46619 tone) was abolished by pretreatment with 10 μM indomethacin.

Discussion

The exact mechanism(s) by which EETs and DHETs function remains unclear, despite their discovery more than 25 years ago (Capdevila et al., 1981). The aim of the present study was to elucidate the mechanism of EET vasodilation using both genetic manipulation and pharmacological tools. Since one of the most recognized hypotheses for EET function is through either direct or indirect interactions with ion channels, namely, BKCa (Archer et al., 2003; Larsen et al., 2006) and TRPV4 (Earley et al., 2005; Vriens et al., 2005), the vasodilatory properties of 14,15-EET were assessed in arteries from mice lacking either gene. Surprisingly, the vasodilatory effects of 14,15-EET were refractory to genetic deletion...
of either BK<sub>Ca</sub> or TRPV4. Likewise, pharmacological inhibition of the BK<sub>Ca</sub> channel with iberiotoxin did not alter the vasodilatory effects of 14,15-EET in mouse aortae. As such, EETs are capable of functioning as efficacious vasodilators independently of BK<sub>Ca</sub> and/or TRPV4 channel activation.

Although such observations do not support a predominant role for BK<sub>Ca</sub> and/or TRPV4 channels in mediating the vasodilatory effects of EETs in mouse aortae and mesenteric artery, it does not exclude contributions from these channels in other tissues (both vascular and nonvascular) from this and other species. Indeed, such results are in contrast to those generated previously in human arteries where EET-induced vasodilation was iberiotoxin-sensitive (Archer et al., 2003; Larsen et al., 2006). However, an iberiotoxin-resistant

![Vasodilatory effects of 14,15-EET in BK<sub>Ca</sub> knockout and TRPV4 knockout mouse isolated arteries.](image-url)
component was evident in these studies, too. In addition, KCa
blockade with tetraethylammonium and charybdotoxin and
membrane depolarization with high K+ both attenuated EET
dilation in coronary and renal arteries (Campbell et al., 1996;
Zou et al., 1996; Oltman et al., 1998). As such, EETs might
function as EDHFs via interactions with other ion channels
in addition to BKCa and TRPV4 (consistent with EDHF re-

<table>
<thead>
<tr>
<th>TABLE 1</th>
<th>Vasodilatory effects of EETs and DHETs in isolated arteries</th>
</tr>
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<tbody>
<tr>
<td>EET/DHET Regioisomer</td>
<td>IC50 EET (µM)</td>
</tr>
<tr>
<td>Rat aorta</td>
<td></td>
</tr>
<tr>
<td>5,6-</td>
<td>—</td>
</tr>
<tr>
<td>8,9-</td>
<td>—</td>
</tr>
<tr>
<td>11,12-</td>
<td>—</td>
</tr>
<tr>
<td>14,15-</td>
<td>—</td>
</tr>
<tr>
<td>Mouse mesenteric resistance artery</td>
<td></td>
</tr>
<tr>
<td>5,6-</td>
<td>1.7 ± 0.5</td>
</tr>
<tr>
<td>8,9-</td>
<td>0.6 ± 0.1</td>
</tr>
<tr>
<td>11,12-</td>
<td>0.6 ± 0.2</td>
</tr>
<tr>
<td>14,15-</td>
<td>0.8 ± 0.1</td>
</tr>
</tbody>
</table>

*P < 0.05 and **P < 0.01. Statistical comparisons of EETs to their corresponding DHETs were made using a two-tailed t-test.
—: full concentration-response curves could not be generated due to low compound potency (i.e., incomplete vasodilation at 10 µM, the highest concentration of compound tested); thus, IC50 values could not be defined.

Fig. 2. Spasmogen-dependent vasodilatory effects of 14,15-EET and 14,15-DHET in rat and mouse isolated conduit and resistance arteries. 14,15-EET reverses tone established in rat isolated aortae, with EC50 concentrations of U-46619 (A) but not phenylephrine (B), endothelin-1 (C), or KCl (D). Likewise, in mouse isolated mesenteric resistance arteries, both 14,15-EET (E) and 14,15-DHET (F) reverse tone established with U-46619 but not phenylephrine. Statistical comparisons versus vehicle treatment were made using a paired, two-tailed t test, and differences are considered significant at P < 0.05.
The ability of the four regioisomers of EET and their corresponding DHETs to reverse contraction elicited by either U-46619 (TP receptor agonist) or phenylephrine (α-adrenoceptor agonist) was assessed in rat isolated aorta and mouse mesenteric resistance artery. The majority of the vasodilatory responses to EETs and DHETs were similar within arteries. Surprisingly, whereas every EET and DHET regioisomer elicited a relaxant response when tissues were preconstricted with U-46619, the preponderance of EET and DHET isoforms failed to reverse contraction established with phenylephrine.

### Table 2: Selective inhibitory effects of 14,15-EET on TP receptor-mediated function

<table>
<thead>
<tr>
<th>Receptor</th>
<th>Assay Preparation</th>
<th>Response Measured</th>
<th>Agonist</th>
<th>14,15-EET (10 μM)</th>
<th>( K_D ) (nM)</th>
<th>Positive Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>TP</td>
<td>Rat Aorta</td>
<td>Constriction</td>
<td>U-46619</td>
<td>( 1.3 \pm 0.6 )</td>
<td>( 2.1 \pm 0.2 )</td>
<td>Positive Control</td>
</tr>
<tr>
<td>TP</td>
<td>Rat 3° bronchus</td>
<td>Constriction</td>
<td>U-46619</td>
<td>( 2.0 \pm 0.7 )</td>
<td>( 2.6 \pm 1.2 )</td>
<td>Positive Control</td>
</tr>
<tr>
<td>TP</td>
<td>Rat Fundus</td>
<td>Constriction</td>
<td>Fluprostanol</td>
<td>&gt;10</td>
<td>( 1.8 \pm 0.6 )</td>
<td>Positive Control</td>
</tr>
<tr>
<td>IP</td>
<td>Rat Aorta</td>
<td>Dilation</td>
<td>Iloprost</td>
<td>&gt;10</td>
<td>&lt;0.1</td>
<td>Positive Control</td>
</tr>
<tr>
<td>EP₁</td>
<td>Mouse Fundus</td>
<td>Contraction</td>
<td>17-PGf₁</td>
<td>&gt;10</td>
<td>( 0.7 \pm 0.3 )</td>
<td>Positive Control</td>
</tr>
<tr>
<td>EP₂</td>
<td>Guinea pig Trachea</td>
<td>Dilation</td>
<td>Butaprost</td>
<td>&gt;10</td>
<td>( 13.5 \pm 3.7 )</td>
<td>Positive Control</td>
</tr>
<tr>
<td>EP₃</td>
<td>Mouse Fundus</td>
<td>Contraction</td>
<td>Sulprostone</td>
<td>&gt;10</td>
<td>(EP₃ KO mouse)</td>
<td>Positive Control</td>
</tr>
<tr>
<td>DP₁</td>
<td>Human Platelets</td>
<td>Aggregation</td>
<td>BW245C</td>
<td>&gt;10</td>
<td>(64.2%(^{e})</td>
<td>Positive Control</td>
</tr>
</tbody>
</table>

*Positive controls were as follows: a SQ-29548 (0.1 μM); b AL-8810 (10 μM); c CAY10441 (10 μM); d SC-51322 (3 μM); e AH6809 (30 μM); f Lack of sulprostone-mediated activity in EP₃ KO mouse fundus served as the positive control for selective EP₃ function; and g BWA868C (11 μM) attenuated the inhibitory effects of 1 nM BW245C (EC₅₀) on ADP-induced platelet activation by 64.2%.

Fig. 3. 14,15-EET is a competitive, selective TP antagonist in isolated vascular and respiratory smooth muscle. Pretreatment of rat isolated aortae (A) with either 14,15-EET (10 μM) or the tool TP receptor antagonist SQ-29548 (0.1 μM) caused significant rightward shifts to the U-46619 concentration-response curve (\( P < 0.001 \)) without altering maximal contraction (\( P > 0.05 \)), whereas 14,15-EET fails (\( P > 0.05 \)) to alter phenylephrine contraction (B). Likewise, in rat isolated 3° bronchi (C), both 14,15-EET and SQ-29548 caused significant rightward shifts to the U-46619 concentration-response curve (\( P < 0.05 \)) without altering maximal contraction (\( P > 0.05 \)), but carbachol-induced contraction is not altered (\( P > 0.05 \)) by 14,15-EET (D). Statistical comparisons of EC₅₀ and Eₘ₅ₒ responses were made using a paired, two-tailed t test or ANOVA (Dunnett’s post test analysis).

The ability of the four regioisomers of EET and their corresponding DHETs to reverse contraction elicited by either U-46619 (TP receptor agonist) or phenylephrine (α-adrenoceptor agonist) was assessed in rat isolated aorta and mouse mesenteric resistance artery. The majority of the vasodilatory responses to EETs and DHETs were similar within arteries. Surprisingly, whereas every EET and DHET regioisomer elicited a relaxant response when tissues were preconstricted with U-46619, the preponderance of EET and DHET isoforms failed to reverse contraction established with phenylephrine.

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\( P \) values indicate statistical significance.

\( K_D \) values indicate the dissociation constant for the agonist-receptor interaction.

\( \alpha \)-adrenoceptor refers to the α-adrenoceptor.

\( \text{EC}_{50} \) refers to the concentration of the agonist required to elicit a response halfway between the baseline and maximum response.

\( \text{E}_{\text{max}} \) refers to the maximum response achievable with the agonist.

\( \text{TP} \) refers to the thromboxane receptor.

\( \text{EP} \) refers to the epoxyprostanate receptor.

\( \text{FP} \) refers to the florphenicol receptor.

\( \text{DP} \) refers to the dipyrone receptor.

\( \text{ADP} \) refers to adenosine diphosphate.

\( \text{BW245C} \) refers to butaprost.

\( \text{SOQ} \) refers to sulprostone.

\( \text{SC} \) refers to stilboestrol.

\( \text{CAY} \) refers to CAY10441.

\( \text{AL} \) refers to AL-8810.

\( \text{SQ} \) refers to SQ-29548.
phenylephrine under identical conditions [any reversal of phenylephrine but not U-46619 tone was abolished by pretreatment with indomethacin, consistent with the observations that these EET and DHET analogs can serve as substrates for COX (Carroll et al., 1993) or modulate its function (Kozak et al., 2003)]. Consistent with the inability to reverse phenylephrine contractile tone, 14,15-EET also failed to reverse contractile tone

Table 3

<table>
<thead>
<tr>
<th>Receptor</th>
<th>Radiolabeled Ligand</th>
<th>Competing Ligand (K&lt;sub&gt;i&lt;/sub&gt;)</th>
<th>14,15-EET</th>
<th>14,15-DHET</th>
<th>Positive Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Prostanoid</td>
<td></td>
<td></td>
<td>µM</td>
<td>nM</td>
<td></td>
</tr>
<tr>
<td>TP</td>
<td>[H]SQ-29548</td>
<td></td>
<td>3.3 ± 0.3</td>
<td>10.5 ± 1.6††</td>
<td>4.5 ± 1.5*</td>
</tr>
<tr>
<td>FP</td>
<td>[H]PGF&lt;sub&gt;2α&lt;/sub&gt;</td>
<td></td>
<td>5.3 ± 1.0</td>
<td>—</td>
<td>0.7 ± 0.1*</td>
</tr>
<tr>
<td>IP</td>
<td>[H]Iloprost</td>
<td>≥24.7 ± 5.3*</td>
<td>—</td>
<td>&gt;50&lt;sup&gt;‡‡&lt;/sup&gt;</td>
<td>10.0 ± 2.0*</td>
</tr>
<tr>
<td>EP&lt;sub&gt;1&lt;/sub&gt;</td>
<td>[H]PGE&lt;sub&gt;2α&lt;/sub&gt;</td>
<td>39.7 ± 2.9&lt;sup&gt;‡‡&lt;/sup&gt;</td>
<td>—</td>
<td>&gt;50&lt;sup&gt;‡&lt;/sup&gt;</td>
<td>64.7 ± 48.0&lt;sup&gt;‡&lt;/sup&gt;</td>
</tr>
<tr>
<td>EP&lt;sub&gt;2&lt;/sub&gt;</td>
<td>[H]PGE&lt;sub&gt;2α&lt;/sub&gt;</td>
<td>18.4 ± 2.2&lt;sup&gt;‡&lt;/sup&gt;</td>
<td>&gt;50&lt;sup&gt;‡&lt;/sup&gt;</td>
<td>&gt;50&lt;sup&gt;‡&lt;/sup&gt;</td>
<td>163.5 ± 41.5&lt;sup&gt;‡‡&lt;/sup&gt;</td>
</tr>
<tr>
<td>EP&lt;sub&gt;3&lt;/sub&gt;</td>
<td>[H]PGE&lt;sub&gt;2α&lt;/sub&gt;</td>
<td>11.1 ± 2.2&lt;sup&gt;‡‡&lt;/sup&gt;</td>
<td>≥41.4 ± 6.2&lt;sup&gt;‡‡&lt;/sup&gt;</td>
<td>0.5 ± 0.2&lt;sup&gt;‡&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>EP&lt;sub&gt;4&lt;/sub&gt;</td>
<td>[H]PGE&lt;sub&gt;2α&lt;/sub&gt;</td>
<td>17.3 ± 4.4&lt;sup&gt;‡&lt;/sup&gt;</td>
<td>&gt;50&lt;sup&gt;‡‡&lt;/sup&gt;</td>
<td>54.8 ± 15.2&lt;sup&gt;‡&lt;/sup&gt;</td>
<td></td>
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<tr>
<td>DP</td>
<td>[H]PGD&lt;sub&gt;2&lt;/sub&gt;</td>
<td>6.1 ± 0.3</td>
<td>26.0 ± 6.0&lt;sup&gt;‡&lt;/sup&gt;</td>
<td>3.8 ± 0.6&lt;sup&gt;‡&lt;/sup&gt;</td>
<td></td>
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<tr>
<td>Leukotriene</td>
<td></td>
<td></td>
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<tr>
<td>BLT&lt;sub&gt;1&lt;/sub&gt;</td>
<td>[H]LTB&lt;sub&gt;4&lt;/sub&gt;</td>
<td>≥30&lt;sup&gt;‡‡&lt;/sup&gt;</td>
<td>&gt;30&lt;sup&gt;‡‡&lt;/sup&gt;</td>
<td>1.0 ± 0.2&lt;sup&gt;‡&lt;/sup&gt;</td>
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<td>CysLT&lt;sub&gt;1&lt;/sub&gt;</td>
<td>[H]LTD&lt;sub&gt;4&lt;/sub&gt;</td>
<td>≥30&lt;sup&gt;‡‡&lt;/sup&gt;</td>
<td>—</td>
<td>1.2 ± 0.1&lt;sup&gt;‡&lt;/sup&gt;</td>
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<tr>
<td>CysLT&lt;sub&gt;2&lt;/sub&gt;</td>
<td>[H]LTD&lt;sub&gt;4&lt;/sub&gt;</td>
<td>≥30&lt;sup&gt;‡‡&lt;/sup&gt;</td>
<td>—</td>
<td>17.0 ± 3.8&lt;sup&gt;‡&lt;/sup&gt;</td>
<td></td>
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</table>

*<i>P</i> < 0.05 and †<i>P</i> < 0.01 versus TP and ‡<i>P</i> < 0.01 versus 14,15-EET. Statistical comparisons were made using a two-tailed t-test or ANOVA analysis.

—, <i>K</i><sub>i</sub> values were not assessed.

Positive controls were as follows: a SQ-29548; b PGF<sub>2α</sub>; c, d iloprost; e butaprost; f sulprostone; g misoprostone; h BWA868C; i CP-105696; j LTD<sub>4</sub>; and k LTC<sub>4</sub>.

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*Fig. 4. Effects of 14,15-EET on prostanoid receptor-mediated isolated tissue function. Pretreatment with 14,15-EET (30 min; 10 µM) failed to alter IP-mediated vasodilation of rat aorta (A), FP-mediated contraction or rat myometrium (B), EP<sub>2</sub>-mediated dilation of guinea pig trachea (C), and EP<sub>1</sub>-mediated contraction of mouse fundus (D). In contrast, antagonist positive controls significantly inhibited prostanoid receptor-mediated function in each assay (10 µM CAY10441 (A); 10 µM AL 8810 (B); 30 µM AH6809 (C); and 3 µM SC-51322 (D); <i>P</i> < 0.05). Statistical comparisons of EC<sub>50</sub>, IC<sub>50</sub>, and E<sub>max</sub> responses were made using ANOVA (Dunnett’s post test analysis).---
pre-established with endothelin-1 and KCl (in contrast to sodium nitroprusside; data not shown). As such, the TP receptor agonist U-46619 was the only vasoconstrictor consistently susceptible to EET/DHET dilation. Such results are in accord with a study by Weintraub et al. (1997) in which 11,12-EET and 14,15-EET reversed U-46619 contraction of endothelium-intact porcine coronary artery but failed to relax tissues contracted with KCl, acetylcholine or phorbol 12,13-dibutyrate.

Because U-46619 typically maintains contractile tone more effectively than most other vasoconstrictors, it is typically used as the contractile spasmogen when studying vasodilator agonists. As such, the phenomenon observed in the current study (i.e., reversal of U-46619 but not phenylephrine, endothelin-1 or KCl contraction) might have been overlooked previously. Indeed, U-46619 has been used as the spasmogen in the majority of isolated tissue studies investigating EET or DHET dilation. Although there are a few examples of EET-induced reversal of a non-TP receptor spasmogen (e.g., endothelin-1 or phenylephrine) (Zou et al., 1996; Oltman et al., 1998; Larsen et al., 2006), such examples might be a result of downstream effects on COX rather than a direct receptor/ion channel interaction. For example, 5,6-EET and 8,9-EET are substrates for COX (forming vasoactive prostaglandin analogs) (Carroll et al., 1993; Homma et al., 1993), and 11,12-EET has been shown to inhibit COX-2 in a time- and concentration-dependent manner (Kozak et al., 2003).

The reversal of U-46619 but not KCl tone is consistent with the hypothesis that EETs function involves activation of a _K_2a channel(s). In contrast, however, the absence of any EET vasodilatory effect when tissues were precontracted with either endothelin-1 or phenylephrine is not. Alternatively, since U-46619, endothelin-1, and phenylephrine all modulate vasoconstriction through a common signal transduction pathway (G_q/phospholipase C/inositol 1,4,5-triphosphate/Ca^{2+} activation via distinct GPCRs), we hypothesized that the EETs and DHETs functioned as direct TP receptor antagonists. To test this hypothesis, the ability of 14,15-EET to inhibit U-46619 contraction was assessed using rat isolated aorta. Consistent with competitive antagonism, pretreatment with 14,15-EET resulted in concentration-dependent, rightward, parallel shifts in the U-46619 concentration-response curves, with a _pA_2 value of 5.89 (consistent with its direct vasodilatory potency observed in this tissue). This action is not limited to the vasculature and, as predicted, is a general, smooth muscle phenomenon observed across species and tissue types. Pretreatment with 14,15-EET (10 μM) caused a significant rightward shift to the U-46619 concentration-response curves in both rat isolated 3rd bronchus and stomach fundus. Both the vascular (aorta) and nonvascular (bronchus and fundus) effects were TP-selective because 14,15-EET (10 μM) did not affect tissue contraction elicited by phenylephrine, carbachol, or serotonin, respectively.

Although 14,15-EET was functioning as a competitive TP receptor antagonist, many prostanoid receptor ligands exhibit appreciable affinity for multiple prostanoid receptors (Abramovitz et al., 2000). As such, the functional effects of 14,15-EET on non-TP prostanoid receptor function were also assessed using an array of isolated tissue/cell biochemical assays. In contrast to the inhibitory effects at the TP receptor, 14,15-EET (∼10 μM) failed to demonstrate any agonist or antagonist effects on FP, IP, EP_{1/2/3}, and DP_{1} receptor-mediated function. As such, 14,15-EET seems to function as a selective and competitive TP receptor antagonist in multiple species (mouse and rat) and tissues (conduit and resistance arteries, 3rd bronchi, and fundus).

To further validate the functional data, the ability of 14,15-EET and 14,15-DHET to compete for binding at prostanooid and nonprostanoid receptors was assessed. In accord with the functional data, 14,15-EET and 14,15-DHET binding was ∼2 to >12-fold selective for the TP receptor over all other prostanooid receptors. The TP receptor binding affinity (_K_ value of 3.3 μM) of 14,15-EET is not inconsistent with those determined previously in which radiolabeled EET agonists [^3H]14,15-EET and 20,125I-14,15-epoxyeicosanoic-8(Z)-enoic acid competed for binding with 14,15-EET at guinea pig monocytes and U937 cell membranes, with _K_ values of 226 and 40 nM, respectively (Wong et al., 2000; Yang et al., 2008). In addition, the TP receptor binding affinity observed in the present study is also consistent with the notion that the EET binding site is likely a GPCR.

Interestingly, however, 14,15-EET binding affinity for both the FP and DP receptors was relatively potent (_K_ values of 5.3–6.1 μM; only ∼2-fold less than the TP _K_ value), but the ligand showed no functional activity (either as an agonist or antagonist) at either the FP or DP receptor. Such results might be due to the use of native (functional assays) versus recombinant (binding assays) receptor. In addition, species variability might also be a factor comparing the FP assays since the functional and binding assays used rat and human receptors, respectively. In contrast to the prostanoid receptors, neither 14,15-EET or DHET showed any appreciable affinity for leukotriene receptors (CysLT_{1A} and BLT_{1}; _K_ values >30 μM) or for 50 distinct nonprostanoid GPCRs, ion channels, and transporters (<30% inhibition at 10 μM). Such results at the adrenergic (α_1), endothelin-1 (ET_{A}), muscarinic (M_3), and serotonin (5-HT_{2A}) receptors are consistent with functional data where 14,15-EET failed to alter vascular or nonvascular tissue contraction elicited by phenylephrine, endothelin-1, carbachol, or serotonin.

Although not unprecedented, the identification of endogenous factors that function as GPCR antagonists is extremely rare. Indeed, few examples exist in the literature, including 5-HT moduline (an endogenous allosteric antagonist of serotonin receptors; Massot et al., 1996) and agouti protein (an endogenous inverse agonist of melanocortin receptors; Ollmann et al., 1997).

The ability of EETs to function as selective TP antagonists could explain their role as anti-inflammatory agents. In this setting, EETs may function to “fine tune” the prostanoid pathway-mediated inflammatory process, inhibiting nuclear factor-κB-mediated expression of proinflammatory adhesion molecules (vascular cell adhesion molecule-1, intercellular adhesion molecule-1, and E-selectin) and leukocyte adhesion following arachidonic acid mobilization (Node et al., 1999). Indeed, increasing EET levels through in vivo sEH inhibition has been demonstrated to attenuate lipopolysaccharide-induced thermal hyperalgesia and allodynia in a rodent model of inflammatory pain (Inceoglu et al., 2006).

In summary, EETs/DHETs may function as novel endogenous G protein-coupled receptor competitive antagonists, inducing vaso/broncho dilation, and, potentially, additional anti-inflammatory actions via direct TP inhibition. Such responses are preferential for the TP receptor, since, using an array of isolated tissue/cell assays, 14,
15-EEET failed to alter FP-, IP-, EP1/2/3-, and DP-mediated function and an array of other nonprostanoid receptors, ion channels, and transporters. Although such observations do not rule out the possibility for additional complimentary mechanisms (including direct or indirect ion channel activation) in other tissues/species, the current findings might provide mechanistic insight into why elevating EET concentrations constitutes a novel strategy for treatment of disease processes characterized by a significant inflammatory component.

Acknowledgments

We thank Mark Pullen for excellent technical assistance and Terry Kenakin and Daniel Krosky for insightful comments during the preparation of this manuscript.

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EETs are produced by the cytochrome P450 epoxygenase, which catalyzes the oxygenation of polyunsaturated fatty acids at the C14-C15 double bond. This reaction generates two major metabolites: 14,15-EET and 11,12-EET. These compounds have been shown to modulate a variety of cellular responses, including vasodilation, antithrombotic effects, and anti-inflammatory actions. The mechanisms by which EETs modulate these responses are complex and involve interactions with multiple intracellular signaling pathways.


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