The biological actions of 11,12-epoxyeicosatrienoic acid in endothelial cells are specific to the R/S enantiomer and require the Gs protein.

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Recommended section assignment: Cardiovascular
Abstract

Cytochrome P450 (CYP)-derived epoxides of arachidonic acid, i.e., the epoxyeicosatrienoic acids (EETs), are important lipid signaling molecules involved in the regulation of vascular tone and angiogenesis. Since many actions of 11,12-EET are dependent on the activation of protein kinase A (PKA), the existence of a cell surface Gs-coupled receptor has been postulated. To assess whether the responses of endothelial cells to 11,12-EET are enantiomer specific and linked to a potential G protein-coupled receptor, we assessed 11,12-EET-induced, PKA-dependent translocation of transient receptor potential (TRP) C6 channels as well as angiogenesis. In primary cultures of human endothelial cells, (±)-11,12-EET led to the rapid (30 seconds) translocation a TRPC6V5 fusion protein, an effect reproduced by 11(R),12(S)-EET, but not 11(S),12(R)-EET or (±)-14,15-EET. Similarly, endothelial cell migration and tube formation were stimulated by (±)-11,12-EET and 11(R),12(S)-EET while 11(S),12(R)-EET and 11,12-dihydroxyeicosatrienoic acid were without effect. The effects of (±)-11,12-EET on TRP channel translocation and angiogenesis were sensitive to EET antagonists and TRP channel trafficking was also prevented by a PKA inhibitor. The siRNA-mediated downregulation of Gs in endothelial cells had no significant effect on responses stimulated by VEGF or a PKA activator but abolished responses to (±)-11,12-EET. The down regulation of Gq/11 failed to prevent 11,12-EET-induced TRPC6 channel translocation or the formation of capillary like structures. Taken together, our results suggest that a Gs-coupled receptor in the endothelial cell membrane responds to 11(R),12(S)-EET, mediates the PKA-dependent translocation and activation of TRPC6 channels, as well as angiogenesis.
Introduction

Cytochrome P450 (CYP) enzymes are membrane-bound, heme-containing terminal oxidases. Even though the majority of CYP enzymes are primarily expressed in the liver, several can be detected in the cardiovascular system and in inflammatory cells. Most is known about the cardiovascular actions of proteins belonging to the CYP4A, CYP2C, and CYP2J families. While \( \omega \)-hydroxylases like the CYP4A enzymes utilize arachidonic acid to generate the vasoconstrictor 20-hydroxyeicosatetraenoic acid, which is implicated in the regulation of myogenic tone and inflammation, the CYP2C and CYP2J epoxygenases generate epoxyeicosatrienoic acids (EETs) which possess vasodilator and anti-inflammatory properties (Spector, 2009; Imig, 2012; Imig, 2013).

In endothelial cells, CYP epoxygenases can generate four regioisomeric EETs: 5,6-EET, 8,9-EET, 11,12-EET, and 14,15-EET. Each regioisomer can exist as S/R and R/S enantiomers and both the production and vascular activity of these enantiomers can vary between vascular beds and species (Daikh, et al., 1994; Zeldin, et al., 1995). For example, 11(R),12(S)-EET is a more potent activator of renal artery calcium–activated potassium (K\(_{Ca}\)) (Zou, et al., 1996) and tracheal epithelial cells (Pascual, et al., 1998) than 11(S),12(R)-EET. This is, however, not a universal observation as the opposite has been reported regarding the selectivity of cardiac ATP-sensitive K\(^+\) channels (Lu, et al., 2002).

One characteristic of many EET-induced cellular responses such as cell proliferation, gap junctional communication or transient receptor potential (TRP) channel translocation is the dependence on protein kinase A (PKA) activation (Wong, et al., 2000; Fukao, et al., 2001; Popp, et al., 2002; Fleming, et al., 2007). Putting these findings together with reports of a high affinity binding site on cell membranes (Wong, et al., 1993; Wong, et al., 1997; Snyder, et al., 2002) the existence of a Gs-coupled, membrane bound EET receptor has been postulated (Yang, et al., 2008; Chen, et al., 2009; Chen, et al., 2011). The fact that a series of stable and specific EET agonists and antagonists has been generated (Gauthier, et al., 2002; Falck, et al., 2003a; Yang, et al., 2008) provides strong, indirect evidence supporting the concept of a membrane-bound EET receptor that recognizes defined structural components.
within the EETs. Moreover, biochemical studies measuring GTP binding to G proteins in endothelial cells confirm the importance of a G protein and indicate that 11,12-EET increases GTP\textsubscript{Y}\textsubscript{35S} binding to Gs, but not Gi proteins (Node, et al., 2001). The aim of this study, therefore, was to determine whether different effects of 11,12-EET observed in endothelial cells, i.e., on TRP channel translocation, migration and tube formation, demonstrate enantiomer specificity and whether or not these responses are dependent on Gs protein expression.

Materials and Methods

Materials. Cell culture media were purchased from Gibco (Invitrogen; Darmstadt, Germany). Growth factor-reduced Matrigel was obtained from BD Biosciences (Bedford, MA, USA). Adenosine 3',5'-cyclic monophosphorothioate, Rp-Isomer (Rp-cAMPS) and Sp-Isomer (Sp-cAMPS) were purchased from Alexis Biochemicals (Grünberg, Germany). 11,12-EET, 11,12-dihydroxyeicosatrienoic acid (DHET), 14,15-EET and 14,15-DHET were purchased from Cayman Chemical Co. (Ann Arbor, MI, USA). The soluble epoxide hydrolase (sEH) inhibitor trans-4-[4-(3-adamantan-1-yl-ureido)-cyclohexyloxy]-benzoic acid (t-AUCB) was kindly provided by Dr. Bruce Hammock (UC Davis, CA). All other chemicals were purchased from Sigma (Deisenhofen, Germany).

The stereo-isomers of 11,12-EET were prepared by resolving a racemic mixture (±) of 11,12-EET (Cayman Chemical, Ann Arbor, MI, USA) by chiral-phase HPLC on a Chiralcel OD column (Daicel, Illkirch, France) as described (Zhang and Blair, 1994). The recovered 11(R),12(S)-EET and 11(S),12(R)-EET were then quantified by LC-MS/MS (Arnold, et al., 2010). The EET antagonists 14,15-epoxyeicosa-5(Z)-enoic acid (14,15-EEZE) and 11,12,20-trihydroxy-eicosa-8(Z)-enoic acid (THE8ZE) were synthesized as described (Falck, et al., 2003b; Bukhari, et al., 2012).

Endothelial Cells. Human umbilical vein endothelial cells were isolated and purified using VE-cadherin (CD144) antibody-coated magnetic beads (Dynal Biotech, Hamburg, Germany) and cultured as reported previously (Bess, et al., 2011). The human umbilical cords were
obtained from local hospitals in Frankfurt am Main and the use of human material in this study conforms to the principles outlined in the Declaration of Helsinki. The isolation of human cells was approved by the ethics committee at the Goethe University in Frankfurt am Main.

HUV-EC-c cells were purchased from American Type Culture Collection (Manassas, VA, USA) and cultured in Ham’s F-12K medium containing 8% fetal calf serum, ECGS (12 μg/ml) and heparin (100 μg/ml; PromoCell, Heidelberg, Germany), penicillin (1 unit/ml), streptomycin (1 μg/ml) and gentamicin (4 μg/ml).

TRPC6-V5 Overexpression. The generation of the adenoviral expression vector (pAdTrack-CMV), the recombination with the adenoviral backbone and the amplification and propagation of the viruses were performed as described (He, et al., 1998; Fleming, et al., 2007). After 40 hours, the cells were transferred to serum-free medium and stimulated as described in the results section.

Immunohistochemistry. Cultured endothelial cells were grown in 8 well-chambers (Ibidi, Martinsried, Germany) coated with fibronectin and stimulated as described in the results section. Thereafter, cells were fixed in paraformaldehyde (4% in PBS, 15 minutes), permeabilized with Triton X-100, blocked and stained with mouse anti-V5 monoclonal antibody (Invitrogen) and the respective secondary antibodies (Alexa546 conjugated anti-goat, Invitrogen). In some experiments endogenously expressed TRPC6 was visualized with an appropriate antibody (Sigma; dilution 1:50). The preparations were viewed using a confocal microscope (LSM510 META, Zeiss).

siRNA-Mediated Downregulation of G Proteins. To target Gs protein expression, a mixture of two oligonucleotides (Hs_GNAS_5 and Hs_GNAS_6, Qiagen, Hilden, Germany) were used, while Gq and G11 proteins were each targeted with one specific siRNA (siRNAGq and siRNAG11; Qiagen). In all experiments, a scrambled siRNA probe (Ambion, Darmstadt, Germany) was used as control. Transfection was performed using lipofectamine RNAiMax (Invitrogen, Darmstadt, Germany) according to the manufacturer’s protocol. After 48 hours in culture, the cells were used to study either migration or tube formation. In some
experiments, the endothelial cells were adenovirally transduced with GFP/TRPC6-V5 4 hours after siRNA treatment.

**Isolation of RNA and RT-qPCR.** Total RNA from cultured primary cells and cell line was extracted using a RNA easy kit (Qiagen, Hilden, Germany) and equal amounts (1 µg) of total RNA were reverse transcribed (Superscript III, Invitrogen, Heidelberg, Germany). mRNA levels were determined using primers as shown below and were detected using SYBR Green (Absolute QPCR SYBR Green Mix; Thermo Scientific, Hamburg, Germany). The relative expression level of mRNA was normalized against GAPDH mRNA expression ($\Delta\Delta$CT method).

<table>
<thead>
<tr>
<th>Forward</th>
<th>Reverse</th>
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<tbody>
<tr>
<td>Gs</td>
<td>5'-GCAGAAGGACAAGCAGGTCT-3'</td>
</tr>
<tr>
<td>Gq</td>
<td>5'-GACTACTTCCCAGAATATGATGGAC-3'</td>
</tr>
<tr>
<td>G11</td>
<td>5'-GCATCCAGGAATGCTACGAC-3'</td>
</tr>
<tr>
<td>GAPDH</td>
<td>5'-ATGACATCAAGAAGGTGGTG-3'</td>
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**Immunoblotting.** For immunoblotting, cells were lysed in Triton X-100 buffer, and cell supernatants were heated with SDS-PAGE sample buffer and separated by SDS-PAGE as described (Fleming, et al., 2005).

**Scratch Wound Migration Assay.** A wound maker (Essen Bioscience; Welwyn Garden City, United Kingdom) was used to generate a uniform wound in the endothelial cell monolayer in 96 well plates, and cell migration was recorded by an automated microscope system (IncuCyte, Essen Bioscience) for up to 24 hours. Wound images were analyzed using IncuCyte 2010A software.

**Tube Formation.** The ability of endothelial cells to form capillary-like structures was assessed as previously described (Zippel, et al., 2013). Briefly, primary cultures of endothelial cells (1x10⁴ cells) where seeded onto angiogenesis microscope slides (µ-Ibidi, Martinsried, Germany) coated with Matrigel. Cells were cultured in EBM supplemented with 5% FCS and incubated at 37°C, 5% CO₂ for 24 hours in an IncuCyte imaging system (Essen
Bioscience) that took photographs automatically every 30 minutes. The formation of vessel-like tubes at 4 hours after cell seeding was assessed by AxioVision Rel.4.8 software (Carl Zeiss, Jena, Germany).

**Statistical Analysis.** Data are expressed as the mean ± SEM. Statistical evaluation was performed with Student’s t test for unpaired data, one-way ANOVA followed by a Bonferroni t test, or ANOVA for repeated measures where appropriate. Values of $P<0.05$ were considered statistically significant.

**Results**

**Pharmacological Characteristics of 11,12-EET-induced TRPC6 Translocation.** As a model system for detecting the activation of the putative 11,12-EET receptor, we assessed the rapid (30 seconds) translocation of the TRPC6 channel from the perinuclear Golgi apparatus to the plasma membrane. In primary cultures of endothelial cells that were transduced with an adenovirus encoding GFP as well as a TRPC6-V5 fusion protein, the channel localized exclusively to the perinuclear Golgi apparatus, as previously described (Fleming, et al., 2007). The application of a racemic mixture of 11,12-EET (1 µM, 30 seconds) induced the rapid translocation of the TRPC6 channel from the perinuclear site to the plasma membrane (Fig. 1A), specifically to clusters in the plasma membrane that also co-stained for the caveolar marker protein caveolin 1 (Supplemental Figure 1A). The response elicited by (±)-11,12-EET was reproduced by 11(R),12(S)-EET while 11(S),12(R)-EET and (±)-14,15-EET were without effect (Fig. 1B). A similar rapid translocation of the endogenous TRPC6 channel was also detected in primary human endothelial cells (Supplemental Figure 1B).

The (±)-11,12-EET-induced TRPC6 channel translocation was abolished by the EET antagonist, 14,15-epoxyeicosa-5(Z)-enoic acid (14,15-EEZE) as well as by the CYP inhibitor miconazole (Fig. 1C), that was recently found to compete with EET for binding to a specific membrane site and thus act as a receptor antagonist (Chen, et al., 2009; Chen, et al., 2011). 11,12-EET-induced TRPC6 channel translocation was abolished in presence of the competitive PKA inhibitor; Rp-cAMPs. Similar results were obtained using the HUV-EC-c cell
line (Supplemental Figure 2) which expressed neither CYP2C enzymes nor the sEH, and again 14,15-EEZE, miconazole and Rp-cAMPs prevented the (±)11,12-EET-induced TRPC6 channel translocation.

**Pharmacological Characteristics of 11,12-EET-induced Angiogenesis.** CYP-derived EETs promote angiogenesis in vitro and in vivo (Medhora, et al., 2003; Michaelis, et al., 2003; Michaelis, et al., 2005). To investigate the potential role of an 11,12-EET receptor in the actions of the epoxide, endothelial cell migration and the ability to form tube-like structures on Matrigel were determined.

In a scratch-wound model (±)-11,12-EET stimulated endothelial cell migration to an extent comparable with that of vascular endothelial cell growth factor (VEGF). Again, 11(R),12(S)-EET elicited a similar response while 11(S),12(R)-EET and 11,12-DHET were without effect (Fig. 2A). As the (±)-11,12-EET is a 50:50 mixture of the R/S and S/R enantiomers, a more detailed concentration–response analysis was performed. This revealed that the maximal response was obtained using a concentration of 30 nM 11(R),12(S)-EET while 100 nM and higher concentrations of (±)-11,12-EET were required to elicit a comparable effect (Supplemental Figure 3). Also in this case, the 11,12-EET antagonists; 11,12,20-THE8ZE (Bukhari, et al., 2012), and miconazole effectively prevented the 11,12-EET-induced endothelial cell migration, as did PKA inhibition (Fig. 2B). 14,15-EEZE slightly attenuated endothelial cell migration in the presence of solvent and also prevented the 11,12-EET-induced migration.

Next, the angiogenic effect of 11,12-EET was assessed by studying the formation of capillary-like endothelial cell tubes on Matrigel. When (±)-11,12-EET was administered to primary cultures of human endothelial cells there was a significant increase in the formation of capillary-like structures that was comparable with the response elicited by VEGF. The latter effect could be reproduced using 11(R),12(S)-EET while 11(S),12(R)-EET and (±)-11,12-DHET were ineffective (Fig. 3A). Consistent with the previous results on TRPC6 channel translocation, miconazole, 14,15-EEZE, 11,12,20-THE8ZE and Rp-cAMPs effectively prevented the (±)-11,12-EET-induced tube formation (Fig. 3B).
EETs are rapidly hydrolyzed to their corresponding diols by soluble epoxide hydrolase (sEH), and stereoselectivity for hydration has been reported with 11(S),12(R)-EET being hydrolyzed faster than 11(R),12(S)-EET (Zeldin, et al., 1995). To determine whether or not the inactivity of 11(S),12(R)-EET could be attributed to its more rapid hydration, experiments were repeated in the presence of a sEH inhibitor (t-AUCB). sEH inhibition failed to influence the formation of capillary-like structures by any of the 11,12-EET preparations used (Supplemental Figure 4).

Consequences of Gs Downregulation on 11,12-EET-induced TRPC6 Channel Translocation. To determine whether or not the 11,12-EET-induced translocation of TRPC6 channels was dependent on the expression of Gs proteins, primary cultures of endothelial cells were treated with either a control siRNA or siRNAs directed against Gs, or Gq/11. Forty eight hours after transfection, there was a significant knockdown of the target mRNAs and proteins (Fig. 4A&B). When TRPC6-V5-expressing cells treated with the control siRNA were exposed to (±)-11,12-EET, a rapid translocation of TRPC6 channels was observed (Fig. 4C). While the downregulation of Gq/11 had no effect on the response to (±)-11,12-EET, no translocation was detected in cells lacking the Gs protein. Even in cell batches where there was a very marked overexpression of the TRPC6-V5 fusion protein, the channel remained absent from the membrane (Supplemental Figure 5). The PKA activator, Sp-cAMPs, was just as effective as 11,12-EET in inducing TRPC6 channel translocation under control conditions and was unaffected by the downregulation of either Gs or Gq/11 (Fig. 4C). Comparable responses were recorded using the HUV-EC-c cell line (Supplemental Figure 6).

Consequences of Gs Downregulation on 11,12-EET-induced Angiogenesis. To determine whether or not 11,12-EET-induced angiogenesis was also dependent on the expression of Gs, primary cultures of human endothelial cells were treated with either control siRNA or siRNAs against Gs or Gq/11, then stimulated with (±)-11,12-EET or VEGF.

While a control siRNA or siRNA-directed against Gq/11 had no effect on endothelial cell migration, the downregulation of Gs slightly attenuated wound healing under control conditions. However, the increase in migration elicited by (±)-11,12-EET that was observed in
the control siRNA and siRNA treated cells was not observed in cells lacking the Gs protein (Fig. 5A). VEGF-stimulated endothelial cell migration was observed in all three treatment groups. Similarly, the downregulation of Gs attenuated the 11,12-EET-induced formation of endothelial cell tubes, while the knockdown of Gq/11 had no effect (Fig. 5D, E&F). In this assay, none of the treatments influenced the response to VEGF.

Discussion

The results of this study demonstrate that the translocation of TRPC6 channels as well as the migration and tube formation elicited by 11,12-EET in endothelial cells are a selective response to the 11(R),12(S)-EET enantiomer and sensitive to 11,12-EET antagonists. Moreover, all of these responses were dependent on the expression of the Gs protein and the activation of PKA and thus display the pharmacological sensitivity expected with the activation of the putative 11,12-EET receptor.

Vascular biologists and physiologists became increasingly interested in the CYP-derived epoxides of arachidonic acid as vasodilators underlying nitric oxide- and prostacyclin-independent vasodilatation in resistance sized arteries (Garland, et al., 2011). KCa channels were the first reported targets of the EETs and both 11,12-EET and 14,15-EET were shown to increase the open probability of large conductance KCa (BK) channels in vascular smooth muscle cells - ultimately resulting in smooth muscle cell hyperpolarization and relaxation (Campbell, et al., 1996). Thereafter, followed demonstrations that the inhibition and down-regulation of CYP2C epoxygenases attenuated the CYP-dependent vasodilator responses in conductance (Fisslthaler, et al., 1999) as well as resistance arteries (Bolz, et al., 2000).

Putting together evidence from a series of studies, it seems that the EET-induced activation of BK channels is not simply the result of the direct binding of the epoxide to an extracellular domain of the channel. For example, while 11,12-EET activates BK channels in cell-attached patches of smooth muscle cells and endothelial cells, it is without effect in inside-out patches. Such observations imply that a cytosolic component or cellular signaling pathway that is absent from inside-out patches is required for EET-stimulated responses. The missing components may well be guanine nucleotide binding (G) proteins and GTP as
the addition of GTP to the cytoplasmic surface of inside-out patches restored the ability of 11,12-EET to open the BK channel. Moreover, BK channel activation by EETs can be prevented by the G protein inhibitor GDPβS as well as by an antibody directed against the Gs protein (Li and Campbell, 1997; Hayabuchi, et al., 1998; Fukao, et al., 2001).

There is a wealth of additional information that supports the concept of a Gs coupled transmembrane receptor for the EETs: (1) High affinity EET binding sites have been described on the surface of some cells (Yang, et al., 2008; Chen, et al., 2009; Pfister, et al., 2010; Chen, et al., 2011). (2) There are differences in the ability of different EET stereoisomers, and not just the different regio-isomers, to elicit biological effects. (3) It has been possible to design and generate a series of stable and specific EET agonists and antagonists (Gauthier, et al., 2002; Falck, et al., 2003a; Yang, et al., 2008). (4) many of the biological actions of the EETs are dependent on the activation of PKA (Wong, et al., 2000; Fukao, et al., 2001; Popp, et al., 2002; Fleming, et al., 2007; Loot, et al., 2012).

The EETs exert a wide variety of effects on vascular endothelial cells including enhanced intracellular communication and cellular processes associated with angiogenesis. Indeed, increased EET production seems to be required for full responsiveness to VEGF (for review see Michaelis and Fleming, 2006). These actions have been linked to the transactivation of the epidermal growth factor receptor (Chen, et al., 2002; Michaelis, et al., 2003), as well as different kinases such as extracellular regulated kinases 1/2 (Wang, et al., 2005), Akt (Chen, et al., 2001; Fleming, et al., 2001; Pozzi, et al., 2005), the AMP-activated protein kinase (Webler, et al., 2008; Xu, et al., 2010), and the forkhead factors FOXO1 and FOXO3a (Potente, et al., 2003). Whether or not these effects can be attributed to the activation of a specific EET receptor or are the consequence of the activation of an intracellular EET-accessible molecule are unknown. In our experience 11,12-EET exerts more consistent effects on endothelial cell signaling and angiogenesis than other regio-isomers such as 14,15-EET. Thus, the current investigation was focused on determining whether 11,12-EET-dependent responses in endothelial cells were specifically activated by one regio-isomer.
Since a receptor-mediated effect of 11,12-EET would be expected to be rapid, it was essential to choose a quick and robust read out of cell activation. Assessing changes in cAMP levels proved unsuitable as, although many of the effects of the EETs rely on PKA activation, the 11,12-EET-induced changes in cAMP tend to be small and inconsistent – particularly in endothelial cells. However, the 11,12-EET stimulated translocation of the TRPC3 and TRPC6 channels in endothelial cells as well as pulmonary vascular smooth muscle cells is a rapid event, i.e., evident within 10 to 30 seconds after cell stimulation (Fleming, et al., 2007; Keserü, et al., 2008). Therefore, TRPC6 channel translocation was chosen as a biological indicator of the activation of the proposed 11,12-EET receptor. Because of the notorious unselectively of available TRP channel antibodies, experiments were performed in endothelial cells overexpressing a TRPC6-V5 fusion protein that can be easily detected via the V5 tag. Also, as the expression of G protein coupled receptors is not always stable in cell culture, experiments were mostly performed using primary cultures of human endothelial cells and confirmed in an endothelial cell line. Using this experimental system, it was possible to demonstrate that the translocation of TRPC6 can be induced by a racemic mixture of 11,12-EET as well as the purified 11(R),12(S)-EET enantiomer while 11(S),12(R)-EET, 14,15-EET and 11,12-DHET were ineffective. Moreover, the caveolar translocation of TRPC6 induced by (±)-11,12-EET was prevented by two structural EET homologues that act as specific EET antagonists. As responses were also abolished by a cAMP analogue, the 11,12-EET-induced translocation of TRP channels bears all the hallmarks of a response requiring a Gs coupled structure that recognizes specific structural aspects within the fatty acid epoxide. To address this further, responses to (±)-11,12-EET were studied in endothelial cells following the siRNA-mediated downregulation of Gs or Gq/11 proteins and, while the lack of Gq/11 failed to alter response to the EET, TRPC6 channel translocation was abolished in the absence of Gs proteins. The cells studied were able to respond normally to other stimuli as the TRP channel translocation induced by a PKA activator was unaffected by the downregulation of either Gq/11 or Gs.
While a rapid effect on an ion channel may be mediated by a specific EET receptor, the longer term effects on endothelial cell migration and angiogenesis may be mediated by alternative mechanisms and perhaps the involvement of intracellular fatty acid receptors such as the peroxisome proliferator activated receptors (Liu, et al., 2005; Ng, et al., 2007; Wray, et al., 2009)? To address this, the effects of the 11,12-EET enantiomers, EET antagonists and PKA inhibitor on endothelial cell migration and tube forming capacity were assessed. Also in these models, endothelial cell activation was restricted to 11(R),12(S)-EET. The EETs generated by the CYP epoxygenases are tightly regulated by the activity of the sEH which metabolizes the epoxides to the corresponding diols and stereoselectivity for hydration has been reported (Zeldin, et al., 1993; Zeldin, et al., 1995). Therefore, to determine whether or not the lack of effect of the 11(S),12(R)-EET could be attributed to its rapid removal, experiments were repeated in the presence of a sEH inhibitor. Preventing EET metabolism did not render 11(S),12(R)-EET capable of stimulating endothelial cell tube formation and failed to potentiate responses to 11(R),12(S)-EET. The angiogenesis response stimulated by (±)-11,12-EET were also prevented by PKA inhibition, the antagonists 14,15-EZEE and THE8ZE as well as by the CYP inhibitor miconazole, that was recently found to compete with EET for binding to a specific membrane site and, thus, act as a potential receptor antagonist (Chen, et al., 2009; Chen, et al., 2011). Moreover, the down regulation of Gs proteins failed to affect migration and tube formation in endothelial cells stimulated by VEGF but abolished responses to (±)-11,12-EET. All of these observations suggest that the 11,12-EET-activated angiogenic response, like that of the TRP channel translocation, requires the presence of a specific Gs coupled receptor for 11,12-EET.

While the molecular identity of the putative 11,12-EET receptor is currently unknown, it already seems possible that the effects of different members of the family CYP/sEH-derived lipid signaling mediators may activate different receptors. For example, the observation that the inhibition of the forskolin-stimulated increase in cAMP could be prevented by high concentrations of 11,12-EET via a mechanism that was dependent on the activity of the sEH and Gi proteins, indicates that 11,12-DHET may antagonize the effects of its parent epoxide.
via a Gi protein coupled mechanism (Abukhashim, et al., 2011). Are then the biological responses elicited by 11,12- EETs a balance of the activation of an epoxide activated Gs-coupled receptor and a Gi coupled 11,12-DHET receptor? It will be interesting to determine whether or not diseases such as hypertension, in particular hypertension associated with the activation of the renin angiotensin system and increased sEH expression, reflect a shift towards Gi-dependent signaling. However, in the current study 11,12-DHET consistently failed to affect 11,12-EET-induced angiogenesis in endothelial cells and is, thus, not able to account for the observations outlined above.
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Authorship Contributions.

Participated in research design: Ding, Frömel, Fleming.

Conducted experiments: Ding, Frömel, Popp.

Contributed new reagents or analytic tools: Falck, Schunck.

Performed data analysis: Ding, Frömel, Popp.

Wrote or contributed to the writing of the manuscript: Ding, Falck, Schunck, Fleming.
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Footnotes

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Figure legends

Fig 1. Characterization of the 11,12-EET induced translocation of TRPC6-V5 in endothelial cells. (A) Representative images showing the localization of GFP (green) and V5 (red) in primary cultures of human endothelial cells adenovirally transduced with GFP and TRPC6-V5 before exposure to solvent (Sol; 0.3% DMSO) or (±)-11,12-EET (1 µM) for 30 seconds; blue = phalloidin, bar = 20µm. (B) Comparison of the abilities of (±)-11,12-EET, 11(R),12(S)-EET (R/S), 11(S),12(R)-EET (S/R) and 14,15-EET (all 1 µM) to induce the translocation of TRPC6-V5. (C) Effect of 14,15-EEZE (1 µM), miconazole (3 µM) and Rp-cAMPs (10 µM) on the (±)-11,12-EET-induced translocation of the TRPC6 channel. The graphs summarize the data from 3 different cell batches, each studied in triplicate and analyzing 100 cells per repetition; **P<0.01 versus solvent.

Fig. 2. Characterization of the 11,12-EET-induced cell migration in endothelial cells. (A) Effects of solvent (Sol; 0.3%DMSO), (±)-11,12-EET, 11(R),12(S)-EET (R/S), 11(S),12(R)-EET (S/R), 11,12-DHET (all 5 µM) and VEGF (30 ng/ml) for 24 hours on the migration of primary cultures of endothelial cells in a scratch wound assay. The representative images show the size of the initial wound (black), the remaining wound (white) and the distance migrated after 12 hours (gray). (B) Effects of miconazole (Micon; 10 µM), 14,15-EEZE (EEZE; 5 µM), 11,12,20-THE8ZE (ZHE8ZE; 5 µM) and Rp-cAMPs (10 µM) on (±)-11,12-EET-induced cell migration (24 hours). The graphs summarize the data from 3-4 different cell batches, each studied in triplicate; **P<0.01, ***P<0.001 versus solvent; #P<0.05, ##P<0.01, ###P<0.001 versus (±)-11,12-EET.

Fig. 3. Characterization of 11,12-EET-induced tube formation in endothelial cells. (A) Comparison of the ability of solvent (Sol; 0.3% DMSO) (±)-11,12-EET, 11(R),12(S)-EET (R/S), 11(S),12(R)-EET (S/R), 11,12-DHET (each 5 µM) and VEGF (18 ng/ml) for 4 hours to induce tube formation in endothelial cells. (B) Effect of miconazole (10 µM), 14,15-EEZE (5 µM), 11,12,20-THE8ZE (5 µM) and Rp-cAMPs (10 µM) on (±)-11,12-EET induced tube formation (4 hours). The graphs summarize the data from 3-6 different cell batches, each studied in triplicate; **P<0.01, ***P<0.001 versus solvent.
Fig. 4. Effect of Gs and Gq/11 downregulation on the 11,12-EET-induced translocation of TRPC6 channels. Effect of a control siRNA (siCTL) or siRNA directed against Gs (siGs) or Gq/11 (siGq/11) on G protein (A) mRNA and (B) protein expression in primary cultures of endothelial cells. (C) TRPC6-V5 localisation in cells treated with solvent (Sol; 0.3% DMSO), (±)-11,12-EET (1 μM, 30 seconds) or Sp-cAMPs (10 μM, 1 hour); green = GFP; red = V5; blue = phalloidin; bar = 10 μm. The graphs summarize the data from 3 independent experiments, each performed in triplicate; **P<0.01, ***P<0.001 versus siCTL or the respective solvent-treated group.

Fig. 5. Consequences of Gs and Gq/11 downregulation on 11,12-EET induced endothelial cell migration and tube formation in vitro. (A) Effects of solvent (Sol; 0.3% DMSO), (±)-11,12-EET, and VEGF (30 ng/ml) for 24 hours in cells pre-treated with either a control siRNA (siCTL) or siRNA directed against Gs (siGs) or Gq/11 (siGq/11) on the migration of primary cultures of endothelial cells in a scratch wound assay. The representative images show the size of the initial wound (black), the remaining wound (white) and the distance migrated after 12 hours (gray). (B) Effects of control siRNA or the downregulation of Gs or Gq/11 proteins on the tube formation recorded 4 hours after stimulation with solvent, (±)-11,12-EET (5 μM) or VEGF (18 ng/ml). The graphs summarize data from 3-6 different cell batches, each studied in triplicate; *P<0.05, ***P<0.001 versus the appropriate Sol/siCTL group.
Fig. 1

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Fig. 2

A

Solvent (±)-EET

VEGF R/S-EET

DHET S/R-EET

B

Sol ±11,12-EET VEGF

Wound closure (×Sol)

Sol Micon EEZE THE8ZE Rp-cAMPS

VEGF

Wound closure (×Sol)

Sol Micon EEZE THE8ZE Rp-cAMPS VEGF

Wound closure (×Sol)
Fig. 3

(A) Sol, (±)-11,12-EET, DHET, VEGF, R/S-EET, S/R-EET.

(B) Sol, (±)-11,12-EET, Micon, 14,15-EEZE, THE8ZE, Rp-cAMPs.

Tube Length (mm)

Sol, VEGF, ±, RS, SR, DHET.

Sol, Rp, Micon, EEZE, THE8ZE.

** ** ***
A

![Graph A](image)

B

![Graph B](image)

C

![Images C](image)

Fig. 4
Fig. 5