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# Functional screening for G protein-coupled receptor targets of 14,15-epoxyeicosatrienoic acid

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ABSTRACT

Epoxyeicosatrienoic acids (EETs) are potent vasodilators that play important roles in cardiovascular physiology and disease, yet the molecular mechanisms underlying the biological actions of EETs are not fully understood. Multiple lines of evidence suggest that the actions of EETs are in part mediated via G protein-coupled receptor (GPCR) signaling, but the identity of such a receptor has remained elusive. We sought to identify 14,15-EET-responsive GPCRs. A set of 105 clones were expressed in *Xenopus* oocyte and screened for their ability to activate cAMP-dependent chloride current. Several receptors responded to micromolar concentrations of 14,15-EET, with the top five being prostaglandin receptor subtypes ($PTGER_2$, $PTGER_4$, PTGFR, PTGDR, $PTGER_3$IV). Overall, our results indicate that multiple low-affinity 14,15-EET GPCRs are capable of increasing cAMP levels following 14,15-EET stimulation, highlighting the potential for cross-talk between prostanoid and other ecosanoid GPCRs. Our data also indicate that none of the 105 GPCRs screened met our criteria for a high-affinity receptor for 14,15-EET.



**Contents**



*Abbreviations:* 14,15-EET (EET), 14,15-epoxyeicosatrienoic acids; 14,15-EEZE, 14,15-epoxyeicosa-5(Z)-enoic acid; VSMCs, vascular smooth muscle cells; GPCR, G protein-coupled receptor; CFTR, Cystic Fibrosis Transmembrane Conductance Regulator; PKA, Protein Kinase A; PTG, prostaglandin; $PGE_2$, prostaglandin E2; IMC, indomethacin; CF172, $CFTR_{inh}$-172 (5-[(4-carboxyphenyl)methylene]-2-thioxo-3-[(3-trifluoromethyl)phenyl-4-thiazolidinone; IBMX, 3-isobutyl-1-methylxanthine; TEVC, two-electrode-voltage clamp.

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## 1. Introduction

Epoxyeicosatrienoic acids (EETs) are endogenous vasodilators in multiple vascular beds [1,2]. Potency of 14,15-EET as a vasodilator varies depending on the tissue and vessel size. For example, in bovine coronary arteries and rat mesenteric arteries, 14,15-EET has been shown to induce vasodilation in micromolar range [2–8] while high potency (low picomolar) vasodilation by EETs has been observed in canine and porcine coronary microvessels [1,9]. We have previously demonstrated that 14,15-EET is abundant in brain tissue, playing critical roles in both normal function and disease [10].

Multiple ligand binding studies suggest the existence of a high affinity (low nanomolar) receptor for 14-15-EET [8,11–14]. To identify this putative high affinity receptor, we selected 150 candidate GPCRs based on their similarities to other lipid-sensing GPCRs. Only a subset of 105 receptors were successfully cloned and shown to display cell surface expression in *Xenopus* oocyte, which were subsequently screened for their ability to increase cAMP-dependent chloride current.

We focused on cAMP-based activity because it has been suggested that 14,15-EET exerts its vasodilator effect via Gs-coupled signaling leading to increased cAMP [8,12]. To monitor changes in cAMP in real time, we used the CFTR (Cystic Fibrosis Transmembrane Conductance Regulator) channel as a functional reporter of increased cAMP and activation of Protein Kinase A (PKA), as opening of CFTR channel requires phosphorylation by PKA [15,16]. In this case, the CFTR channel was co-expressed in an oocyte expression system along with each GPCR of interest. The utility of this method in studying GPCR signaling has been demonstrated previously [17–19]. We also employed ERK activation assay and a β-arrestin recruitment assay as alternative methods to detect GPCR activation by 14,15-EET. Our results indicate that while none of the candidate receptors tested met our criteria for a high affinity Gs-coupled receptor for 14,15-EET, we were able to identify several previously unknown low affinity 14,15-EET receptors.

## 2. Materials and methods

### 2.1. Mouse mesenteric artery diameter measurement

Mouse mesenteric arteries were isolated, cut into 1–2 mm in length and mounted on a single vessel chamber (CH-1 Living Systems Instrumentation, Burlington, VT), secured between two glass micropipettes and tied with two knots on each end. Vessels were maintained at a constant pressure (80 mmHg) using a pressure servo system (Living Systems Instrumentation, Burlington, VT), and superfused continuously with MOPS buffered solution (in mM: 144 NaCl, 3.0 KCl, 2.5 $CaCl_2$, 1.5 $MgSO_4$, 2.0 MOPS, 5.0 glucose, 2.0 Pyruvate, 0.02 EDTA, 1.2 $NaH_2PO_4$) with a flow rate of 2.5 mL/min. The temperature of the perfusion chamber was maintained at 37 °C using both stationary (TC-095, Living Systems Instrumentation, Burlington, VT) and inline heater (TC-344B, Warner Instruments, Hamden, CT). Vessel diameter was continuously monitored using a video dimension analyzer (Living Systems Instrumentation, Burlington, VT), digitized and recorded using AxonScope data acquisition software (Molecular Devices, Sunnyvale, CA)

### 2.2. HEK293 cell culture and transfection

Human Embryonic Kidney-293 cells (HEK 293; American Type Culture Collection, Manassas, VA) were grown in Dulbecco's modified Eagle's medium (DMEM, Invitrogen) supplemented with 10% fetal bovine serum (FBS). Cells were thawed, maintained in 5% $CO_2$ at 37 °C and used through passage 12. Cells grown in 12-well plates were transfected with 2.5 μg GPCR plasmids and Lipofectamine 2000 reagent (Invitrogen) at 50–70% confluency in media lacking antibiotics. Cells transfected with transfection solution alone without the plasmid served as controls.

### 2.3. GPCR vector construction

GPCR clones were obtained from DNASU Plasmid Repository (The Biodesign Institute, Arizona State University. Tempe, AZ) and UMR cDNA Resource Center (University of Missouri-Rolla, Rolla, MO). They were subcloned into a modified pcDNA3.1 vector containing both CMV and T7 promotors to permit both mammalian expression and in vitro transcription of each candidate receptor for oocyte expression. The expression vector also contains an in-frame hemagglutinin (HA) epitope tag that resulted in amino terminus tagging of each candidate receptor.

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