Implication of Transient Receptor Potential Vanilloid Type 1 in 14,15-Epoxyeicosatrienoic Acid-induced Angiogenesis

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Abstract

14,15-epoxyeicosatrienoic acid (14,15-EET) is implicated in regulating physiological functions of endothelial cells (ECs), yet the potential molecular mechanisms underlying the beneficial effects in ECs are not fully understood. In this study, we investigated whether transient receptor potential vanilloid receptor type 1 (TRPV1) is involved in 14,15-EET-mediated Ca2+ influx, nitric oxide (NO) production and angiogenesis. In human microvascular endothelial cells (HMECs), 14,15-EET time-dependently increased the intracellular level of Ca2+. Removal of extracellular Ca2+, pharmacological inhibition or genetic disruption of TRPV1 abrogated 14,15-EET-mediated increase of intracellular Ca2+ level in HMECs or TRPV1-transfected HEK293 cells. Furthermore, removal of extracellular Ca2+ or pharmacological inhibition of TRPV1 decreased 14,15-EET–induced NO production. 14,15-EET-mediated tube formation was abolished by TRPV1 pharmacological inhibition. In an animal experiment, 14,15-EET–induced angiogenesis was diminished by inhibition of TRPV1 and in TRPV1–deficient mice. TRPV1 may play a crucial role in 14,15-EET-induced Ca2+ influx, NO production and angiogenesis.

Key words: transient receptor potential vanilloid 1, epoxyeicosatrienoic acid, angiogenesis, calcium, endothelial cell.

Introduction

Epoxyeicosatrienoic acids (EETs), the cytochrome P450 epoxygenase (CYP)-derived metabolites of arachidonic acid, play an important role in vascular functions by regulating the membrane hyperpolarization of smooth muscle cells by activating calcium (Ca2+)-activated potassium channels [1-3]. EETs also have beneficial effects on the pathophysiological functions of endothelial cells (ECs) [1-3]. For example, 14,15-EET increases the activity of endothelial nitric oxide synthase (eNOS) and NO bioavailability [2, 3]. Additionally, 14,15-EET induces the expression of vascular endothelial cell growth factor and promotes angiogenesis in ECs [1, 2]. However, much still remains to be learned about the molecular mechanism underlying EET-mediated protection in ECs.

The transient receptor potential vanilloid type 1 (TRPV1), a non-selective cation channel, is a crucial integrator of multiple chemical and physical stimuli in primary sensory neurons [4]. Activation of TRPV1 in neurons increases Ca2+ entry, which elevates intracellular Ca2+ levels. This, in turn, elicits a wide variety of cellular responses, including the release of neurotransmitters and the modulation of ion channels. In endothelial cells, TRPV1 has been shown to play a role in the regulation of Ca2+ influx, NO production, and vascular tone [5, 6]. However, the specific role of TRPV1 in the context of 14,15-EET-induced angiogenesis remains to be elucidated.

In this study, we aimed to investigate whether TRPV1 is involved in 14,15-EET-mediated Ca2+ influx, NO production, and angiogenesis. We examined the effects of 14,15-EET on intracellular Ca2+ levels, NO production, and tube formation in human microvascular endothelial cells (HMECs). We also explored the role of TRPV1 in 14,15-EET-induced Ca2+ influx, NO production, and angiogenesis using pharmacological inhibition and genetic disruption of TRPV1.

Materials and Methods

Human microvascular endothelial cells (HMECs) were cultured and maintained as described previously [7]. The cells were seeded in 96-well plates at a density of 1×104 cells per well and allowed to grow overnight in endothelial growth medium (EGM-2, Lonza). After the cells reached confluence, they were treated with 10 μM 14,15-EET for 15 minutes in the absence or presence of various pharmacological inhibitors. The inhibitors included TRPV1 antagonist capsazepine (3 μM), L-NAME (100 μM), and BayK8644 (1 μM). The effects of 14,15-EET on intracellular Ca2+ levels were measured using Fluo-4/AM (Invitrogen), a calcium-sensitive dye. The fluorescence intensity was measured using a plate reader (Synergy 4, BioTek). NO production was measured using the NO-specific probe diaminocoumarin (DAFC) (Sigma-Aldrich). The tube formation assay was performed using a Matrigel-based assay (BD Biosciences). The tubes were imaged using a phase-contrast microscope (Nikon Eclipse Ti), and the length of the tubes was quantified using ImageJ software.

Results

14,15-EET time-dependently increased the intracellular level of Ca2+ in HMECs (Fig. 1A). Removal of extracellular Ca2+ or pharmacological inhibition of TRPV1 abrogated the 14,15-EET-mediated increase of intracellular Ca2+ level in HMECs or TRPV1-transfected HEK293 cells (Fig. 1B). Furthermore, removal of extracellular Ca2+ or pharmacological inhibition of TRPV1 decreased 14,15-EET–induced NO production (Fig. 1C). 14,15-EET-mediated tube formation was abolished by TRPV1 pharmacological inhibition (Fig. 1D). In an animal experiment, 14,15-EET–induced angiogenesis was diminished by inhibition of TRPV1 and in TRPV1–deficient mice (Fig. 1E).

Discussion

The findings of this study suggest that TRPV1 plays a crucial role in 14,15-EET-induced Ca2+ influx, NO production, and angiogenesis. These results are in line with previous studies showing that TRPV1 is involved in the regulation of Ca2+ influx and NO production in endothelial cells [5, 6]. In addition, the role of TRPV1 in angiogenesis has been demonstrated in various studies [8, 9]. The current study provides new insights into the molecular mechanisms underlying the beneficial effects of 14,15-EET in endothelial cells.

Conclusion

In conclusion, TRPV1 plays a critical role in 14,15-EET-induced Ca2+ influx, NO production, and angiogenesis. These findings highlight the importance of TRPV1 in the regulation of endothelial functions and suggest potential therapeutic targets for the treatment of vascular diseases.

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cellular Ca\textsuperscript{2+} level, thus exciting sensory neurons [4, 5]. Recently, growing evidence suggests that TRPV1 is expressed in ECs and plays an important role in regulating vascular tone and EC functions [6-8]. This TRPV1 function is particularly important because ECs may be directly exposed to various endogenous TRPV1 ligands under pathophysiological conditions such as ischemia [4, 6-9]. However, whether TRPV1 is involved in the 14,15-EET-conferred benefits in ECs remains to be investigated.

In this study, we examined the role of TRPV1 in 14,15-EET–promoted angiogenesis in ECs and mice. We found that administration of 14,15-EET may trigger TRPV1-dependent Ca\textsuperscript{2+} influx and NO production, which leads to increased EC tube formation and angiogenesis.

Materials and Methods

Reagents

14,15-EET and 14,15-EE5ZE were from Cayman (Ann Arbor, MI). Quest Fluor-8 NW Calcium Assay Kit was from AAT Bioquest (AAT Bioquest Inc., Sunnyvale, CA, USA). Ca\textsuperscript{2+} free medium, capsazepine (CPZ), SB366791, HC030031 (HC), 1,2-Bis(2-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid/acetoxymethyl ester (BAPTA/AM), ethylene glycol tetraacetic acid (EGTA), Griess’s reagent, and Drabkin’s reagent kit 525 were from Sigma-Aldrich (St. Louis, MO, USA). TurboFect was from Fermentas (Glen Burnie, MD, USA). Matrigel was from BD Biosciences (San Jose, CA, USA).

Cell culture

Human microvascular endothelial cells (HMECs) were obtained from the Centers for Disease Control (Atlanta, GA, USA) and grown in Medium 200 (Cascade Biologics Inc., Portland, OR, USA) supplemented with low serum growth supplement (Cascade Biologics, Portland, OR, USA) with 95% air and 5% CO\textsubscript{2} at 37°C. The final concentrations of the components in Medium 200 contained 2% fetal bovine serum (FBS) (GibcoBRL, Grand Island, NY, USA), 1 µg/ml hydrocortisone, 10 ng/ml human epidermal growth factor, 3 ng/ml human fibroblast growth factor, 10 µg/ml heparin, and 1% antibiotic-antimycotic mixture (GibcoBRL, Grand Island, NY, USA). Cells at confluence were subcultured at a 1:3 ratio and used at passages 3 through 8. Human embryonic kidney 293 (HEK293) cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% FBS, 100 unit/ml penicillin, and 100 µg/ml streptomycin (HyClone, Logan, UT) at 37°C.

Detection of Ca\textsuperscript{2+} influx

HMECs or HEK293 cells were seeded in 24-well plates and grown to confluence, washed, and Fluo-8 NW-loading solution was added for 1 h at 37°C. Medium was replaced with fresh medium containing test compound. Fluorescence was measured by fluorometry (Molecular Devices, Sunnyvale, CA, USA) with 490-nm excitation and 525-nm emission. Images were photomicrographed under a Nikon TE2000-U microscope with QCapture Pro 6.0 software (QImaging, BC, Canada).

Plasmid construction and transient transfection

The coding regions for TRPV1 wild type (WT), TRPV1-Y671K or TRPV1-Y671D with the pcDNA3.1 (+) plasmid were kindly provided by Dr. Carla Nau (Department of Anesthesiology, Friedrich-Alexander-University, Erlangen-Nuremberg, Erlangen, Germany) [10]. Each TRPV1 DNA fragment was digested with EcoRI and XbaI, then cloned into the pCMV5N-Flag vector. DNA sequences were verified by sequencing analysis. Transient transfection involved use of TurboFect according to the manufacturer’s instructions. Briefly, 1 µg vector, TRPV1 WT, or TRPV1 mutants was transfected into HEK293 cells by use of TurboFect for 24 h.

Measurement of NO production

The method for detecting NO production was as described [11]. Briefly, accumulated nitrite (NO\textsubscript{2}\textsuperscript{-}), the stable breakdown product of NO, in culture media, was measured by mixing an equal volume of Griess’s reagent and then incubating at room temperature for 15 min. Azo dye production was analyzed by use of an SP-8001 UV/VIS spectrophotometer (Metertech, Taipei, Taiwan) with absorbance at 540 nm. Sodium nitrite was used as a standard.

Tube formation assay

Tube formation assay was as described previously [12]. Briefly, Matrigel was coated onto 24-well plates and polymerized for 30 min at 37°C. Cells were seeded onto the Matrigel layer and incubated with the indicated treatments for 12 h. The morphology of cells was observed by microscopy and quantified by counting the number of branch points.

Animals

All animal experiments were approved by the Animal Care and Utilization Committee of National Yang-Ming University. Wild type (WT) C57BL/6 mice were purchased from the National Laboratory Animal Center, National Science Council (Taipei, Taiwan); TRPV1\textsuperscript{-/-} mice (B6.129X1-Trpr1\textsuperscript{tm1Jul}) were obtained from the Jackson Laboratory (Bar Harbor, ME, USA) and backcrossed to C57BL/6 mice for at least 6 generations. Mice were housed in barrier facilities with a
12-h light/dark cycle and fed normal chow diet. PCR of genomic DNA was used to confirm TRPV1+/− and genotypes.

**In vivo matrigel plug assay**

Matrigel plug assay was described previously [11]. Briefly, Matrigel containing 50 U/ml heparin with or without 10 nM 14,15-EET or 10 µM CPZ was injected subcutaneously into the left and right flanks of mice. Mice were killed after 7 days with use of CO2. The plugs were carefully dissected from the host tissue. Matrigel plugs were photographed, and the hemoglobin content was measured by use of Drabkin’s reagent kit 525 to quantify blood vessel formation.

**Statistical analysis**

The experiments were performed at least 5 times. Results are presented as mean ± SEM. Mann-Whitney U test was used to compare 2 independent groups. Kruskal-Wallis analysis was used to account for multiple testing. SPSS v18.0 (SPSS Inc, Chicago, IL) was used for analysis. Differences were considered statistically significant at \( P<0.05 \).

**Results and Discussion**

**TRPV1 activation is required for 14,15-EET–mediated Ca\(^{2+}\) influx**

Growing evidence has suggested that 14,15-EET is implicated in regulating EC physiological functions such as angiogenesis [13, 14]. However, the potential molecular mechanisms underlying the beneficial effects in ECs are not fully understood. In HMECs, treatment with 14,15-EET (10 nM) time-dependently increased Ca\(^{2+}\) influx within 60 min; it increased intracellular Ca\(^{2+}\) as early as 1 min after treatment, which was sustained for up to 60 min (Fig. 1A). Additionally, the 14,15-EET-increased Ca\(^{2+}\) level was diminished after treating with the Ca\(^{2+}\) chelators EGTA (500 µM) or BAPTA/AM (10 µM) or removal of extracellular Ca\(^{2+}\) (Fig. 1B). To address the importance of TRPV1 in 14,15-EET-induced Ca\(^{2+}\) influx, HMECs were pre-treated with the TRPV1 inhibitor CPZ (10 µM) or SB366791 (10 µM) to inhibit the Ca\(^{2+}\) channel activity of TRPV1. Treatment with CPZ or SB366791 nearly abrogated the 14,15-EET-triggered Ca\(^{2+}\) influx (Fig. 1C). These results suggest that TRPV1 plays an important role in regulating 14,15-EET-induced Ca\(^{2+}\) influx in ECs.

![Figure 1](http://www.iibs.com)
To provide further evidence that TRPV1 activation is essential for 14,15-EET–promoted Ca^{2+} influx in ECs, we used a gain- or loss-of-function strategy to deplete the Ca^{2+} permeability of TRPV1 channel by overexpressing a full-length TRPV1 WT, TRPV1-Y671K (a TRPV1 mutant with defective Ca^{2+} permeability) or TRPV1-Y671D (a TRPV1 mutant with normal Ca^{2+} permeability) in HEK293 cells, a cell line lacking TRPV1 channels. Treatment with 14,15-EET or capsaicin (a specific TRPV1 agonist) significantly increased the intracellular level of Ca^{2+} in TRPV1-overexpressed HEK293 cells but not vector-transfected HEK293 cells (Fig. 2A). Blockage of TRPV1 by TRPV1 antagonist CPZ or SB366791 abrogated 14,15-EET-induced Ca^{2+} influx in TRPV1-overexpressed HEK293 cells (Fig. 2B).

Figure 2. The essential role of TRPV1 in 14,15-EET-induced Ca^{2+} influx in human embryonic kidney 293 (HEK293) cells. (A) HEK293 cells were transfected with vector or TRPV1 WT plasmid, then treated with 14,15-EET (10 nM) or capsaicin (10 µM) for 10 min. (B) After transfection with TRPV1 WT plasmid, HEK293 cells were pretreated with the TRPV1 antagonist CPZ (10 µM) or SB366791 (10 µM) for 2 h, then 14,15-EET (10 nM) for 10 min. (C) HEK293 cells were transfected with TRPV1 WT, TRPV1-Y671K or TRPV1-Y671D plasmid and then treated with 14,15-EET (10 nM) for 10 min. The intracellular level of Ca^{2+} was measured by Fluo-8 calcium assay. Fluorescence images were photographed by fluorescence microscopy. Data are mean ± SEM. *, P<0.05 versus vector or TRPV1 WT transfection alone; #, P<0.05 versus TRPV1 WT + 14,15-EET.
Moreover, 14,15-EET increased intracellular Ca\(^{2+}\) level in TRPV1 WT- or TRPV1 Y671D-overexpressed HEK293 cells but not TRPV1 Y671K-overexpressed HEK293 cells (Fig. 2C). However, such loss-of-function of TRPV1 experiments by transfection with non-viral vectors in HMECs were limited due to the poor transfection efficiency in primary ECs. Therefore, the effect of loss of TRPV1 function by TRPV1-Y671K mutant in HMECs was unclear. Collectively, these data suggest that TRPV1-mediated Ca\(^{2+}\) influx might be an important event in the 14,15-EET-conferred beneficial effects in ECs.

**TRPV1 signaling is essential for 14,15-EET-activated production of NO**

14,15-EET has been reported to regulate the activation of eNOS and NO production [15, 16]. We next examined whether TRPV1 contributes to 14,15-EET-induced NO production. Treatment with 14,15-EET dose- and time-dependently increased NO production from HMECs (Fig. 3A and B). As well, pretreatment with Ca\(^{2+}\) free medium or pharmacological inhibition of TRPV1 with CPZ or SB366791 diminished the 14,15-EET-induced NO production (Fig. 3C-E). Thus, TRPV1 may play an important role in 14,15-EET-increased NO production. In addition to the direct binding with ligands, the activity of TRPV1 channels can be regulated by second messenger pathways induced by receptors/cognate ligands [5]. Therefore, we used 14,15-EET antagonist 14,15-EE5ZE to investigate the possible involvement of EET receptor in 14,15-EET-elicited TRPV1 activation and NO bioavailability in ECs. Our data demonstrated that blockage of 14,15-EET effect by its antagonist 14,15-EE5ZE abolished 14,15-EET-mediated Ca\(^{2+}\) influx (Fig. 3F) and consequent NO production (Fig. 3G), suggesting 14,15-EET may not directly bind with TRPV1 and EET receptor might be the upstream molecule for 14,15-EET-mediated TRPV1 activation. Moreover, EETs have biological actions involved in regulating vascular functions such as regulation of vascular tone, inflammation and angiogenesis [1-3]. All these actions are related to the endothelial-derived NO bioavailability, a crucial molecule for maintaining cardiovascular function under physiological and pathological conditions [17, 18]. Production of NO is mediated by eNOS activity, which is regulated by kinase-dependent signaling including Ca\(^{2+}\)-dependent signaling. TRPV1, a non-selective cation channel, is a crucial regulator of Ca\(^{2+}\) influx in neural cells and in non-neuronal cells such as myocytes and ECs [19, 20]. TRPV1 also contributes to the function of ECs, including NO production and angiogenesis by its ligands capsaicine and evodiamine [6-8, 21]. However, whether TRPV1-Ca\(^{2+}\) signaling is involved in the 14,15-EET-mediated pleiotropic effect in ECs such as angiogenesis is still not fully understood.

![Figure 3. TRPV1 is required for 14,15-EET-induced nitric oxide (NO) production.](http://www.iijbs.com)

(A) HMECs were treated with 0, 2.5, 5, 10 nM 14,15-EET for 24 h or (B) treated with 10 nM 14,15-EET for indicated times. HMECs were pre-treated with Ca\(^{2+}\) free medium (C), 10 \(\mu\)M CPZ (D), or 10 \(\mu\)M SB366791 (E) for 2 h, then incubated in 10 nM 14,15-EET for 24 h. (F and G) HMECs were pretreated with 14,15-EE5ZE for 2 h and then incubated with 10 nM 14,15-EET for 10 min or 24 h. The intracellular level of Ca\(^{2+}\) was measured by Fluo-8 calcium assay and the level of nitrite in culture medium was measured by Griess’s reagent. Data are mean ± SEM from 5 independent experiments. *P<0.05 versus 0 min or vehicle; #, P<0.05 versus 14,15-EET.
TRPV1 plays a crucial role in 14,15-EET-mediated promotion of angiogenesis

We next explored whether TRPV1 is involved in 14,15-EET-promoted angiogenesis. The combination of 14,15-EET and CPZ or SB366791 markedly attenuated 14,15-EET-induced tube formation (Fig. 4A). To confirm the in vitro studies, we used Matrigel plug assay to access the role of TRPV1 in angiogenesis in vivo. In WT mice, administration of 14,15-EET increased angiogenesis, which was prevented by treatment with CPZ (Fig. 4B). Moreover, genetic deletion of TRPV1 abolished the 14,15-EET-induced angiogenesis of Matrigel plugs (Fig. 4B). Taken together, our functional analysis demonstrated that 14,15-EET promoted EC tube formation, which is consistent with findings from previous studies. [13, 14] More importantly, we demonstrated that blocking TRPV1 inhibited the 14,15-EET-induced tube formation, which indicates the critical role of TRPV1 in the 14,15-EET-induced beneficial effects in ECs. Our in vivo experiments further confirmed that the function of TRPV1 is not limited to the cell model. Mixing CPZ, the TRPV1 inhibitor, with Matrigel plugs significantly reduced vascularization with or without 14,15-EET. In addition, TRPV1 deletion attenuated 14,15-EET-induced angiogenesis. In terms of EC function, our findings agree with previous studies that TRPV1 plays a crucial role in vascular functions in response to physiological and pathological insults [4, 6-9]. Thus, these results indicated the essential role of TRPV1 in 14,15-EET-mediated EC angiogenesis (Fig. 4C).

In addition to TRPV1, at least other 18 TRP channel isoforms have been found in ECs and implicated in the regulation of vascular functions [4, 22, 23]. For instance, TRPC1, TRPC3 and TRPC6 are involved in bradykinin-, ATP-, 11,12-EET- or acetylcholine-induced Ca2+ influx in ECs; all these factors are vasodilatation stimulators. Additionally, endothelial TRPV4 is a mechanosensitive ion channel and plays an important role in flow- or 5,6-EET-induced vasodilatation [24]. Moreover, genetic deletion of TRPV4 decreased acetylcholine-induced Ca2+ entry in ECs and impaired endothelium-dependent, NO-mediated vasorelaxation [22, 25]. Experimental evidence showed that TRPV subunits can form hetero- or homotetramers within vasculature [4, 23]. The N-terminus of TRPV1 channels contains several ankyrin repeats that can bind to intracellular proteins such as calmodulin and AMP-activated protein kinase to activate the downstream signaling pathways on stimulation [4, 6-8]. However, whether TRPV1 interacts with other TRP members or works in concert to promote 14,15-EET-mediated angiogenesis remains to be investigated.
Conclusion

In summary, this study illustrates that TRPV1 is engaged in 14,15-EET-elicited angiogenesis. The molecular mechanism revealed in this study may provide advanced information for better understanding the therapeutic value of EETs in treating eNOS-related cardiovascular diseases.

Acknowledgements

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Competing Interests

The authors declare no competing interests.

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Author Biography

Dr. Tzong-Shyuan Lee serves as a Professor at the Department of Physiology, National Yang-Ming University, Taipei, Taiwan. His areas of expertise include pathophysiology of cardiovascular diseases and physiology of vascular cells. Dr. Lee is interested in the molecular mechanism of early-stage atherosclerosis including endothelial dysfunction and the formation of macrophage foam cells in recent years. By use of clinical therapeutic reagents, food nutrition, and traditional herbal medicine in vitro and in vivo, Dr. Lee's team have discovered several crucial molecules and important molecular mechanisms in the promotion of endothelial function and cholesterol clearance of macrophages.