20-\(^{125}\)Iodo-14,15-Epoxideicosanoic Acid: a High-Affinity Radioligand Used to Characterize the Epoxideicosatrienoid Acid Antagonist Binding Site

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ABSTRACT

Epoxideicosatrienoid acids (EETs) are cytochrome P450 epoxide metabolites of arachidonic acid. They relax vascular smooth muscle by membrane hyperpolarization. These actions are inhibited by the EET antagonist, 14,15-epoxideicosanoid-5(Z)-enoic acid (14,15-EE5ZE). We synthesized 20-\(^{125}\)Iodo-14,15-EE5ZE (20-\(^{125}\)I-14,15-EE5ZE), a radiolabeled EET antagonist, and characterized its binding to cell membranes. 14,15-EET (10 \(^{-8}\)-10 \(^{-5}\)M) caused a concentration-related relaxation of the preconstricted bovine coronary artery and phosphorylation of p38 in U937 cells. 20-\(^{125}\)I-14,15-EE5ZE binding to U937 cells reached equilibrium within 5 min and remained unchanged for 30 min. The binding was saturable and reversible, and it exhibited \(K_d\) and \(B_{\text{max}}\) values of 1.11 \pm 0.13 nM and 1.13 \pm 0.04 pmol/mg protein, respectively. Guanosine 5\(^\prime\)-O-(3-thio)triphosphate (10 \(\mu\)M) did not change the binding, indicating antagonist binding of the ligand. Various EETs and EET analogs (10 \(^{-10}\)-10 \(^{-5}\)M) competed for 20-\(^{125}\)I-14,15-EE5ZE binding with an order of potency of 11,12-EET = 14,15-EET > 8,9-EET = 14,15-EE5ZE > 15-hydroxyeicosatetraenoic acid = 14,15-dihydroxyeicosatrienoid acid. 8,9-Dihydroxyeicosatrienoid acid and 11-hydroxyeicosatetraenoic acid did not compete for binding. The soluble and microsomal epoxide hydrodase inhibitors (1-cyclohexyl-3-dodecyl-urea, elaidamide, and 12-hydroxy-elaidamide) and cytochrome P450 inhibitors (sulfaphenazole and probadifen) did not compete for the binding. However, two cytochrome P450 inhibitors, N-methylsulfonfyl-6-(2-propargyloxyphenyl)hexanalamide (MS-PPOH) and miconazole competed for binding with \(K_i\) of 1558 and 315 nM, respectively. Miconazole and MS-PPOH, but not probadifen, inhibited 14,15-EET-induced relaxations. These findings define an EET antagonist’s binding site and support the presence of an EET receptor. The inhibition of binding by some cytochrome P450 inhibitors suggests an alternative mechanism of action for these drugs and could lead to new drug candidates that target the EET binding sites.

ABBREVIATIONS: EET, epoxideicosatrienoid acid; 14,15-EE5ZE, 14(S),15(R)-cis-epoxy-eicosa-8(Z)-enoic acid; 14,15-EE5ZE, 14(S),15(R)-cis-epoxy-eicosa-5(Z)-enoic acid; EH, epoxide hydrodase; 20-I-14,15-EE5ZE, 20-iodo-14,15-epoxideicosanoid-8(Z)-enoic acid; OTs, 20-tosyl-14,15-EE5ZE, 14,15-Epoxyeicosa-15,16-dihydroxy-11-ethyl-10,11-epoxyeicosatetraenoic acid; 15-HETE, 15-hydroxyeicosatetraenoic acid; 14,15-DHET, 14,15-dihydroxyeicosatrienoid acid; 14,15-DHESZE, 14,15-dihydroxy-eicosa-5(Z)-enoic acid; 8,9-DHET, 8,9-dihydroxyeicosatrienoid acid; 11-HETE, 11-hydroxyeicosatetraenoic acid; MS-PPOH, N-methylsulfonfyl-6-(2-propargyloxyphenyl)hexanalamide; GTP\(\gamma\)S, guanosine 5\(^\prime\)-O-(3-thio)triphosphate; MAP, mitogen-activated protein; PPAR, peroxisomal proliferator-activated receptor; U46619, 9-11-dideoxy-11α,9α-epoxymethano-prostaglandin F\(_{2\alpha}\); NS1619, 1,3-dihydro-1,2-hydroxy-5-(trifluoromethyl)phenyl)-5-(trifluoromethyl)-2H-benzimidazol-2-one; BF\(_{Ca}\), large-conductance Ca\(^{2+}\)-activated K\(^+\) channel; K\(_{ATP}\), ATP-sensitive K\(^+\) channel.
tributed, but not limited, to several signal transduction pathways including G protein-coupled to large-conductance, calcium-activated potassium (BK_{Ca}) channels (Li and Campbell, 1997), nuclear factor κB (Nodé et al., 1999), epidermal growth factor receptor-Src-kinase (Chen et al., 1999), mitogen-activated protein (MAP) kinases (Fleming et al., 2001), and phosphatidylinositol 3-kinase (Chen et al., 2001).

Although many downstream molecules and pathways have been identified, the initiation step in EET signaling pathways is still not clear. Low-affinity EET-binding proteins have been proposed to mediate EET action. These binding proteins include fatty acid-binding protein (Widstrom et al., 2001), peroxisomal proliferator-activated receptor (PPAR)-α (Cowart et al., 2002), PPAR-γ (Liu et al., 2005), and ATP-sensitive K channels (Lu et al., 2006). Although EETs may exert some actions through some of these proteins, the micromolar affinity of EETs for these proteins cannot explain physiological responses that occur with nanomolar concentrations of EETs. High-affinity EET binding proteins or receptors still require identification.

Several lines of evidence suggest that EETs act through a specific binding site. Falk et al. (2003a) tested a series of 14,15-EET analogs for their ability to relax the bovine coronary artery. 14(S),15(R)-cis-Epoxyeicosanoic acid was the simplest structure with full agonist activity. The requirement for a specific stereoisomer of the epoxide suggested a specific binding site for the EET. On vascular smooth muscle, 14,15-EET that was tethered to silica beads could not enter the cell but inhibited arachidate activity to a similar extent as 14,15-EET (Snyder et al., 2002). Thus, 14,15-EET acted on the cell surface and not intracellularly. A high-affinity EET binding site was described in intact cells and membrane preparations from guinea pig monocellular cells and human U937 cells. By use of [3H]14,15-EET as a radioligand, specific and saturable binding with a K_D of 5.7 nM was determined in guinea pig monocytes and a K_D of 13.84 nM in U937 cells (Wong et al., 1993, 1997, 2000). This binding site was further defined in the cell membranes by Yang et al. (2008) by use of 20-125I-14,15-epoxyeicosanoic-8Z-enolic acid (20,125I-14,15-EE5ZE). 20-125I-14,15-EE5ZE bound U937 membranes in a specific, saturable, and reversible manner with a K_D of 11.8 nM. EET analogs, but not prostaglandins or lipoxygenase metabolites, displaced the 14,15-EET radioligands from their binding site. This binding site was down-regulated by cAMP-protein kinase A pathway activation and GTPγS suggesting a possible G protein-coupled receptor (GPCR) (Wong et al., 1997; Yang et al., 2008). These studies suggested that EETs act via a cell surface receptor. U937 cells are good model systems for studying a high-affinity EET binding site/receptor(s).

Radiolabeled ligands have been key tools for receptor identification, signal transduction pathway investigation, drug discovery, and mapping amino acid residues in ligand binding sites. [3H]-labeled ligands, in general, have low specific activity and are expensive to synthesize (Wong et al., 1993, 1997, 2000). [125I]-labeled EET agonist ligands have been synthesized (Yang et al., 2007, 2008), but antagonist radio-ligands are traditionally favored in drug screening. Furthermore, antagonists are proposed to occupy a different, but overlapping, binding pocket than agonists. An antagonist EET radioligand may be used to map an antagonist’s binding pocket of the EET-binding protein(s). Here, we have modified the structure of the first EET antagonist, 14,15-epoxyeicosanoic acid (14,15-EE5ZE), synthesized, and characterized the first EET antagonist radioligand, 20-125I-14,15-EE5ZE (Gauthier et al., 2002).

Materials and Methods

Synthesis of 20-125I-14,15-EE5ZE. 20-125I-14,15-EE5ZE is synthesized from the corresponding 20-tosyl (OTs)-14,15-EE5ZE as reported previously (Prestwich et al., 1988; Yang et al., 2008). The syntheses of nonradiolabeled (cold) 20-14,15-EE5ZE and 20-OTs-14,15-EE5ZE are described in the Supplemental Data (Mosset et al., 1989; Cai et al., 2006; Yang et al., 2008). Here, the synthesis of 20-125I-14,15-EE5ZE is described. To 2 μl of 20-OTs-14,15-EE5ZE in acetone (640 nmol). The reaction was carried out at 37°C for 4 days, with shaking 2 to 3 times daily, and stopped by 10 μl of a saturated Na2S2O3 solution. The reaction mixture was added to a Bio-Sil A (Bio-Rad Laboratories, Hercules, CA) silica acid column. The column was then eluted by 2 volumes of hexane/ethyl acetate (90%:10%) and 2 volumes of hexane/ethyl acetate (80%:20%). The eluent was dried under N2 and purified by high-performance liquid chromatography with use of a C18 reverse-phase column (Nucleosil; 5 μM; 4.6 × 250 mm; Phenomenex, Torrance, CA). A linear gradient of 50 to 100% solvent B in solvent A (solvent B: acetonitril/glacial acetic acid = 999:1; solvent A: water) over 40 min was used to elute 20-125I-14,15-EE5ZE. 20-14,15-EE5ZE was used as a chromatographic standard and detected in the column effluent by UV absorbance at 205 nm. The specific activity of 20-125I-14,15-EE5ZE was 17.68 Ci/mmol.

Culture of U937 Cells. U937 cells were cultured in suspension in RPMI 1640 medium (Invitrogen, Carlsbad, CA) containing 10% fetal bovine serum (HyClone Laboratories, Logan, UT), 25 mM HEPES, 2 mM l-glutamine, and 1 mM sodium pyruvate, 100 U/ml penicillin, 100 μg/ml streptomycin, and 0.25 μg/ml amphotericin B (Yang et al., 2007, 2008). Culture medium was changed every 2 to 3 days. Cells were cultured at 37°C in a 5% CO2 in air-humidified atmosphere and harvested after reaching a density of 5 to 10 × 10^6 cells/ml.

Measurement of Phospho-p38 and p38 in U937 Cells. U937 cells (10^6 cells/ml) were suspended in phosphate-buffered saline containing SKF525A (10 μM), triacsin C (20 μM), and 12-(3-adamantan-1-yl-ureido)-dodecanoic acid (1 μM) to inhibit cytochrome P450, esterification, and epoxide hydrolyse (EH), respectively (Yang et al., 2008). Cells were incubated for 10 min at 37°C with vehicle, 14,15-EET (100 nM), 14,15-EET-5ZE (10–1000 nM), or 14,15-EET and 20-14,15-EE5ZE (10–1000 nM). Subsequently, the cell suspension was centrifuged for 5 min at 4°C. The cell pellet was resuspended in lysis buffer (150 mM NaCl, 10 mM HEPES, 1 mM EDTA, 1 mM EGTA, 1 mM Na2S2O3, pH 7.5, containing 1% Triton X-100 and Roch proteinase inhibitor mix) and incubated for 10 min on ice. Proteins were separated by electrophoresis, and phospho-p38 and p38 were detected by Western immunoblotting.

Western Blotting. U937 cellular lysates were mixed with reducing buffer and heated at 95°C for 10 min to denature proteins (Yang et al., 2008). The above samples were separated by electrophoresis on a 12% polyacrylamide Redi-Gel (Bio-Rad Laboratories) and transferred to a nitrocellulose membrane (Bio-Rad Laboratories) for immunoblotting with anti-phospho-p38 antibody (Cell Signaling Technology, Danvers, MA). The nitrocellulose membrane was reprobed with anti-p38 antibody (Santa Cruz Biotechnology, Santa Cruz, CA).

Vascular Reactivity of Bovine Coronary Arteries. Fresh bovine hearts were obtained from a local slaughterhouse. The left anterior descending branch of coronary artery was dissected, cleaned of connective tissue, and cut into 3-mm-long rings of 1.5–3.0-mm diameter (Campbell et al., 1996; Falk et al., 2003a). The arterial rings were suspended in a water-jacketed tissue chamber containing Krebs’ buffer (119 mM NaCl, 4.8 mM KCl, 24 mM NaHCO3, 0.2 mM...
KH$_2$PO$_4$, 0.2 mM MgSO$_4$, 11 mM glucose, 0.02 mM EDTA, and 3.2 mM CaCl$_2$ in 5% CO$_2$ and 95% O$_2$ environment at 37°C. Ring tension was recorded with a model FT-03C force transducer (Grass Instruments, Milford, MA), ETH-400 bridge amplifier, and MacLab 8e A/D converter controlled by a Macintosh computer. The arterial rings were stretched gradually to a tension of 3.5 g and equilibrated for 1.5 h. KCl (40–60 mM) was repeatedly added and washed away until reproducible stable contractions were reached. The thromboxane mimetic U46619 (20 nM) was added to increase basal contraction to 50 to 75% KCl. Increasing concentrations of 14,15-EET or the BK$_{	ext{Ca}}$ channel activator, NS1619, were added and relaxations recorded. To block the 14,15-EET effects, rings were preincubated with vehicle, 20-I-14,15-EE5ZE (10$^{-6}$ M), proadifen (20$^{-6}$ M), miconazole (20$^{-6}$ M), or MS-PPOH (20$^{-6}$ M) for 10 min, and the 14,15-EET relaxation was recorded. Similar experiments using miconazole (20$^{-6}$ M) and MS-PPOH (20$^{-6}$ M) were repeated with the BK$_{	ext{Ca}}$ channel opener NS1619 as the agonist (Gauthier et al., 2002). Results are expressed as the percentage of relaxation of the U46619-treated rings, with 100% relaxation representing basal tension.

**U937 Membrane Preparation.** Cell and membrane preparations were kept in ice or in the cold room. Cells were pooled and centrifuged at 1000 rpm for 5 min (Yang et al., 2007, 2008). Cell pellets were combined, washed with 10 ml of phosphate-buffered saline, pH 7.4, twice, and resuspended with Hanks’ balanced salt solution containing protease inhibitor cocktail (Roche Diagnostics, Indianapolis, IN). After sonicating for 20 s, the lysate was centrifuged at 1000 g for 10 min. The supernatants were centrifuged at 110,000 g for 45 min, and the pellet was resuspended in binding buffer consisting of 10 mM HEPES, 5 mM CaCl$_2$, 5 mM MgCl$_2$, and 5 mM EGTA, pH 7.4. Protein concentration was determined by the Bradford method (Bio-Rad Laboratories).

**20-125I-14,15-EE5ZE Binding Assays.** 20-125I-14,15-EE5ZE binding assays were performed with a Brandel 48-well harvester system (Brandel Inc., Gaithersburg, MD) at 4°C (Yang et al., 2007, 2008). Binding was determined in triplicate and repeated on three to four membrane preparations. Fifty micrograms of protein was incubated in binding buffer (see U937 Membrane Preparation for composition) with various concentrations of 20-125I-14,15-EE5ZE for various times. The binding was stopped by filtration through GF/A glass filter paper. After washing five times with 3 ml of binding buffer each, the radioactivity on the filter paper was counted by a γ-scintillation counter. Nonspecific binding was measured in the presence of 20 μM 14,15-EE5ZE. Specific binding was calculated from total binding minus nonspecific binding. The data were analyzed using Prism software as reported previously (Yang et al., 2007, 2008).

Time course of binding was determined by incubating 2.9 nM radioligand with the membranes for various times (0–30 min) (Yang et al., 2008). Saturation of binding was carried out by use of a 15-min incubation time with different concentrations of the radioligand. To determine the reversibility of ligand binding, 1 or 20 μM 11,12-EET was incubated with membranes for various times (0–60 min) after 10 min of preincubation with radioligand (2.9 nM). For ligand competition, 20-125I-14,15-EE5ZE (1–2 nM) was incubated in presence of different concentrations of competing ligands for 15 min. Binding obtained in the presence of vehicle was defined as 100%. To determine the effect of GTP$\gamma$S on ligand binding, the membranes were preincubated with 10 μM GTP$\gamma$S or vehicle for 15 min before incubation with various concentrations of the radioligand for 15 min.

**Statistical Analysis.** The data are expressed as means ± S.E.M. Statistical evaluation of the data were performed by a one-way analysis of variance followed by the Student-Newman-Keuls multiple comparison test when significant differences were present. P < 0.05 was considered statistically significant.

**Results**

**Chemical Structures of EETs, EET Analogs, Cytochrome P450 Inhibitors, and Epoxide Hydrolase Inhibitors.** Figure 1A shows the structures of EET regioisomers, EET analogs, cytochrome P450 inhibitors, and epoxide hydrolase inhibitors that were studied.

**Synthesis of 20-125I-14,15-EE5ZE.** Cumulative synthesis and structure-activity relationships have revealed the basic
structural requirements for EET agonist and antagonist activity (Gauthier et al., 2002, 2003; Falck et al., 2003a, 2003b). 14,15-EE8ZE has all of the structural features of a full agonist whereas 14,15-EE5ZE is the first EET receptor antagonist. We have previously synthesized a 125I-labeled EET agonist, 20-125I-14,15-EE8ZE (Yang et al., 2008). In a similar manner, we synthesized 20-125I-14,15-EE5ZE as a radiolabeled antagonist.

**Antagonist Activity of 20-I-14,15-EE5ZE.** We tested whether 20-I-14,15-EE5ZE is an antagonist similar to 14,15-EE5ZE in rings of bovine coronary arteries. 14,15-EET relaxed U46619 preconstricted bovine coronary artery rings with EC_{50} value of approximately 2 μM (Fig. 2A). Pretreatment with 10 μM 20-I-14,15-EE5ZE reduced 14,15-EET-induced relaxations. These results indicate that 20-I-14,15-EE5ZE inhibits the action of 14,15-EET.

To further confirm the antagonist activity of 20-I-14,15-EE5ZE, 14,15-EET-induced p38 MAP kinase activity was monitored in U937 cells with immunoblotting. The phosphorylation of p38 in U937 cells was stimulated in concentration-dependent manner by 14,15-EET (0.1–100 nM) (Fig. 3A). In contrast, the inactive EET thiirane analog (0.1–100 nM) did not alter p38 phosphorylation, indicating specific activation of p38 by 14,15-EET (Falck et al., 2003a). The effect of 20-I-14,15-EE5ZE was tested on 14,15-EET-induced p38 phosphorylation (Fig. 3, B and C). 20-I-14,15-EE5ZE decreased 14,15-EET-stimulated p38 phosphorylation at concentrations from 1 to 1000 nM. This result indicates that 20-I-14,15-EE5ZE is an antagonist of 14,15-EET in U937 cells.

**Characterization of 20-125I-14,15-EE5ZE Binding on U937 Membranes.** Figure 4, A and C, shows the time- and concentration-dependent binding of 20-125I-14,15-EE5ZE to U937 cell membranes. The half-time of association was 0.9 min at 2.9 nM 20-125I-14,15-EE5ZE (Fig. 4A). The specific binding reached equilibrium within 5 min and remained unchanged up to 30 min. Equilibrium binding was performed at an incubation time of 15 min with increasing concentration of radioligand. Specific binding increased with radioligand and was saturable (Fig. 4, B and C). Nonspecific binding increased linearly with increasing concentrations of the radioligand. Scatchard analysis of the saturable binding suggested a single-site binding model (Fig. 4D) (r^2 = 0.95). Binding affinity K_D was 1.11 ± 0.13 nM, and B_max was 1.13 ± 0.04 pmol/mg (n = 4). If we assume association rate constant k_{on} = 6.4 × 10^6 M^{-1} s^{-1} for 20-125I-14,15-EE5ZE, the dissociation rate constant k_{off} will be 0.007 s^{-1} calculated from k_{off} = K_D × k_{on} and the t_{1/2} is 99 s from t_{1/2} = ln 2/k_{off}. This antagonist radioligand has 10 times higher affinity than the agonist radioligand, 20-125I-14,15-EE5ZE (K_D = 11.8 nM and B_max = 5.8 pmol/mg, n = 5) (Yang et al., 2008). This difference in K_D values between the agonist and antagonist ligands was statistically significant (p < 0.0057). The B_max
Previous experiments suggested that the EET receptor in U937 membranes might be a GPCR because GTP\(_\gamma\)S blocked the binding of the agonist radioligand 20-\(^{125}\)I-14,15-EESZ (Yang et al., 2008). 20-\(^{125}\)I-14,15-EES binding to U937 membranes was determined in the present or absence of 10 \(\mu\)M GTP\(_\gamma\)S. The specific binding did not differ in the presence or absence of GTP\(_\gamma\)S (Fig. 5B). This experiment further indicates that 20-I-14,15-EES is a antagonist.

Competition for 20-\(^{125}\)I-14,15-EESZ binding to U937 cell membranes was performed using three EETs, several EET structural analogs, cytochrome P450 inhibitors, and EH inhibitors (Zou et al., 1994; Harder et al., 1995; Wang et al., 1998; Morsissee et al., 1999; Falck et al., 2003a). Figure 6, A and B, and Table 1 show the rank order and \(K_i\) values of competing compounds for 20-\(^{125}\)I-14,15-EES binding (11,12-EET = 14,15-EET > 8,9-EET = 14,15-EET-mSA = 14,15-EESZ > 15-GIHET = thirane > 14,15-DHE5Z). 8,9-DHET and 11-HETE did not displace 20-\(^{125}\)I-14,15-EES from its binding site (Table 1). These results suggest that the binding site is specific for three EETs (8,9-EET, 11,12-EET, and 14,15-EET) but not for 15-GIHET, 14,15-DHET, thirane of 14,15-EET, or 14,15-DHE5Z. This competition rank order of EETs and EET analogs is similar to the rank order previously report for 20-\(^{125}\)I-14,15-EES suggesting the same binding site (Yang et al., 2008).

Cytochrome P450s metabolize arachidonic acid to EETs and EH metabolizes EETs to DHETs (Morsissee et al., 1999; Spector et al., 2004). Several of the inhibitors of these enzymes have structures similar to fatty acids and the EETs. For this reason, cytochrome P450 and EH inhibitors were also tested. The cytochrome P450 inhibitors sulfaphenazole and proadifen and the soluble and microsomal EH inhibitors 1-cyclohexyl-3-dodecyl-urea, elaidamide, and 12-hydroxyl-elaideamide did not compete with 20-\(^{125}\)I-14,15-EET, indicating that this binding site is not a cytochrome P450 or EH (Table 1). Three cytochrome P450 inhibitors inhibited 20-\(^{125}\)I-14,15-EES binding to U937 membranes. Miconazole and MS-PPOH inhibit with \(K_i\) of 315 and 1558 nM, respectively (Fig. 6B). In contrast, ketoconazole is less effective in inhibiting binding with approximately 50% inhibition at the highest concentration tested, 50 \(\mu\)M. These findings suggest that the three compounds are cytochrome P450 and EET receptor dual inhibitors with principle structures unrelated to EETs or EET analogs. To test this possibility, we examined their effects of 14,15-EET-induced relaxation of coronary arteries. 14,15-EET caused a concentration-related relaxation of preconstricted arterial rings (Fig. 2). Both miconazole (20 \(\mu\)M) and MS-PPOH (20 \(\mu\)M) inhibited the EET-induced relaxations (Fig. 2, E and C). In contrast, proadifen (20 \(\mu\)M) was without effect (Fig. 2B). NS1619, a BK\(_{ca}\) channel opener, relaxed coronary arteries in a concentration-related manner (Gauthier et al., 2002) (Fig. 2, D and F). The relaxations to NS1619 were not altered by MS-PPOH but were reduced slightly, and significantly, by miconazole at the highest concentrations of NS1619. Thus, the blockade of 14,15-EET-induced relaxations by MS-PPOH is due to a blocking EET binding to its receptor and not inhibition of the BK\(_{ca}\) channel. The blockade of the EET relaxations by miconazole is predominantly due to inhibition of EET binding; however, a component is due to a reduction in BK\(_{ca}\) channel activation.
Discussion

EETs are synthesized by the vascular endothelium and have a number of cardiovascular actions (Rosolowsky and

Fig. 4. Time- and concentration-dependent binding of 20-125I-14,15-EESZE to U937 membranes. A, time-dependent binding of 20-125I-14,15-EESZE to U937 membranes. 20-125I-14,15-EESZE (2.9 nM) was incubated with 50 μg of total U937 membrane for indicated times at 4°C (n = 4). Specific binding was determined in the presence of 20 μM 14,15-EESZE. B, effect of 20-125I-14,15-EESZE concentration on total, nonspecific, and specific binding (n = 4). C, specific binding of 20-125I-14,15-EESZE expanded from B. D, Scatchard analysis of data from B. Each value represents the mean ± S.E.M. (n = 4).

Fig. 5. 20-125I-14,15-EESZE binding to U937 membranes. A, reversibility: U937 membranes were incubated with 2.9 nM 20-125I-14,15-EESZE for 10 min to reach binding equilibrium. 11,12-EET (1 or 20 μM) was added, and binding was terminated at the indicated times. Specific binding was determined. B, effect of GTPγS. Membranes were preincubated with or without 10 μM GTPγS for 15 min. Indicated concentrations of 20-125I-14,15-EESZE were added, and the incubation was continued for 15 min. The specific binding was determined. Each value represents the mean ± S.E.M. (n = 4).

Fig. 6. Inhibition of 20-125I-14,15-EESZE binding to U937 membranes by EETs (A), EET analogs (B), and cytochrome P450 inhibitors (C). 20-125I-14,15-EESZE (2.9 nM) was incubated with increasing concentrations of EETs, EET analogs or inhibitors, and U937 membrane for 15 min. Specific binding was determined in the presence or absence of 20 μM 14,15-EESZE. Specific binding obtained in the presence of vehicle represents 100% binding. Each value represents the mean ± S.E.M. (n = 4).
We synthesized and characterized 20-125I-14,15-EE5ZE as an antagonist radioligand. It showed specific, saturable binding to U937 membranes, and the specific binding was reversed by the addition of an excess of 11,12-EET. The antagonist radioligand bound with higher affinity than did the agonist radioligand. 20-125I-14,15-EE5ZE had a Kᵢ of 1.11 nM, whereas 20-125I-14,15-EE8ZE had a Kᵢ of 11.8 nM (Yang et al., 2008). The reason for the lower Kᵢ for the antagonist radioligand than the agonist radioligand is the faster kᵦ (0.5 versus 6.4 × 10⁴ M⁻¹ s⁻¹ for agonist versus antagonist) and slower kᵣ (0.06 versus 0.007 s⁻¹ for agonist versus antagonist) (Yang et al., 2008). The higher affinity of 20-125I-14,15-EE5ZE is an advantage over previously studied radioligands providing a higher sensitivity for ligand binding studies.

The binding of 20-125I-14,15-EE5ZE was displaced by EETs and some EET analogs. The active EET agonists and EET antagonist displaced the radioligand with a lower Kᵢ than the inactive analogs or EET metabolites (Falck et al., 2003a, 2003b). Likewise, Wong et al. (1993) demonstrated that 14,15-EET displaced [3H]14,15-EET binding to monocytes, but the ligand was not displaced by thromboxane, platelet-activating factor, leukotriene B₄, or leukotriene D₄. Inceoglu et al. (2007) showed that the EETs did not alter binding to neurokinin, cannabinoid, benzodiazepine, or dopamine receptors. It is interesting that the ranking order of the Kᵢ values of EETs and EET analogs was similar with 20-125I-14,15-EE8ZE and 20-125I-14,15-EE5ZE, suggesting that the agonist and antagonist radioligands label the same binding protein on U937 membranes.

Several lines of evidence suggest that the EET binding site is a GPCR. 11,12-EET increased KCa channel activity in cell-attached patches of coronary artery smooth muscle cells (Li and Campbell, 1997). However, in inside-out patches of the same cells, the EET was without effect unless GTP was added to the bath. The ability of 11,12-EET to increase KCa channel activity in inside-out patches with GTP was inhibited by the G protein antagonist GDPβS or an anti-Gsα antibody (Li and Campbell, 1997). These studies indicate that 11,12-EET activates KCa channels via a membrane-delimited mechanism involving activation of a G protein, possibly Gsα. Likewise, EETs increase GTPγS binding to Gs, but not Gi, to endothelial cell membranes (Node et al., 2001). Radioligand-binding studies confirmed these findings. The binding of 20-125I-14,15-EE5ZE to membranes of U937 was decreased in a concentration-related manner by the GTP analog, GTPγS (Yang et al., 2008). Because this ligand is an EET agonist, the decreased binding indicates that the EET binding site is coupled to a G protein. In contrast, 20-125I-14,15-EE5ZE is an EET antagonist, and, as would be predicted with an antagonist, GTPγS did not affect binding of the radioligand. These results further supported the notion that the EET receptor is coupled to a G protein.

Cytochrome P450 inhibitors and EH inhibitors are commonly used to estimate the contribution of endogenous EETs to physiological or pathological processes (Harder et al., 1995; Fisslthaler et al., 1999; Campbell and Falck, 2007; Fisslthaler et al., 2007; Fisslthaler et al., 2007; Gross et al., 2008). Considering these diverse actions, it is important to understand the mechanism of action of the EETs.

In screening a series of 14,15-EET analogs for relaxation of bovine coronary artery rings, we discovered that 14,15-EE5ZE blocked the relaxations by all four regioisomeric bovine coronary artery rings, we discovered that 14,15-EE5ZE was a selective EET antagonist. It is interesting that the ranking order of the Kᵢ values of EETs and EET analogs was similar with 20-125I-14,15-EE8ZE and 20-125I-14,15-EE5ZE, suggesting that the agonist and antagonist radioligands label the same binding protein on U937 membranes.

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<tr>
<th>Competitor</th>
<th>Kᵢ (95% Confidence Intervals)</th>
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<tr>
<td>11,12-EET</td>
<td>0.990 (0.658–1.49)</td>
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<tr>
<td>14,15-EET</td>
<td>1.35 (0.76–2.39)</td>
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<tr>
<td>8,9-EET</td>
<td>9.26 (4.48–19.1)</td>
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<td>14,15-EET-mSA</td>
<td>15.4 (11.2–21.2)</td>
</tr>
<tr>
<td>14,15-EE5ZE</td>
<td>15.3 (10.2–23.8)</td>
</tr>
<tr>
<td>15-HETE</td>
<td>1243 (680–2269)</td>
</tr>
<tr>
<td>14,15-DHET</td>
<td>2531 (1812–3534)</td>
</tr>
<tr>
<td>14,15-Thiirane</td>
<td>3091 (1868–5116)</td>
</tr>
<tr>
<td>14,15-DHE5ZE</td>
<td>&gt;5 × 10⁴</td>
</tr>
<tr>
<td>8,9-DHET</td>
<td>&gt;5 × 10⁴</td>
</tr>
<tr>
<td>11-HETE</td>
<td>&gt;5 × 10⁴</td>
</tr>
<tr>
<td>1-Cyclohexyl-3-dodecyl-urea</td>
<td>&gt;2 × 10⁴</td>
</tr>
<tr>
<td>Elaidamide</td>
<td>&gt;2 × 10⁴</td>
</tr>
<tr>
<td>12-Hydroxyl-elaidamide</td>
<td>&gt;2 × 10⁴</td>
</tr>
<tr>
<td>Sulfaphenazole</td>
<td>&gt;2 × 10⁴</td>
</tr>
<tr>
<td>Proadifen</td>
<td>&gt;2 × 10⁴</td>
</tr>
<tr>
<td>Miconazole</td>
<td>315 (145–685)</td>
</tr>
<tr>
<td>MS-PPOH</td>
<td>1558 (659–3684)</td>
</tr>
<tr>
<td>Ketoconazole</td>
<td>&gt;5 × 10⁴</td>
</tr>
</tbody>
</table>

TABLE 1

Comparison of Kᵢ of different EETs, EET analogs, cytochrome P450 inhibitors, and EH inhibitors.

Campbell, 1996; Campbell and Falck, 2007; Spector and Norris, 2007). They have been implicated as endogenous mediators of vasodilation, cardioprotection, and angiogenesis and inhibitors of inflammation, thrombosis, and platelet aggregation (Node et al., 1999; Krotz et al., 2003; Gauthier et al., 2007; Gross et al., 2008). Considering these diverse actions, it is important to understand the mechanism of action of the EETs.
some EH inhibitors to activate PPARs (Liu et al., 2005), they are without effect on EET binding, which supports their activity as specific EH inhibitors. The cytochrome P50 inhibitors proadifen and sulfaphenazole also failed to alter binding. Thus, this high-affinity binding site for 20,12R,14,15-
EE5ZE is unique from other previous known lipid receptors and EET-related enzymes.

Three cytochrome P450 inhibitors, miconazole, MS-PPOH, and ketocozazole, displace 20,12R,14,15-EE5ZE from the EET receptor. They represent the first group of ligands structurally unrelated to the EETs. Miconazole inhibits cytochrome P450 and EET binding with similar \( K_i \) values (300 and 315 nM, respectively) (Zou et al., 1994; Harder et al., 1995). MS-PPOH has a lower \( K_i \) value for inhibition of EET binding than for inhibition of cytochrome P450 (1.6 and 13 
\( \mu \)M, respectively) (Wang et al., 1998). Ketocozazole was the least effective inhibitor of EET binding and inhibited binding by approximately 50% at 50 
\( \mu \)M. The \( K_i \) for ketocozazole inhibition of cytochrome P450 epoxygenase is approximately 10 
\( \mu \)M (Zou et al., 1994; Harder et al., 1995). Thus, these structures differ widely in their specificity for cytochrome P450 and EET binding. To determine whether binding predicts EET antagonist activity, we tested the effects of these inhibitors on 14,15-EET-induced relaxation of the bovine coronary artery. Both MS-PPOH and miconazole inhibited the relaxations to 14,15-EET, whereas proadifen, which did not affect binding, was without effect on EET relaxations. MS-PPOH did not alter the relaxations to the BK\(_{Ca}\) channel opener NS1619, indicating that it does not block BK\(_{Ca}\) channel activation. Although miconazole (10 \( \mu \)M) failed to alter BK\(_{Ca}\) channel activity (Campbell et al., 1996), miconazole (20 \( \mu \)M) partially inhibited the relaxations to NS1619. However, this degree of BK\(_{Ca}\) channel inhibition could not account for the blockade of EET-induced relaxation by miconazole. Thus, MS-PPOH acts as an EET antagonist, and miconazole acts predominantly as an EET antagonist. The structural differences in these nonlipid inhibitors may lead to the design of EET receptor ligands with improved water solubility and kinetic properties that are useful for future animal and human applications. These dual cytochrome P450 inhibitors and EET receptor ligands, and possibly other cytochrome P450 inhibitors, inhibit EET signaling pathways at two different sites of action. Careful interpretation of previous publications using these dual inhibitor/ligands may be needed. Variations on the structures of miconazole and/or MS-PPOH may lead to the identification of specific, noneicosanoid EET antagonists devoid of cytochrome P450 inhibition. Such antagonists may be useful for studies in vivo and provide new insights into the endogenous roles of the EETs.

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References


Chen JK, Wang DW, Falck JR, Capdevila J, and Harris RC (1999) Transection of an active site in cytochrome P450 arachidonic acid epoxygenase indicates that 14,15-


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