The Biological Actions of 11,12-Epoxyeicosatrienoic Acid in Endothelial Cells Are Specific to the *R/S*-Enantiomer and Require the G_s Protein^[S]

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ABSTRACT

Cytochrome P450–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. Because many actions of 11,12-*cis*-epoxyeicosatrienoic acid (EET) are dependent on the activation of protein kinase A (PKA), the existence of a cell-surface G_s-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 TRPC6-V5 fusion protein, an effect reproduced by 11(*R*),12(*S*)-EET, but not by 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, whereas 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 small interfering RNA-mediated down-regulation of G_s in endothelial cells had no significant effect on responses stimulated by vascular endothelial growth or a PKA activator but abolished responses to (±)-11,12-EET. The down-regulation of G_q/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 G_s-coupled receptor in the endothelial cell membrane responds to 11(*R*),12(*S*)-EET and mediates the PKA-dependent translocation and activation of TRPC6 channels, as well as angiogenesis.

Introduction

Cytochrome P450 (P450) enzymes are membrane-bound, heme-containing terminal oxidases. Even though most P450 enzymes are expressed primarily 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. Whereas ω -hydroxylases such as the CYP4A enzymes use arachidonic acid to generate the vasoconstrictor 20-hydroxy-eicosatetraenoic 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, 2013).

In endothelial cells, P450 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 is 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

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ABBREVIATIONS: 14,15-EEZE, 14,15-epoxyeicosa-5(*Z*)-enoic acid; BK, large conductance calcium-activated K⁺ channels; DHET, *vic*-dihydroxyeicosatrienoic acid; EET, *cis*-epoxyeicosatrienoic acids; GFP, green fluorescent protein; K_{Ca} channels, calcium-activated potassium channels; P450, cytochrome P450; PKA, protein kinase A; sEH, soluble epoxide hydrolase; siRNA, small interfering RNA; *t*-AUCB, *trans*-4-[4-(3-adamantan-1-yl-ureido)-cyclohexyloxy]-benzoic acid; THE8ZE, 11,12,20-trihydroxy-eicosa-8(*Z*)-enoic acid; TRP, transient receptor potential; VEGF, vascular endothelial growth factor.

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, 1997; Snyder et al., 2002), the existence of a G_s-coupled, membrane-bound EET receptor has been postulated (Yang et al., 2008; Chen, et al., 2009, 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 [³⁵S]guanosine 5'-O-(3-thio)triphosphate binding to G_s, but not G_i proteins (Node et al., 2001). Therefore, the aim of this study 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 these responses are dependent on G_s protein expression.

Materials and Methods

Cell culture media were purchased from Gibco (Invitrogen, Darmstadt, Germany). Growth factor-reduced Matrigel was obtained from BD Biosciences (Bedford, MA). Rp-isomer cAMPs and Sp-isomer 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). 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 (University of California Davis, Davis, CA). All other chemicals were purchased from Sigma-Aldrich (Deisenhofen, Germany).

The stereoisomers of 11,12-EET were prepared by resolving a racemic mixture (±) of 11,12-EET (Cayman Chemical) by chiral-phase high-performance liquid chromatography 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 liquid chromatography–tandem mass spectrometry (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.

A human umbilical vein endothelial cell–based cell line (HUV-EC-c cells) were purchased from American Type Culture Collection (Manassas, VA) and cultured in Ham's F-12K medium containing 8% fetal calf serum, ECGS (12 μg/ml), heparin (100 μg/ml; PromoCell, Heidelberg, Germany), penicillin (1 U/ml), streptomycin (1 μg/ml), and gentamicin (4 μg/ml).

TRPC6-V5 Overexpression. The generation of the adenoviral expression vector (pAd-Track-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 eight-well chambers (Ibidi, Martinsried, Germany) coated with

fibronectin and stimulated as described under *Results*. Thereafter, cells were fixed in paraformaldehyde (4% in phosphate-buffered saline, 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 (dilution 1:50; Sigma-Aldrich). The preparations were viewed using a confocal microscope (LSM510 META; Zeiss, Jena, Germany).

Small Interfering RNA-Mediated Downregulation of G Proteins. To target G_s protein expression, a mixture of two oligonucleotides (Hs_GNAS_5 and Hs_GNAS_6; Qiagen, Hilden, Germany) were used, whereas G_q and G11 proteins were each targeted with one specific small interfering (si) RNA (siRNA_{Gq} and siRNA_{G11}; Qiagen). In all experiments, a scrambled siRNA probe (Ambion, Darmstadt, Germany) was used as control. Transfection was performed using Lipofectamine RNAiMax (Invitrogen) 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 green fluorescent protein (GFP)/TRPC6-V5 4 hours after siRNA treatment.

Isolation of RNA and Reverse Transcription-Quantitative Polymerase Chain Reaction. Total RNA from cultured primary cells and cell line was extracted using a RNA easy kit (Qiagen), and equal amounts (1 μg) of total RNA were reverse-transcribed (Superscript III; Invitrogen). Messenger RNA levels were determined using the primers described in Table 1. They were detected using SYBR Green (Absolute QPCR SYBR Green Mix; Thermo Fisher Scientific, Hamburg, Germany). The relative expression level of mRNA was normalized against glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA expression (ΔΔC_T method).

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, UK) 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 (1 × 10⁴ cells) were seeded onto angiogenesis microscope slides (μ-Ibidi, Martinsried, Germany) coated with Matrigel. Cells were cultured in endothelial cell basal medium supplemented with 5% fetal calf serum 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 4 hours after cell seeding was assessed by AxioVision Rel.4.8 software (Carl Zeiss GmbH, Jena, Germany).

Statistical Analysis. Data are expressed as the mean ± S.E.M. Statistical evaluation was performed with Student's *t* test for unpaired data, one-way analysis of variance, followed by Bonferroni's *t* test or analysis of variance for repeated measures where appropriate. Values of *P* < 0.05 were considered statistically significant.

Results

Pharmacologic 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

TABLE 1

Messenger RNA levels were determined using the primers G_s, G_q, G11, and GAPDH

Primers	Forward	Reverse
G _s	5'-GCAGAAGGACAAGCAGGTCT-3'	5'-CAATGGTTTCAATCGCCTCT-3'
G _q	5'-GACTACTTCCCAGAATATGATGGAC-3'	5'-GGTTCAGGTCCACGAACATC-3'
G11	5'-GCATCCAGGAATGCTACGAC-3'	5'-GGTCAACGTCGGTCAGGTAG-3'
GAPDH	5'-ATGACATCAAGAAGGTGGTG-3'	5'-CATACCAGGAAATGAGCTTG-3'

Golgi apparatus, as previously described (Fleming et al., 2007). The application of a racemic mixture of 11,12-EET (1 μ M, 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 contained for the caveolar marker protein caveolin 1 (Supplemental Fig. 1A). The response elicited by ($\pm$)-11,12-EET was reproduced by 11(*R*),12(*S*)-EET, whereas 11(*S*),12(*R*)-EET and ($\pm$)-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 Fig. 1B).

The ($\pm$)-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 P450 inhibitor miconazole (Fig. 1C), which 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, 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 Fig. 2), which expressed neither CYP2C enzymes nor the sEH and, again, 14,15-EEZE, miconazole, and Rp-cAMPs prevented the ($\pm$)-11,12-EET-induced TRPC6 channel translocation.

Pharmacological Characteristics of 11,12-EET-Induced Angiogenesis. P450-derived EETs promote angiogenesis in vitro and in vivo (Medhora et al., 2003; Michaelis et al., 2003,

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 tubelike structures on Matrigel were determined.

In a scratch-wound model ($\pm$)-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, whereas 11(*S*),12(*R*)-EET and 11,12-DHET were without effect (Fig. 2A). As the ($\pm$)-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, whereas 100 nM and higher concentrations of ($\pm$)-11,12-EET were required to elicit a comparable effect (Supplemental Fig. 3). In addition, 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 ($\pm$)-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

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% dimethylsulfoxide) or ($\pm$)-11,12-EET (1 μ M) for 30 seconds; blue = phalloidin, bar = 20 μ m. (B) Comparison of the abilities of ($\pm$)-11,12-EET, 11(*R*),12(*S*)-EET (*R/S*), 11(*S*),12(*R*)-EET (*S/R*), and 14,15-EET (all 1 μ M) to induce the translocation of TRPC6-V5. (C) Effect of 14,15-EEZE (1 μ M), miconazole (3 μ M), and Rp-cAMPs (10 μ M) on the ($\pm$)-11,12-EET-induced translocation of the TRPC6 channel. The graphs summarize the data from three 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% dimethylsulfoxide), (±)-11,12-EET, 11(R),12(S)-EET (R/S), 11(S),12(R)-EET (S/R), 11,12-DHET (all 5 μ M), 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 μ M), 14,15-EEZE (EEZE; 5 μ M), 11,12,20-THE8ZE (THE8ZE; 5 μ M), and Rp-cAMPs (10 μ M) on (±)-11,12-EET-induced cell migration (24 hours). The graphs summarize the data from three or four 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.

latter effect could be reproduced using 11(R),12(S)-EET, whereas 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 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 Fig. 4).

Consequences of G_s Downregulation on 11,12-EET-Induced TRPC6 Channel Translocation. To determine whether the 11,12-EET-induced translocation of TRPC6 channels was dependent on the expression of G_s proteins, primary cultures of endothelial cells were treated with either a control siRNA or siRNAs directed against G_s or $G_q/11$. Forty-eight hours after transfection, there was a significant knockdown of the target mRNAs and proteins (Fig. 4, A and 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). Although the downregulation of $G_q/11$ had no effect on the response to

(±)-11,12-EET, no translocation was detected in cells lacking the G_s protein. Even in cell batches where there was a marked overexpression of the TRPC6-V5 fusion protein, the channel remained absent from the membrane (Supplemental Fig. 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 G_s or $G_q/11$ (Fig. 4C). Comparable responses were recorded using the HUV-EC-c cell line (Supplemental Fig. 6).

Consequences of G_s Downregulation on 11,12-EET-Induced Angiogenesis. To determine whether 11,12-EET-induced angiogenesis was also dependent on the expression of G_s , primary cultures of human endothelial cells were treated with either control siRNA or siRNAs against G_s or $G_q/11$ and then stimulated with (±)-11,12-EET or VEGF.

Although a control siRNA or siRNA-directed against $G_q/11$ had no effect on endothelial cell migration, the downregulation of G_s 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 G_s protein (Fig. 5A). VEGF-stimulated endothelial cell migration was observed in all three treatment groups. Likewise, the downregulation of G_s attenuated the 11,12-EET-induced formation of endothelial cell tubes, whereas the knockdown of $G_q/11$ had no effect (Fig. 5, D–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 these responses were dependent on the expression of the G_s 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 P450-derived epoxides of arachidonic acid as vasodilators underlying nitric oxide- and prostacyclin-independent vasodilation in resistance sized arteries (Garland et al., 2011). K_{Ca} 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 K_{Ca} (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 downregulation of CYP2C epoxygenases attenuated the P450-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, whereas 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

Fig. 3. Characterization of 11,12-EET-induced tube formation in endothelial cells. (A) Comparison of the ability of solvent [Sol; 0.3% dimethylsulfoxide] (±)-11,12-EET, 11(*R*),12(*S*)-EET (*R/S*), 11(*S*),12(*R*)-EET (*S/R*), 11,12-DHET (each 5 μ M), and VEGF (18 ng/ml) for 4 hours to induce tube formation in endothelial cells. (B) Effect of miconazole (10 μ M), 14,15-EEZE (5 μ M), 11,12,20-THE8ZE (5 μ M), and Rp-cAMPs (10 μ M) on (±)-11,12-EET-induced tube formation (4 hours). The graphs summarize the data from three to six different cell batches, each studied in triplicate. ** P < 0.01; *** P < 0.001 versus solvent.

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 GDP β S, as well as by an antibody directed against

the G_s 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 G_s-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.,

Fig. 4. Effect of G_s and G_q/11 downregulation on the 11,12-EET-induced translocation of TRPC6 channels. Effect of a control siRNA (siCTL) or siRNA directed against G_s (siGs) or G_q/11 (siGq/11) on G protein mRNA (A) and protein expression (B) in primary cultures of endothelial cells. (C) TRPC6-V5 localization in cells treated with solvent (Sol; 0.3% dimethylsulfoxide), (±)-11,12-EET (1 μ M, 30 seconds) or Sp-cAMPs (10 μ M, 1 hour); green = GFP; red = V5; blue = phalloidin; bar = 10 μ m. The graphs summarize the data from three independent experiments, each performed in triplicate. ** P < 0.01; *** P < 0.001 versus siCTL or the respective solvent-treated group.

Fig. 5. Consequences of G_s and G_{q/11} down-regulation on 11,12-EET induced endothelial cell migration and tube formation in vitro. (A) Effects of solvent (Sol; 0.3% dimethylsulfoxide), (±)-11,12-EET, and VEGF (30 ng/ml) for 24 hours in cells pretreated with either a control siRNA (siCTL) or siRNA directed against G_s (siGs) or G_{q/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 G_s or G_{q/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 three to six different cell batches, each studied in triplicate. **P* < 0.05; ****P* < 0.001 versus the appropriate Sol/siCTL group.

2009, 2011; Pfister et al., 2010). 2) There are differences in the ability of different EET stereoisomers and not just the different regioisomers, to elicit biologic 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 biologic 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 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 is unknown. In our experience, 11,12-EET exerts more consistent effects on endothelial cell signaling and angiogenesis than other regioisomers such as 14,15-EET. Thus, the current investigation focused on determining whether 11,12-EET-dependent responses in endothelial cells were specifically activated by one regioisomer.

Because a receptor-mediated effect of 11,12-EET would be expected to be rapid, it was essential to choose a quick and robust readout 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 10–30 seconds after cell stimulation) (Fleming et al., 2007; Keserü

et al., 2008). Therefore, TRPC6 channel translocation was chosen as a biologic indicator of the activation of the proposed 11,12-EET receptor. Because of the notorious unselectivity 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. By use of 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, whereas 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 homologs that act as specific EET antagonists. As responses were also abolished by a cAMP analog, the 11,12-EET-induced translocation of TRP channels bears all the hallmarks of a response requiring a G_s-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 after the siRNA-mediated downregulation of G_s or G_{q/11} proteins, and although the lack of G_{q/11} failed to alter response to the EET, TRPC6 channel translocation was abolished in the absence of G_s 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 G_{q/11} or G_s.

Although 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. In these models, endothelial cell activation was restricted to 11(*R*),12(*S*)-EET. The EETs generated by the P450 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, 1995). Therefore, to determine whether 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 ($\pm$)-11,12-EET were also prevented by PKA inhibition, the antagonists 14,15-EZEE and THE8ZE, as well as by the P450 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, 2011). Moreover, the downregulation of G_s proteins failed to affect migration and tube formation in endothelial cells stimulated by VEGF but abolished responses to ($\pm$)-11,12-EET. All these observations suggest that the 11,12-EET-activated angiogenic response, similar to that of the TRP channel translocation, requires the presence of a specific G_s -coupled receptor for 11,12-EET.

Although 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 P450/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 G_i proteins, indicates that 11,12-DHET may antagonize the effects of its parent epoxide via a G_i protein-coupled mechanism (Abukhashim et al., 2011). Are then the biologic responses elicited by 11,12-EETs a balance of the activation of an epoxide activated G_s -coupled receptor and a G_i -coupled 11,12-DHET receptor? It will be interesting to determine whether diseases such as hypertension, in particular hypertension associated with the activation of the renin angiotensin system and increased sEH expression, reflect a shift toward G_i -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 herein.

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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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