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20-Iodo-14,15-epoxyeicosa-8(*Z*)-enoyl-3-azidophenylsulfonamide: Photoaffinity Labeling of a 14,15-Epoxyeicosatrienoic Acid Receptor

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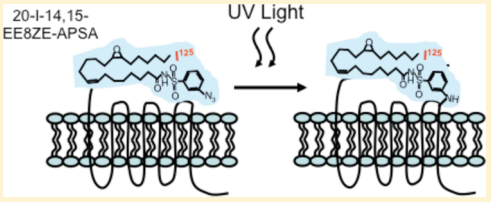
Supporting Information

ABSTRACT: Endothelium-derived epoxyeicosatrienoic acids (EETs) relax vascular smooth muscle by activating potassium channels and causing membrane hyperpolarization. Recent evidence suggests that EETs act via a membrane binding site or receptor. To further characterize this binding site or receptor, we synthesized 20-iodo-14,15-epoxyeicosa-8(*Z*)-enoyl-3-azidophenylsulfonamide (20-I-14,15-EE8ZE-APSA), an EET analogue with a photoactive azido group. 20-I-14,15-EE8ZE-APSA and 14,15-EET displaced 20-^{125}I-14,15-epoxyeicosa-5(*Z*)-enoic acid binding to U937 cell membranes with K_i values of 3.60 and 2.73 nM, respectively. The EET analogue relaxed preconstricted bovine coronary arteries with an ED_{50} comparable to that of 14,15-EET. Using electrophoresis, 20-^{125}I-14,15-EE8ZE-APSA labeled a single 47 kDa band in U937 cell membranes, smooth muscle and endothelial cells, and bovine coronary arteries. In U937 cell membranes, the 47 kDa radiolabeling was inhibited in a concentration-dependent manner by 8,9-EET, 11,12-EET, and 14,15-EET (IC_{50} values of 444, 11.7, and 8.28 nM, respectively). The structurally unrelated EET ligands miconazole, MS-PPOH, and ketoconazole also inhibited the 47 kDa labeling. In contrast, radiolabeling was not inhibited by 8,9-dihydroxyeicosatrienoic acid, 5-oxoeicosatetraenoic acid, a biologically inactive thiirane analogue of 14,15-EET, the opioid antagonist naloxone, the thromboxane mimetic U46619, or the cannabinoid antagonist AM251. Radiolabeling was not detected in membranes from HEK293T cells expressing 79 orphan receptors. These studies indicate that vascular smooth muscle, endothelial cells, and U937 cell membranes contain a high-affinity EET binding protein that may represent an EET receptor. This EET photoaffinity labeling method with a high signal-to-noise ratio may lead to new insights into the expression and regulation of the EET receptor.



Epoxyeicosatrienoic acids (EETs) are cytochrome P450 (CYP) metabolites of arachidonic acid (AA).[1−3] Four regioisomeric EETs (14,15-, 11,12-, 8,9-, and 5,6-EET) are synthesized. Several CYP epoxygenases, including CYP2C and CYP2J, are capable of synthesizing the EETs. EETs have a variety of biological activities. They decrease inflammation, are antinociceptive, promote angiogenesis, protect the heart and brain from ischemic-reperfusion injury, and decrease the level of platelet adhesion.[4−9] Additionally, EETs function as endothelium-derived hyperpolarization factors (EDHF) in the coronary circulation.[10−13] They are synthesized and released by the vascular endothelium in response to agonists such as bradykinin, acetylcholine, cyclic stretch, and shear stress.[13] EETs relax vascular smooth muscle by activating high-conductance, calcium-activated potassium (BK_{Ca}) channels, resulting in membrane hyperpolarization, a reduction in the rate of influx of calcium through voltage-activated calcium channels. 11,12-EET activation of smooth muscle cell BK_{Ca} channels requires intracellular GTP, but not ATP, and is blocked by the guanine nucleotide binding protein (G protein) inhibitor GTPβS and by an anti-Gαs antibody.[14] Thus, a G protein, likely Gαs, mediates EET activation of BK_{Ca} channels.

Several lines of evidence suggest that EETs act through a specific binding site or receptor. Specific structural features are required for 14,15-EET to relax the bovine coronary artery.[15] For full agonist activity, the structure must contain a 20-carbon chain, an (*S*,*R*)-*cis*-epoxide, an 8(*Z*)-olefin, and a carboxyl at carbon 1, i.e., 14(*S*),15(*R*)-*cis*-epoxyeicosa-8(*Z*)-enoic acid (14,15-EE8ZE). Partial reduction of the olefins to produce 14,15-EE5ZE results in an antagonist.[16] The need for a specific enantiomer of the 14,15-epoxide suggests a specific binding site is involved in relaxation. When 14,15-EET was tethered to silica beads to maintain the EET extracellularly, 14,15-EET and the tethered 14,15-EET had similar activity in smooth muscle cells, indicating an extracellular site of action.[17] These studies suggest EETs act on a cell surface binding site or receptor.

The existence of both high- and low-affinity EET receptors or binding sites is supported by many physiological and biochemical experiments. The low-affinity EET receptors and binding sites

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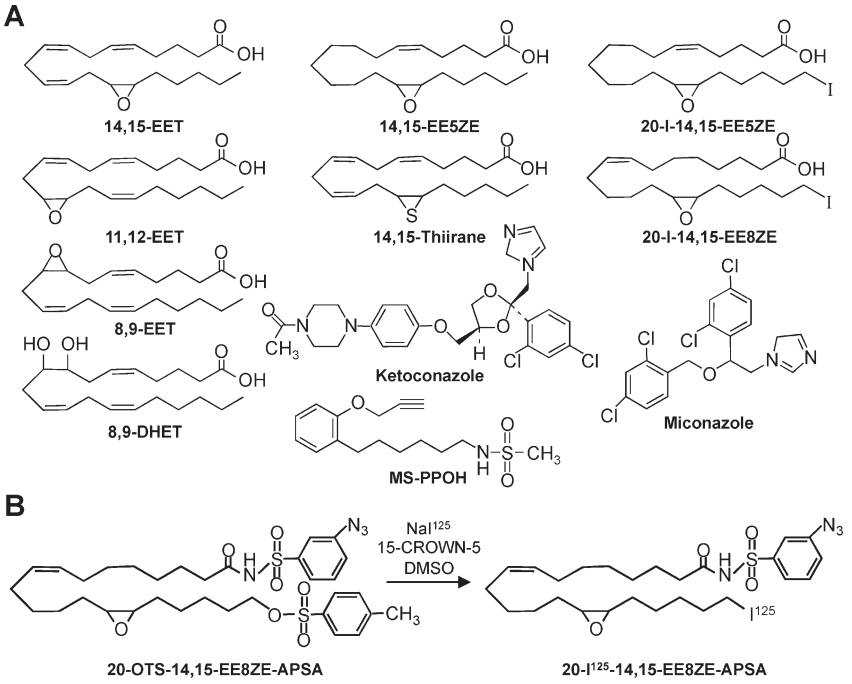

Figure 1. Chemical structures of EETs and EET analogues. (A) Structures of chemicals used in the study. (B) Chemical synthesis of 20-^{125}I-14,15-EE8ZE-APSA (mAPSA isomer).

include fatty acid binding protein,[18] peroxisomal proliferator-activated receptor-α (PPAR-α),[19] PPAR-γ,[20] peripheral benzodiazepine receptor,[21] cannabinoid (CB) receptor,[21] neurokinin receptor,[21] dopamine receptor[21] and ATP-sensitive K channel,[22] prostaglandin E (EP2) receptor,[23] and thromboxane (TP) receptor.[24] However, many physiological responses are elicited by lower concentrations of EETs, suggesting the existence of high-affinity EET receptors.[13] To study high-affinity EET binding, Wong et al. pioneered the use of guinea pig mononuclear and U937 cells for direct radioligand binding studies.[25−27] A high-affinity specific binding site with a K_D of 1−10 nM was characterized using 14,15-EET agonist radioligands such as 14,15-^{3}H-EET and 20-^{125}I-14,15-epoxyeicosa-8(Z)-enoic acid (EE8ZE) and the EET antagonist 20-^{125}I-14,15-epoxyeicosa-5(Z)-enoic acid (EE5ZE).[26,28−30] For example, 20-^{125}I-14,15-EE8ZE exhibited high-affinity, reversible specific binding that was inhibited by the G protein ligand GTPγS.[29] Overall, these studies suggested 14,15-EET acts through an unknown G protein-coupled receptor (GPCR).

Here, we developed and characterized a 14,15-EET agonist photoaffinity probe with a photoactive arylazido (AZ) group attached to the carboxyl end of 20-I-14,15-EE8ZE. This analogue, 20-iodo-14,15-epoxyeicosa-8(Z)-enoyl-3-azidophenylsulfonamide (20-I-14,15-EE8ZE-APSA), may be radiolabeled as described for 20-^{125}I-14,15-EE8ZE.[29] The photoactive group is covalently bound to an EET-targeted receptor or binding site through covalent binding upon UV light-induced photolytic cross-linking. The labeled receptor or binding site is resolved by electrophoresis and detected by radioautography. The photoaffinity labeling method provides a high signal:noise ratio due to protein separation prior to detection; e.g., nonspecifically labeled proteins are separated from specifically labeled proteins. This method also provides insights into the characteristics of the receptor or binding protein such as molecular mass, density, and binding specificity. Using this method, we have characterized a 47 kDa protein that is photolabeled with 20-^{125}I-14,15-EE8ZE-APSA. This photolabeled protein was detected in U937 cells, endothelial cells, and vascular smooth muscle cells.

EXPERIMENTAL PROCEDURES

Materials. EET analogues were synthesized as previously described, and their structures are given in Figure 1A.[15,28,29] Chemicals and solvents were purchased from Sigma-Aldrich (St. Louis, MO).

Synthesis of Carrier Free 20-^{125}I-14,15-EE8ZE-APSA. Carrier free 20-^{125}I-14,15-EE8ZE-APSA (meta isomer) was synthesized from 20-tosyl (OTs)-14,15-EE8ZE-APSA using conditions different from those described for 20-I-14,15-EE8ZE-APSA synthesis (see the Supporting Information). (^{125}I was used in designated areas and with appropriate precautions as designated by the radiation safety office of the Medical College of Wisconsin.) Two millicuries of carrier free Na^{125}I (0.8 nmol, 17.4 Ci/mg) in 20 μL was added to 20 μL of DMSO containing 640 nmol of 20-OTs-14,15-EE8ZE-APSA and 20 μL of 15-crown-5 (Sigma-Aldrich) as a phase transfer reagent. The reaction was conducted at 25 °C for 4−7 days as the mixture was shaken two or three times daily and stopped by addition of 10 μL of a saturated $Na_2S_2O_3$ aqueous solution. The reaction mixture was transferred to a 15 mL conical glass tube and extracted with 0.25 mL of diethyl ether four times. The extract was dried under N_2 and purified by high-performance liquid chromatography (HPLC) using a C18 reverse-phase column [Nucleosil, 5 μm, 4.6 mm × 250 mm (Phenomenex, Torrance, CA)]. A linear gradient from 50 to 100% solvent B in solvent A (solvent B being a 999:1 acetonitrile/glacial acetic acid mixture and solvent A being water) over 40 min was used to elute 20-^{125}I-14,15-EE8ZE-APSA. 20-I-14,15-EE8ZE-APSA was used as a standard to mark the collection time. The specific activity of 20-^{125}I-14,15-EE8ZE-APSA was calculated to be 2000 Ci/mmol.

Vascular Reactivity of Bovine Coronary Arteries. The protocols are the same as previously reported.[10,15] The left anterior descending branch of the bovine coronary artery was used for tension measurements using a model FT-03C force transducer (Grass Instruments, Milford, MA), a MacLab ETH-400 bridge amplifier, and a MacLab 8e A/D converter (AD Instruments, Colorado Springs, CO) controlled by a Macintosh computer. Rings were preconstricted with the thromboxane mimetic U46619 (20 nM), and relaxation responses to increasing concentrations of 14,15-EET or the ortho (o), meta (m), and para (p) isomers of 20-I-14,15-EE8ZE-APSA (oAPSA, mAPSA, and pAPSA, respectively) were measured. Results were expressed as the percent relaxation with the basal tension representing 100% relaxation.

Cell Culture and Membrane Preparation. Endothelial cells and smooth muscle cells were isolated and cultured as previously described.[31,32] Human endothelial cells were obtained from Invitrogen (Carlsbad, CA) and cultured according to the company's recommendations. U937 cells were cultured in suspension in an RPMI 1640 medium (Invitrogen) mixture containing 10% fetal bovine serum (HyClone Laboratories, Logan, UT), 25 mM HEPES, 2 mM L-glutamine, 1 mM sodium pyruvate, 100 units/mL penicillin, 100 μg/mL streptomycin, and 0.25 μg/mL amphotericin B as previously reported[28−30] and harvested after reaching a density of 5−10 $\times$ 10^5 cells/mL.

To prepare cell or tissue mixed membranes, phosphate-buffered saline (pH 7.4) was used to wash the cells or tissue. After being sonicated for 20 s in Hanks balanced salt solution with protease inhibitors (Roche), the lysates were centrifuged at 1000*g* for 10 min, and the supernatants were obtained. These supernatants were centrifuged again at 110000*g* for 45 min, and the pellets were resuspended in binding buffer [10 mM HEPES, 5 mM $CaCl_2$, 5 mM $MgCl_2$, and 5 mM EGTA (pH 7.4)]. This 100000*g* pellet contains a mixture of membranes.[29]

To prepare membranes enriched with plasma membranes, the U937 cell pellet was resuspended in 10 mL of sucrose buffer [10 mM Tris base and 0.25 M sucrose (pH 7.4)] and sonicated for 20 s five times. The homogenate was centrifuged at 700*g* for 15 min, and the supernatant was centrifuged at 66000*g* for 45 min. The pellet was washed, resuspended in 10 mL of sucrose buffer through sonication, and centrifuged again at 66000*g* for 45 min. The pellet was resuspended in 5 mL of buffer, carefully layered atop a sucrose gradient (5.5 mL of 40% sucrose and 5.5 mL of 30% sucrose), and centrifuged at 77000*g* for 90 min. The middle interface was removed, mixed with 3 volumes of sucrose buffer, and centrifuged at 66000*g* for 45 min. The pellet was resuspended in binding buffer.

20-^{125}I-14,15-EE5ZE Binding Assays. Using 20-^{125}I-14,15-EE5ZE, U937 membrane binding assays were performed as previously reported.[30] Briefly, 1−2 nM 20-^{125}I-14,15-EE5ZE, 50 μg of membrane protein, and different concentrations of the oAPSA, mAPSA, or pAPSA isomer of 20-I-14,15-EE8ZE-APSA with or without 20 μM 14,15-EE5ZE were incubated for 15 min at 4 °C. Following filtration with a Brandel 48-well harvester system (Brandel Inc., Gaithersburg, MD), the radioactivity was measured by gamma scintillation counting. The data were analyzed with Prism (Prism Software Co., Orange, CA). Specific binding was calculated as the difference between the absence and presence of 14,15-EE5ZE.

Photoaffinity Labeling Using 20-^{125}I-14,15-EE8ZE-APSA. The photoaffinity probe, 20-^{125}I-14,15-EE8ZE-APSA (mAPSA isomer) (1 nM), was incubated with 400 μg of membranes for 15 min at 4 °C with EET regioisomers or vehicle in a total volume of 100 μL. The reaction mixtures were exposed to UV light at a wavelength of 280 nm for 15 min. After photolysis, the light-induced free radical reactions were stopped by addition of dithiothreitol-containing sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE) loading buffer and heated for 15 min at 100 °C. The protein mixtures were further separated by denaturing SDS−PAGE. SDS−PAGE gels were stained with Biosafe Coomassie Blue for 30 min, destained with distilled water for 2 h, and dried under a vacuum gel dryer for 1 h. The radioactivity on the gel was determined by radioautography using Kodak X-ray film. The film was exposed to the gel at −80 °C for 18−24 h. For concentration-dependent inhibition curves, the intensity of labeling was determined with ImageJ (National Institutes of Health, Bethesda, MD), and the data were normalized and plotted using GraphPad (GraphPad Software, La Jolla, CA) assuming a one-site competitive binding model. The levels of labeling with the highest concentration of the inhibitor and no inhibitor were set to 0 and 100%, respectively.

Overexpression and Photolabeling of GPCRs in HEK293T Cells. HEK293 cells overexpressing GPCR cells were provided by Multispan Inc. (Hayward, CA). Candidate GPCRs proteins were cloned in pMEX vectors and overexpressed in HEK293T cells in 96-well plates. An equal number of U937 cells was used as a positive control, and vector-transfected HEK293T cells were used as a negative control. Each well of cells was treated with 30 μL of lysis buffer [50 mM Tris (pH 7.4), 150 mM NaCl, 0.5% Nonidet P-40, 1% deoxycholic acid, and protease inhibitor cocktail from Roche], incubated on ice for 30 min, and homogenized by sonification. Cell lysates were incubated with 1 nM 20-^{125}I-14,15-EE8ZE-APSA at 4 °C, photolabeled by being exposed to UV light for 15 min, and analyzed by electrophoresis and radioautography by the protocol described above.

Data Analysis. Data are expressed as means ± the standard error of the mean (SEM). Significant differences between individual groups were evaluated with a Student's *t* test or ANOVA followed by a Student−Newman−Keuls multiple-comparison test. *P* values of <0.05 were considered statistically significant.

RESULTS

Synthesis of Carrier Free 20-^{125}I-14,15-EE8ZE-APSA. The structural difference between the photoprobe, 20-^{125}I-14,15-EE8ZE-APSA, and the radioligand, 20-^{125}I-14,15-EE8ZE, is the addition of the phenylsulfonamide group containing a meta photoactive azide.[29] The synthesis of 20-^{125}I-14,15-EE8ZE-APSA utilizes the 20-OTS-14,15-EE8ZE-APSA precursor (Figure 1B). While the synthesis of 20-^{125}I-14,15-EE8ZE was conducted directly in acetone,[29] these reaction conditions failed with 20-^{125}I-14,15-EE8ZE-APSA synthesis. Other conditions were tested. Incubation of 20-OTs-14,15-EE8ZE-APSA with Na^{125}I for 4−7 days in DMSO with the phase transfer reagent 15-crown-5 provided the best reaction yield for 20-^{125}I-EE8ZE-APSA, resulting in a specific activity of 2000 mCi/mmol (Figure 1B).

Agonist Activity of 20-I-14,15-EE8ZE-APSA. Previous structure−activity studies indicated that 20-I-14,15-EE8ZE is an EET receptor agonist.[29] To determine whether the C1 modification of 20-I-14,15-EE8ZE affects agonist activity, relaxation of U46619-preconstricted bovine coronary artery rings to 14,15-EET and 20-I-14,15-EE8ZE-APSA isomers was compared. The oAPSA, mAPSA, and pAPSA isomers of 20-I-14,15-EE8ZE-APSA relaxed

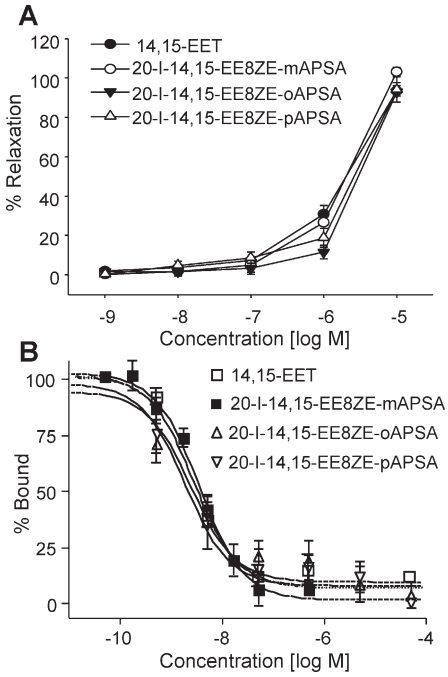

Figure 2. Agonist activity of 20-I-14,15-EE8ZE-APSA isomers. (A) Effect of oAPSA, mAPSA, and pAPSA isomers of 20-I-14,15-EE8ZE-APSA and 14,15-EET on vascular tone in bovine coronary arteries. Bovine coronary arteries were precontracted with U46619. Cumulative concentrations (10^{-9} to 10^{-5} M) of 14,15-EET or 20-I-14,15-EE8ZE-APSA isomers were added, and isometric tension was measured. Each value represents the mean ± SEM for six or seven measurements. (B) Inhibition of the specific binding of 20-^{125}I-14,15-EE5ZE to U937 membranes by 20-I-14,15-EE8ZE-APSA isomers and 14,15-EET. 20-^{125}I-14,15-EE5ZE was incubated with increasing concentrations of 14,15-EET or 20-I-14,15-EE8ZE-APSA isomers for 15 min. Specific binding was assessed in the presence or absence of 20 μM 14,15-EE5ZE. Specific binding obtained in the presence of vehicle represents 100% binding. Each value is the mean ± SEM for four measurements.

the arterial rings to an extent similar to that of 14,15-EET, with the agonists having similar EC_{50} values of approximately 2 μM (Figure 2A). Thus, the position of the AZ group did not affect activity. This experiment indicates that the photoprobe 20-I-14,15-EE8ZE-APSA is an EET agonist. These results are consistent with the agonist activity of other sulfonamide analogues of 14,15- and 11,12-EETs.[15,28,33,34]

Effect of 20-I-14,15-EE8ZE-APSA on 20-^{125}I-14,15-EE5ZE Binding to Membranes of U937 Cells. A high-affinity G protein-coupled EET receptor exists on U937 cell membranes. The receptor has been characterized by several EET agonist and antagonist radioligands such as ^{3}H-14,15-EET (agonist), 20-^{125}I-14,15-EE8ZE (agonist), 14,15-EET-phenyl-^{125}I-sulfonamide (agonist), and 20-^{125}I-14,15-EE5ZE (antagonist).[25,28−30] To test whether the photoprobe 20-I-14,15-EE8ZE-APSA binds to the EET receptor, 20-^{125}I-14,15-EE5ZE was incubated with U937 membranes with and without various concentrations of 20-I-14,15-EE8ZE-APSA isomers or 14,15-EET (Figure 2B). The specific binding of 20-^{125}I-14,15-EE5ZE was assessed in the presence and absence of 14,15-EE5ZE. The IC_{50} values were 3.60, 2.4, 1.9, and 2.73 nM for the mAPSA, oAPSA, and pAPSA isomers of 20-I-14,15-EE8ZE-APSA and 14,15-EET, respectively. This indicates

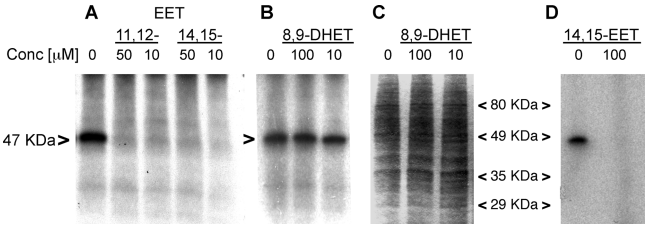

Figure 3. Photoaffinity labeling of U937 cell mixed membranes (A−C) and plasma membranes (D) with 20-^{125}I-14,15-EE8ZE-APSA. 20-^{125}I-14,15-EE8ZE-APSA (mAPSA isomer) (1 nM) was incubated with membranes; photo-cross-linking was performed with UV light, and proteins were separated by SDS−PAGE. Concentrations of EETs (nanomolar) are indicated. Vehicle was used as a control. The photolabeled 47 kDa bands are indicated with >. (A) Photolabeling of a 47 kDa protein in mixed membranes and inhibition by EET receptor agonists 11,12-EET and 14,15-EET. (B and C) Effect of 8,9-DHET on photolabeling of the 47 kDa protein in mixed membranes. Autoradiography (B) and Coomassie blue staining (C) of the same gel. (D) Photolabeling of a 47 kDa protein in plasma membranes and inhibition by 14,15-EET.

that 20-I-14,15-EE8ZE-APSA isomers have affinities for the U937 receptor site similar to that of 14,15-EET despite the reduction of two double bonds and modification of C1 and C20 of the 14,15-EET structure. The mAPSA isomer was used for subsequent studies.

Photoaffinity Labeling by 20-^{125}I-14,15-EE8ZE-APSA. Mixed membranes (Figure 3A−C) and plasma membranes (Figure 3D) of U937 cells were incubated with 20-^{125}I-14,15-EE8ZE-APSA (mAPSA isomer) with and without EETs and cross-linked by exposure to UV light and proteins resolved by electrophoresis. Autoradiography (Figure 3A) detected the radiolabeling of a single 47 kDa band. The labeling of the 47 kDa protein was inhibited by co-incubation of the photoprobe with 11,12-EET or 14,15-EET. It was not inhibited by 8,9-DHET, which was previously shown not to displace 20-^{125}I-14,15-EE5ZE from its binding site[30] (Figure 3B). Coomassie blue staining of the gel shows equal loading of the protein with the treatments (Figure 3C). 20-^{125}I-14,15-EE8ZE-APSA similarly labeled a 47 kDa protein in plasma membranes (Figure 3D). The photolabeling was inhibited by co-incubation of the photoprobe with 14,15-EET. These findings suggest specific labeling of a 47 kDa EET binding site by the photoprobe.

Characterization of 47 kDa Labled Protein as a High-Affinity EET Receptor. To determine whether the labeled 47 kDa protein is a high-affinity binding site for EETs, we used EET agonists (8,9-EET, 11,12-EET, and 14,15-EET), an EET antagonist (14,15-EE5ZE), and a structurally similar but biologically inactive analogue (14,15-thiirane) to compete for photoaffinity labeling (see Figure 1 for structures of the compounds).[15,16] Panels A−E of Figure 4 are representative autoradiographs, and Figure 4F is a summary showing the concentration-dependent competition (10^{-10} to 10^{-5} M) for photolabeling. 14,15-EET, 11,12-EET, 8,9-EET, and 14,15-EE5ZE inhibited the photolabeling of U397 membranes with IC_{50} values of 8.28, 11.2, 443.9, and 1097 nM, respectively (Table 1). The 14,15-thiirane did not alter the photolabeling. It appears that this 47 kDa protein is a high-affinity receptor or binding site specifically for 11,12-EET and 14,15-EET but a lower-affinity site for 8,9-EET and 14,15-EE5ZE.

Structurally Unrelated EET Receptor Antagonists Inhibit Labeling of the 47 kDa Protein. Three cytochrome P450

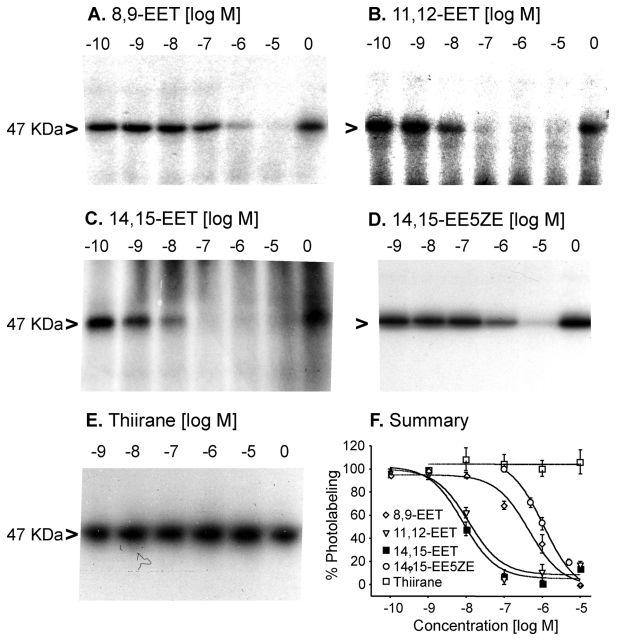

Figure 4. Concentration-dependent inhibition of 20-^{125}I-14,15-EE8ZE-APSA photolabeling of a 47 kDa protein in U937 membranes. U937 membranes were incubated with 20-^{125}I-14,15-EE8ZE-APSA and the indicated amount of the EET or EET analogue followed by the photolabeling protocol described in Experimental Procedures. Labeling of the 47 kDa receptor is indicated with >. Autoradiographs are shown in panels A–E. Inhibition of labeling by 8,9-EET (A), 11,12-EET (B), 14,15-EET (C), 14,15-EE5ZE (D), and the 14,15-thiirane analogue (E). Panel F is a summary of the inhibition of photolabeling EETs and EET analogues: 14,15-EET (■), 11,12-EET (▽), 8,9-EET (◇), 14,15-EE5ZE (○), and 14,15-thiirane (□) (n = 4).

Table 1. Inhibition of 20-^{125}I-14,15-EE8ZE-APSA Photolabeling by EETs and EET Analogues

competitor	K_i (95% confidence interval) (nM)
14,15-EET	8.28 (4.74–14.5)
11,12-EET	11.2 (5.94–22.9)
8,9-EET	443.9 (246.6–799.1)
14,15-EE5ZE	1097 (690–1743)
14,15-thiirane	>1 × 10^4

inhibitors, miconazole, *N*-(methylsulfonyl)-2-(2-propynyloxy)-benzenehexanamide (MS-PPOH), and ketoconazole, also act as EET receptor ligands competing for binding of 20-^{125}I-EE5ZE to U937 membranes.[30] Both miconazole and MS-PPOH block 14,15-EET-mediated relaxation of arterial rings. Thus, besides inhibiting cytochrome P450, these drugs, like 14,15-EE5ZE, act as EET antagonists. As a result, we tested the effect of these drugs on photolabeling. Miconazole completely blocked the photolabeling by 20-^{125}I-14,15-EE8ZE-APSA, while MS-PPOH and ketoconazole partially inhibited photolabeling (Figure 5). The inhibition by three structurally unrelated EET antagonists further suggests that the 47 kDa protein is an EET receptor or binding site.

Identification of the 47 kDa EET Receptor or Binding Site in Vascular Cells and Arteries. EETs relax vascular smooth muscle.[10,15] As with U937 cell membranes, 20-^{125}I-14,15-EE8ZE-APSA labeled a 47 kDa protein in membranes from rabbit

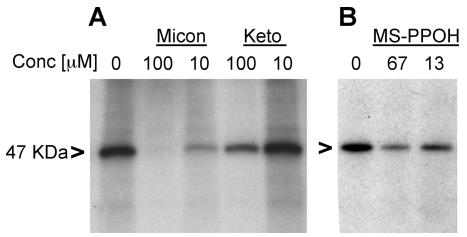

Figure 5. Inhibition of photolabeling by 20-^{125}I-14,15-EE8ZE-APSA of the 47 kDa protein in U937 cell membranes by three structurally unrelated EET receptor ligands, miconazole, ketoconazole, and MS-PPOH. Autoradiographs indicating (A) inhibition of labeling by various concentrations of miconazole and ketoconazole and (B) inhibition of labeling by various concentrations of MS-PPOH. The experiment was repeated four times. The 47 kDa protein is indicated with >.

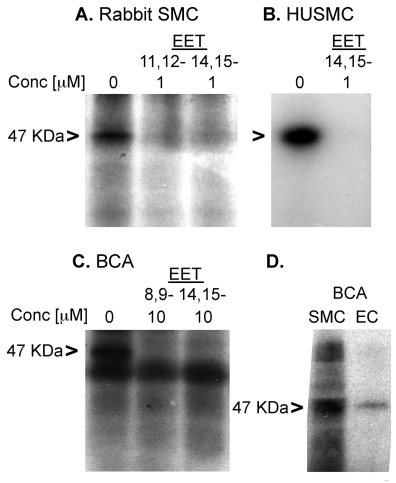

Figure 6. Photoaffinity labeling of the 47 kDa receptor in vascular cells and tissues. Autoradiography indicating photolabeling in (A) rabbit smooth muscle cell membranes (RSMC), (B) bovine coronary artery membranes (BCA), (C) human smooth muscle cells (HUSMC), and (D) bovine coronary artery endothelial cells and smooth muscle cells. 11,12-EET and 14,15-EET were used to inhibit photolabeling. Autoradiographs are shown. The 47 kDa positions are indicated with >.

smooth muscle cells (Figure 6A), human smooth muscle cells (Figure 6B), and bovine coronary artery (Figure 6C). The photolabeling was also inhibited by co-incubation with 8,9-EET, 11,12-EET, or 14,15-EET. A radiolabeled band was observed below the 47 kDa band in bovine coronary arterial membranes (Figure 6C). This labeling was not changed by co-incubation with 8,9-EET or 14,15-EET, so this protein represents a nonspecifically labeled protein (Figure 6C). The photolabeling in the bovine coronary arteries appears to be due to the presence of the binding site in both smooth muscle and endothelial cells. Photolabeling of the 47 kDa protein was detected in cultured bovine coronary endothelial cells and smooth muscle cells; however, the level of expression was much greater in smooth muscle cells than in endothelial cells (Figure 6D). The experiments revealed an EET receptor or binding site in smooth muscle cells and coronary arteries and to a lesser extent in endothelial cells. Photolabeling of the 47 kDa protein was also observed in membranes from the canine heart and rat kidney (data not shown).

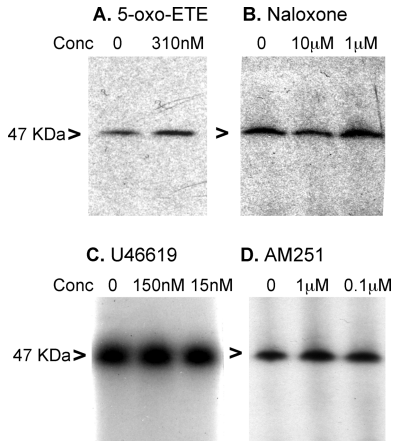

Figure 7. Effect of candidate receptor agonists and antagonists on photolabeling of U937 membranes with 20-^{125}I-14,15-EE8ZE-APSA. Candidate receptor ligands were used to compete with the photoprobe for labeling of the 47 kDa protein. U937 membranes were labeled with 1 nM 20-^{125}I-14,15-EE8ZE-APSA with or without (vehicle control) indicated ligands. Autoradiographs are shown for (A) 5-oxo-ETE, a 5-oxo-ETE receptor agonist, (B) naloxone, an opioid receptor antagonist, (C) U46619, a thromboxane (TP) receptor agonist, and (D) AM251, a cannabinoid (CB)-1 receptor antagonist. The 47 kDa protein is indicated with >.

Use of 20-^{125}I-14,15-EE8ZE-APSA To Screen Candidate Receptors. Ligand competition with 20-^{125}I-14,15-EE8ZE-APSA was used to screen receptors known to be present on U937 cell membranes.[35−38] Some of these receptors have been suggested to mediate the action of EETs.[24,39] Because the EET receptor has the characteristics of a GPCR,[14,29] only ligands for GPCRs were tested. The photolabeling of a 47 kDa protein in U937 cell membranes by 20-^{125}I-14,15-EE8ZE-APSA was not inhibited by 5-oxoeicosatetraenoic acid (a 5-oxo-ETE receptor agonist), naloxone (an opioid antagonist), U46619 (a TP receptor agonist), or AM251 (a CB1 receptor antagonist), suggesting these candidate receptors are not EET receptors (Figure 7).

GPCRs without known ligands or related to receptors with lipid ligands are likely candidates for the EET receptor.[40,41] The photoaffinity labeling method was used to screen 79 such receptors (Table 2). HEK293T cells were transiently transfected with one of 79 N-terminally FLAG-tagged GPCRs, and the expression of the receptor protein was confirmed by flow cytometry using a FLAG specific antibody. Vector-transfected HEK293T cell lysates were used as a blank control. Each of the 79 GPCR cell lysates and blank control were incubated with 20-^{125}I-14,15-EE8ZE-APSA, photolabeled, and analyzed via radioautography. Lysates from an equal number of U937 cells were used as a positive control. No radiolabeled bands were observed in any of the lysates of the 79 GPCR-expressing cells or the blank control. However, under identical conditions, U937 cells showed a single 47 kDa radiolabeled band. Thus, photolabeling detected the endogenous EET receptor in U937 cells but did not detect a receptor in HEK293T cells overexpressing GPCRs. Thus, the 79 GPCRs in Table 2 are not the EET receptor labeled with 20-^{125}I-14,15-EE8ZE-APSA.

DISCUSSION

While many physiological and biochemical activities have been described for the EETs,[4,7,42−44] the initiation step in the actions of EETs has not been defined. Several lines of evidence implicate a cell surface, high-affinity, G protein-coupled receptor as the initial step;[17,26,29,30] however, further characterization and identification of this receptor are needed. Photoaffinity labeling has been a useful approach to receptor identification. These studies describe the characterization of the first EET photoaffinity probe, 20-I-14,15-EE8ZE-APSA, and its use to characterize the EET receptor. 20-I-14,15-EE8ZE-APSA relaxed the bovine coronary artery with an ED_{50} similar to that of 14,15-EET and displaced the EET antagonist radioligand 20-^{125}I-14,15-EE5ZE from its binding site on U937 cell membranes with an IC_{50} similar to that of 14,15-EET. Thus, 20-I-14,15-EE8ZE-APSA is a high-affinity agonist ligand for the EET binding site or receptor. Importantly, this photoactive ligand can be radiolabeled with high specific activity.

When incubated with U937 membranes and cross-linked with UV light, 20-^{125}I-14,15-EE8ZE-APSA radiolabeled a single 47 kDa protein band. The radiolabeling of the 47 kDa protein was specific for the EET structure with EET agonists (8,9-EET, 11,12-EET, and 14,15-EET) and the structurally related EET antagonist (14,15-EE5ZE). In contrast, the inactive EET analogues 14,15-thiirane and 8,9-DHET did not inhibit the radiolabeling. The IC_{50} values of the EET analogues for inhibition of labeling by 20-^{125}I-14,15-EE8ZE-APSA suggest the 47 kDa protein is a high-affinity receptor for 11,12-EET and 14,15-EET but has a lower affinity for 8,9-EET and 14,15-EE5ZE. This affinity ranking order (11,12-EET = 14,15-EET > 8,9-EET = 14,15-EE5ZE and no binding for 8,9-DHET and 14,15-thiirane) is the same as that determined in radioligand binding assays and correlates with agonist activity.[15,29,30]

Epoxide hydrolase and cytochrome P450 inhibitors were screened for binding to the EET receptor using 20-^{125}I-14,

Table 2. Orphan Receptors Tested[a]

ADMR	GPR35	GPR88	GPR172A
CD97	GPR37L1	GPR89	GPR173
EB12	GPR40	GPR97	GPR174
EMR1	GPR41	GPR101	GPR175
ETLD1	GPR43	GPR107	GPR176
G2A	GPR45	GPR114	GPR182
GPR1	GPR52	GPR116	GRCA
GPR3	GPR56	GPR120	MAS1
GPR6	GPR61	GPR133	MAS1L
GPR12	GPR62	GPR135	MRGD
GPR15	GPR63	GPR139	MRGE
GPR18	GPR64	GPR143	MRGF
GPR19	GPR75	GPR146	MRGX3
GPR21	GPR78	GPR148	MRGX4
GPR22	GPR80	GPR151	OPN3
GPR25	GPR82	GPR153	P2RY8
GPR26	GPR83	GPR155	P2Y10
GPR31	GPR84	GPR160	PSGR
GPR32	GPR85	GPR161	RAIG3
GPR34	GPR87	GPR171	TM7SF1

[a] Abbreviations: ADMR, GPR182; EMR1, EGF-like module containing mucin-like hormone receptor-like 1; GPR, G protein-coupled receptor; MAS, Mas receptor; MRG, MAS-related GPR; OPN, opsin; P2RY, purinergic receptor P2Y; PSGR, olfactory receptor 51E2; RAIG3, retinoic acid-induced gene 3, GPR; TM7SF1, transmembrane 7 superfamily member 1.

15-EE5ZE as the radioligand.[30] Miconazole and MSPPOH inhibited binding with K_i values of 350 and 1558 nM, respectively. Ketoconazole (50 μM) inhibited binding by 50%. The concentrations of the drugs that inhibited binding of the EET radioligand differed from the concentrations that inhibited cytochrome P450.[30] Thus, inhibition of EET binding was not associated with cytochrome P450 inhibition. Other cytochrome P450 inhibitors such as sulfaphenazole and proadifen and epoxide hydrolase inhibitors did not alter binding of the radioligand. In addition, miconazole and MSPPOH blocked 14,15-EET-induced relaxation of bovine coronary arteries. Proadifen did not alter 14,15-EET relaxations. These studies indicate that MSPPOH and miconazole, besides inhibiting cytochrome P450, act as EET antagonists like 14,15-EE5ZE. Miconazole, MSPPOH, ketoconazole, and 14,15-EE5ZE inhibited 20-^{125}I-14,15-EE8ZE-APSA photolabeling of the 47 kDa protein. These data are consistent with our previous conclusion that these drugs are EET antagonists and suggest that the 47 kDa protein is the binding site for 20-^{125}I-14,15-EE5ZE and an EET receptor.

The initial characterization of the EET photoprobe used U937 cell membranes because this cell line has been a model system for studying the EET receptor.[26,29,30] However, the photolabeling of the 47 kDa protein by 20-^{125}I-14,15-EE8ZE-APSA was also observed in membranes from rabbit, bovine, and human vascular smooth muscle cells, bovine coronary artery endothelial cells, bovine coronary artery, canine heart, and rat kidney. As with U937 cells, this labeling was inhibited by EET agonists. Because EETs relax vascular smooth muscle,[10] inhibit the expression of adhesion molecules on endothelial cells,[4] alter renal tubular and vascular function,[45] and cause cardioprotection,[8] a protein involved in binding EETs and initiating EET actions would be expected to be present in these cells and tissues.

Several studies indicate the utility of the EET photoprobe in characterizing the EET receptor. These photolabeling experiments reveal for the first time that the EET binding site or receptor has a molecular mass of 47 kDa. This information may be used to eliminate a number of proteins that may bind EETs. For example, CYP2J and CYP2C synthesize EETs, fatty acid binding proteins and PPARs bind EETs, and soluble epoxide hydrolase converts EETs to DHETs.[4,18−20,46] However, these proteins cannot represent the EET binding site labeled by the photoprobe because the molecular masses of CYPs, PPARs, and epoxide hydrolase exceed 47 kDa and the masses of fatty acid binding proteins are less than 47 kDa. By the same logic, BK_{Ca} channel proteins, TRPV4 channels, the EP2 receptor, and the TP receptor can be eliminated[47−49] despite suggestions that these proteins may function as EET receptors.[24,50]

The photoprobe made EET receptor screening simple and applicable on a relatively large scale. With small amounts of protein, candidate EET receptor proteins can be tested directly for photolabeling. Additionally, competition by specific receptor agonists or antagonists for photolabeling by 20-^{125}I-14,15-EE8ZE-APSA can be used to eliminate other known receptors. For example, AM251 (a CB1 receptor antagonist), 5-oxo-ETE (a 5-oxo-ETE receptor agonist), naloxone (an opioid antagonist), and U46619 (a thromboxane receptor agonist) failed to inhibit photolabeling, thereby eliminating these receptors as possible EET receptors. Thus, while high concentrations of EETs may inhibit TP receptors or activate EP2 receptors,[23,24] the TP and EP receptors are not high-affinity EET receptors.

A large number of GPCRs have no known ligand and are termed orphan receptors.[40] Many of these GPCRs may bind lipid mediators.[41] For this reason, a group of 79 GPCRs that bind lipids, are related phylogenetically to lipid binding receptors, or are expressed in EET-responding tissues were tested for 14,15-EET binding by photolabeling. None of the 79 GPCRs were photolabeled by 20-^{125}I-14,15-EE8ZE-APSA. When U937 membranes were treated in an identical manner at the same time, the photoprobe labeled a 47 kDa protein. Because the method is capable of labeling and detecting the endogenous EET receptor in U937 cells, it should certainly detect an overexpressed GPCR that binds 14,15-EET. Thus, it appears that none of the 79 GPCRs represent the high-affinity EET receptor labeled by 20-I-14,15-EE8ZE-APSA. Of particular note, free fatty acid receptors and receptors that bind modified or oxidized fatty acids such as GPR2A, GPR18, GPR40, GPR41, GPR43, GPR84, and GPR120 were not labeled by the photoprobe.[51−53]

The photoprobe that is characterized herein is an analogue of 14,15-EET and may label only an EET receptor or receptors with high affinity for 14,15-EET and possibly 11,12-EET such as the 47 kDa protein. The failure to detect receptor labeling with 20-^{125}I-14,15-EE8ZE-APSA in HEK293T cells overexpressing orphan GPCRs does not exclude the possibility that these GPCRs are receptors for other EET regioisomers or low-affinity EET receptors. Photoprobes for the other EET regioisomers may be needed to identify other EET receptors.

In summary, 20-I-14,15-EE8ZE-APSA is the first EET photoaffinity probe and has EET agonist activity. This photoprobe labeled a 47 kDa high-affinity EET binding protein in U937 cells and vascular cells. Because photolabeling was inhibited by several EET agonists (8,9-EET, 11,12-EET, and 14,15-EET) and EET antagonists (14,15-EE5ZE, miconazole, ketoconazole, and MSPPOH), we propose this 47 kDa protein is a high-affinity EET receptor. Used in a same way as an antibody, the photoprobe could be a molecular marker for the EET receptor in normal cells and tissues and in disease and improve our understanding of the expression and regulation of the EET receptor.

■ ASSOCIATED CONTENT

Supporting Information. Methods for the synthesis of 20-OTs-14,15-EE8ZE-APSA and 20-I-14,15-EE8ZE-APSA. This material is available free of charge via the Internet at http://pubs.acs.org.

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■ ABBREVIATIONS

EET, epoxyeicosatrienoic acid; CYP, cytochrome P450; 14,15-EE8ZE, 14,15-*cis*-epoxyeicosa-8(*Z*)-enoic acid; 14,15-EE5ZE, 14,15-*cis*-epoxyeicosa-5(*Z*)-enoic acid; 20-I-14,15-EE8ZE-APSA, 20-iodo-14,15-epoxyeicosa-8(*Z*)-enoyl-3-azidophenylsulfonamide; 8,9-DHET, 8,9-dihydroxyeicosatrienoic acid; MSPPOH, *N*-(methylsulfonyl)-2-(2-propynyloxy)benzenehexanamide; U46619, 9,11-dideoxy-11α,9a-epoxymethanoprostaglandin F2α; EDRF, endothelium-derived relaxation factor; EDHF, endothelium-derived hyperpolarizing factor; 20-I-14,15-EE8ZE, 20-iodo-14,15-epoxyeicosa-8(*Z*)-enoic acid; OTs, 20-tosyl; HPLC, high-performance liquid chromatography; G protein, guanine nucleotide binding protein; GPCR, G protein-coupled receptor; BK_{Ca}, large conductance Ca^{2+}-activated K^+; TP, thromboxane prostanoid receptor; EP, prostaglandin E prostanaoid receptor; CB, cannabinoid.

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