Molecular mechanisms of activation of endothelial nitric oxide synthase mediated by transient receptor potential vanilloid type 1

Li-Chieh Ching¹, Yu Ru Kou¹, Song-Kun Shyue², Kuo-Hui Su¹, Jeng Wei³, Li-Ching Cheng⁴, Yuan-Bin Yu⁵, Ching-Chian Pan¹, and Tzong-Shyuan Lee¹,6*

¹Department of Physiology, National Yang-Ming University, Taipei 11221, Taiwan; ²Institute of Biomedical Sciences, Academia Sinica, Taipei, Taiwan; ³Heart Center, Cheng-Hsin General Hospital, Taipei, Taiwan; ⁴Department of Nursing, Chang-Gung Institute of Technology, Taoyuan, Taiwan; ⁵Division of Hematology and Oncology, Taipei Veterans General Hospital, Taipei, Taiwan; and ⁶Graduate Institute of Basic Medical Science, China Medical University, Taichung, Taiwan

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Aims
We investigated the molecular mechanism underlying the role of transient receptor potential vanilloid type 1 (TRPV1), a Ca²⁺-permeable non-selective cation channel, in the activation of endothelial nitric oxide (NO) synthase (eNOS) in endothelial cells (ECs) and mice.

Methods and results
In ECs, TRPV1 ligands (evodiamine or capsaicin) promoted NO production, eNOS phosphorylation, and the formation of a TRPV1–eNOS complex, which were all abrogated by the TRPV1 antagonist capsazepine. TRPV1 ligands promoted the phosphorylation of Akt, calmodulin-dependent protein kinase II (CaMKII) and TRPV1, and increased the formation of a TRPV1–Akt–CaMKII complex. Removal of extracellular Ca²⁺ abolished the ligand-induced increase in the phosphorylation of Akt and CaMKII, formation of a TRPV1–eNOS complex, and eNOS activation. Inhibition of PI3K and CaMKII suppressed the ligand-induced increase in TRPV1 phosphorylation, formation of a TRPV1–eNOS complex, and eNOS activation. TRPV1 activation increased the phosphorylation of Akt, CaMKII, and eNOS in the aortas of wild-type mice but failed to activate eNOS in TRPV1-deficient aortas. Additionally, TRPV1 ligand-induced angiogenesis was diminished in eNOS- or TRPV1-deficient mice. When compared with apolipoprotein E (ApoE)-deficient mice, ApoE/TRPV1-double-knockout mice displayed reduced phosphorylation of eNOS, Akt, and CaMKII in aortas but worsened atherosclerotic lesions.

Conclusion
TRPV1 activation in ECs may trigger Ca²⁺-dependent PI3K/Akt/CaMKII signalling, which leads to enhanced phosphorylation of TRPV1, increased TRPV1–eNOS complex formation, eNOS activation and, ultimately, NO production.

Keywords
Evodiamine • TRPV1 • eNOS • PI3K/Akt • CaMKII

1. Introduction
Nitric oxide (NO) derived from endothelial cells (ECs) is a key regulator of vascular tone.¹ It diffuses to the underlying smooth muscle, where it activates soluble guanylyl cyclase to elicit cyclic guanosine monophosphate production, which subsequently causes the relaxation of smooth muscle cells.² NO production in ECs mainly depends on the activity of endothelial NO synthase (eNOS), which is tightly regulated via a complex signalling network, including Ca²⁺- or kinase-dependent signalling pathways such as PI3K/Akt and calmodulin-dependent kinase II (CaMKII).³,⁴ Additionally, the physical interaction of eNOS with various intracellular proteins such as caveolin-1 and heat shock protein 90 (HSP90) or with a membrane channel protein, porin, plays a crucial role in the regulation of eNOS activity.⁵,⁶ Dysregulation of eNOS in ECs has been implicated in the pathogenesis of several cardiovascular diseases such as hypertension and atherosclerosis.²

The transient receptor potential (TRP) vanilloid type 1 (TRPV1), a ligand-gated non-selective cationic channel, is mainly expressed in primary nociceptive sensory neurons.⁷ TRPV1 can be activated by
heat, protons, several endogenous lipid molecules and various exogenous ligands, such as evodiamine (an active ingredient of evodia fruit) and capsacin (an active ingredient of hot pepper). Activation of TRPV1 permits Ca$^{2+}$ entry, which results in an elevated level of intracellular Ca$^{2+}$ and serves as a Ca$^{2+}$ signal to elicit responses in neurons. TRPV1 can interact with several intracellular proteins such as calmodulin and PI3K/Akt, important regulators of eNOS activation. Phosphorylation of TRPV1 by protein kinases, including PI3K and CaMKII, has been reported to regulate TRPV1 channel function in sensory neurons. The role of TRPV1 in the regulation of vascular tone was originally thought to be mediated solely through the release of vasodilator neuropeptides, such as calcitonin gene-related peptide (CGRP) from these sensory fibres after activation of TRPV1 located at these fibre terminals. Recently, increasing evidence suggests that TRPV1 is expressed in ECs and is important in the regulation of vascular tone. In isolated arteries, TRPV1 ligands cause an increase in NO release in the isolated mesenteric bed and induce endothelium- and NO-dependent vasodilation, both of which can be blocked by TRPV1 antagonists. These findings provide indirect evidence of the importance of TRPV1 activation in eNOS activation and NO production in ECs. This TRPV1 function is particularly important because ECs may be directly exposed to high temperature, protons, and various endogenous TRPV1 ligands under pathophysiological conditions such as tissue ischaemia or inflammation.

In the present study, we investigated the role of TRPV1 in eNOS activation and underlying molecular mechanisms in ECs and mice. Here, we report that TRPV1 activation by ligands in ECs may trigger Ca$^{2+}$-dependent PI3K/Akt/CaMKII signalling, which leads to increased eNOS activation and enhanced formation of a TRPV1–eNOS complex also encompassing Akt and CaMKII. The formation of this complex may be vital for the regulation of eNOS activation.

2. Methods

2.1 Reagents

Evodiamine and LY294002 were obtained from Calbiochem (San Diego, CA, USA). Rabbit antibodies for phospho-eNOS at Ser1179, phospho-CaMKII at Thr286, phospho-serine/threonine and mouse antibody for eNOS were from Cell Signaling Technology (Beverly, MA, USA). Rabbit antibodies for eNOS, CaMKII, goat antibody for Akt and protein A/G-Sepharose, scramble small interfering RNA (siRNA) and TRPV1 siRNA were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Mouse antibody for TRPV1 was from Abnova (Taoyuan, Taiwan). Mouse antibody for α-tubulin, NG-Nitro-L-arginine methyl ester (L-NAME), capsaicin, capsazepine (CPZ), KN62, ethylene glycol, ionomycin, sodium nitrite, and heparin gene-related peptide (CGRP) from these sensory fibres after activation of TRPV1 located at these fibre terminals.

2.2 Cell culture

Bovine aortic ECs (BAECs) were obtained from Cell Applications (San Diego, CA, USA) and cultured in Dulbecco’s modified Eagle medium supplemented with 10% foetal bovine serum (FBS), 100 U/mL penicillin, and 100 µg/mL streptomycin (HyClone, Logan, UT, USA). Human aortic ECs (HAECs) were purchased from Cascade Biologics (Portland, OR, USA) and cultured in Medium 200 supplemented with 10% FBS and 1% PEN-STREP-AMPHO SOL (penicillin, 10,000 units/mL; streptomycin, 10 mg/mL; amphotericin B, 0.025 mg/mL) and 2% low-serum growth supplement (Cascade Biologics). Mouse aortic ECs (MAECs) were isolated from the aortas of wild-type (WT) or TRPV1$^{-/-}$ mice and cultured with Medium 200 in Matrigel-coated dishes.

2.3 Determination of nitrite production

NO has an extremely short half-life and quickly metabolites to nitrite and nitrate. Thus, the level of nitrite was determined by Griess assay to reflect NO production. The cell culture medium was mixed with an equal volume of Griess reagent, then azo dye production was determined by absorbance at 540 nm after 15 min incubation at room temperature. Sodium nitrite was used as a standard.

2.4 Mice

The investigation conformed to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996), and all animal experiments were approved by the Animal Care and Utilization Committee of the National Yang-Ming University. WT C57BL/6 mice, eNOS$^{-/-}$, TRPV1$^{-/-}$, and ApoE$^{-/-}$ mice were purchased from Jackson Laboratory (Bar Harbor, MN, USA). To generate ApoE$^{-/-}$TRPV1$^{-/-}$ mice, TRPV1$^{-/-}$ mice were crossed with the ApoE$^{-/-}$ background, and polymerase chain reaction of genomic DNA was used to confirm ApoE$^{-/-}$ and TRPV1$^{-/-}$ genotypes. At the end of experiment, mice were euthanized with CO$_2$ and hearts were fixed with 4% paraformaldehyde, embedded in paraffin, and sectioned. The deparaffinized sections underwent H&E staining and quantification of atherosclerotic lesions at the aortic sinus. The isolated aortas were homogenized and lysates were subjected to western blot analysis.

2.5 Immunoprecipitation assay and western blot analysis

Cells were rinsed with phosphate-buffered saline (PBS), then lysed in immunoprecipitation lysis buffer (50 mmol/L Tris pH 7.5, 5 mmol/L ethylenediaminetetraacetic acid, 300 mmol/L sodium chloride, 1% Triton X-100, 1 mmol/L phenylmethylsulphonyl fluoride, 10 µg/mL leupeptin, and 10 µg/mL aprotonin). Aliquots (1000 µg) of cell lysates were incubated with specific primary antibody overnight, then with protein A/G-Sepharose for 2 h. Immune complexes were collected by centrifugation, washed three times with cold PBS, then eluted in sodium dodecyl sulphate (SDS) lysis buffer (1% Triton, 0.1% SDS, 0.2% sodium azide, 0.5% sodium deoxycholate, 10 µg/mL leupeptin, 10 µg/mL aprotonin). Eluted protein samples were separated on SDS–PAGE after being transferred to membranes, samples were immunoblotted with primary antibodies, then secondary antibodies conjugated with horse-radish peroxidase. Bands were revealed using an enzyme-linked chemiluminescence detection kit (Perkin Elmer, Waltham, MA, USA), and density was quantified using Imagequant 5.2 software (Healthcare Bio-Sciences, PA, USA).

2.6 Small interfering RNA transfection

HAECs were transfected with scramble or TRPV1 siRNA using Lipofectamine for 24 h for the indicated experiments.

2.7 In vivo Matrigel plug angiogenesis assay

To induce the formation of new blood vessels in vivo, Matrigel (8 mg/mL) was mixed with heparin (50 U/mL) and with or without the TRPV1 ligand evodiamine (1 mM/mL), and then were injected subcutaneously in mice. At 7 days post-injection, Matrigel plugs were removed and photographed. The haemoglobin assay was performed after Matrigel plugs were cultured in vivo.
Figure 1 Blockade of TRPV1 abrogates TRPV1 ligand-increased NO production and eNOS phosphorylation in BAECs. (A) BAECs were treated with concentrations of evodiamine (0–1 μmol/L) for 24 h or with 1 μmol/L evodiamine for 0–24 h. Level of nitrite in medium was analysed by Griess assay. (B) The evodiamine-induced NO production was abrogated by pretreatment with L-NAME (400 μmol/L). BAECs treated with 1 μmol/L evodiamine for up to 240 min showed an increase in phosphorylated eNOS at Ser1179. (C) BAECs were pretreated with or without 10 μmol/L capsazepine, a TRPV1 antagonist, for 1 h, and then incubated with evodiamine (1 μmol/L) or capsaicin (10 μmol/L) for an additional 24 h or an additional 15 min. (D) HAECs were transfected with scramble (200 nmol/L) or TRPV1 siRNA (200 nmol/L) for 24 h, then incubated with evodiamine (1 μmol/L) for an additional 24 h or an additional 15 min. Nitrite levels in medium were analysed by Griess assay. Cell lysates were immunoblotted with TRPV1, α-tubulin, phosphorylated eNOS, or eNOS protein. *P < 0.05 vs. non-treated group, #P < 0.05 vs. evodiamine- or capsaicin-treated group.
homogenized and incubated with Drabkin’s reagent for 30 min at room temperature. The haemoglobin concentration was calculated at 540 nm.

**2.8 Statistical analysis**

Results are presented as mean ± SD from five independent experiments. Mann–Whitney U test was used to compare two independent groups. Kruskal–Wallis followed by the Bonferroni post hoc analyses were used to account for multiple testing. SPSS software v. 8.0 (SPSS Inc., Chicago, IL, USA) was used. P < 0.05 was considered statistically significant.

**3. Results**

**3.1 TRPV1 ligand increases eNOS phosphorylation and NO production**

To test whether the TRPV1 ligand can increase NO production in ECs, BAECs were treated with various doses of evodiamine or at the indicated times. Treatment with 0.1 or 1 μmol/L evodiamine for 24 h significantly increased levels of nitrite in cultured medium (Figure 1A). The nitrite level increased as early as 1 h after treatment with 1 μmol/L evodiamine and continued to increase thereafter. However, eNOS protein level was not affected by 0.1 or 1 μmol/L evodiamine with 24 h treatment (data not shown), which suggests that this increase in NO production may be owing to increased activity of eNOS. Indeed, the increase in NO production was prevented by pretreatment with L-NAME, the non-selective inhibitor of NOS (Figure 1B), which indicates that the production was specifically linked to the function of eNOS. Treatment with 1 μmol/L evodiamine significantly increased eNOS phosphorylation, which occurred as early as 10 min after treatment, peaked at 15 min, and gradually decreased to the basal level thereafter (Figure 1B). We next examined whether the ligand-induced increase in eNOS phosphorylation and NO production was specifically owing to TRPV1 activation. Treatment with 1 μmol/L evodiamine or 10 μmol/L capsaicin significantly increased both NO production and eNOS phosphorylation (Figure 1C), both of which were nearly abrogated by pretreatment with TRPV1 siRNA.
with CPZ, a specific TRPV1 antagonist. Moreover, the evodiamine-induced eNOS phosphorylation and NO production were prevented by TRPV1 siRNA treatment in HAECs (Figure 1D).

3.2 \(Ca^{2+}\)-dependent PI3K/Akt/CaMKII signalling is essential for the ligand-induced increase in NO production and eNOS phosphorylation

We next examined whether \(Ca^{2+}\) and PI3K/Akt/CaMKII signalling pathway are involved in the TRPV1 ligand-induced activation of eNOS. Treatment with 1 \(\mu\)mol/L evodiamine increased the phosphorylation of Akt and CaMKII, both of which occurred between 5 and 30 min after treatment (Figure 2A). Pretreatment with LY294002 (a specific inhibitor of PI3K/Akt signalling), KN62 (a CaMKII inhibitor), or EGTA (a \(Ca^{2+}\) chelator) prevented the evodiamine-induced increase in the phosphorylation of eNOS and CaMKII (Figure 2B–D), as well as NO production (Figure 2E). Additionally, the evodiamine-induced phosphorylation of Akt and CaMKII was nearly abolished by TRPV1 siRNA (Figure 2F). These results suggest the essential role of \(Ca^{2+}\)-dependent PI3K/Akt/CaMKII signalling in the TRPV1-mediated activation of eNOS.

3.3 \(Ca^{2+}\)-dependent PI3K/Akt/CaMKII signalling is essential for the ligand-induced increase in TRPV1 phosphorylation and association of TRPV1 and eNOS

Because the physical interaction of eNOS with intracellular proteins is important in the regulation of eNOS activity,\(^5,6\) we determined whether TRPV1 may interact with eNOS. Results from immunoprecipitation assay revealed that TRPV1 was physically associated with eNOS under basal conditions (Figure 3). Treatment with 1 \(\mu\)mol/L evodiamine for 15 min or 10 \(\mu\)mol/L capsaicin for 10 min (Figure 3A) increased the formation of a TRPV1–eNOS complex. Additionally, this ligand-induced increase in TRPV1–eNOS complex formation was abrogated by pretreatment with CPZ or EGTA (Figure 3A), which suggests the importance of TRPV1 activation in the formation of the complex.

Because \(Ca^{2+}\) and PI3K/Akt/CaMKII signalling is important in TRPV1 ligand-induced eNOS phosphorylation, we examined
whether this signalling was also involved in the increase in the formation of a TRPV1–eNOS complex. Treatment with evodiamine increased the formation of a TRPV1 complex with Akt (Figure 3B) and CaMKII (Figure 3C). Pretreatment with LY294002 (Figure 3D) or KN62 (Figure 3E) abolished the evodiamine- or capsaicin-induced increase in the formation of a TRPV1–eNOS complex. Additionally, evodiamine induced TRPV1 phosphorylation (Figure 4A), which was also abrogated by pretreatment with LY294002 (Figure 4B) or KN62 (Figure 4C). These results suggest that Ca^{2+}-dependent PI3K/Akt/ CaMKII signalling is required for the TRPV1 ligand-induced increase in TRPV1 phosphorylation and the formation of a TRPV1–eNOS complex.

### 3.4 Deletion of TRPV1 impairs ligand-induced increase in eNOS phosphorylation and angiogenesis in vivo

To explore whether TRPV1 activation may induce eNOS activation in vivo, we intraperitoneally injected WT mice with a TRPV1 ligand, evodiamine, and measured the phosphorylated levels of Akt, CaMKII, and eNOS in aortas. In WT mice, the increase in eNOS phosphorylation occurred as early as 2 h after treatment, peaked at 4 h, and decreased to the basal level thereafter (Figure 5A). In addition, the phosphorylation of Akt and CaMKII was elevated within 1 h after treatment (Figure 5A). However, activation of TRPV1 causes the release of CGRP from perivascular sensory nerve endings and serves as a vasodilator neuropeptide. To exclude the possible involvement of CGRP on TRPV1 ligand-induced eNOS phosphorylation, WT mice were pretreated with a CGRP antagonist, SB268262, 1 h before subsequent evodiamine administration. Treatment with SB268262 did not alter evodiamine-induced eNOS phosphorylation (Figure 5B), which suggests that activation of TRPV1-induced eNOS phosphorylation is independent of CGRP effects. Additionally, the TRPV1 ligand-induced increase in eNOS phosphorylation in the aortas of TRPV1^{−/−} mice was completely prevented (Figure 5C). Similar results were observed in primary MAECs, whereby deletion of TRPV1 completely abrogated the evodiamine-induced phosphorylation of eNOS, Akt, and CaMKII (Figure 5D).
NO-dependent angiogenesis is a critical remodelling process in the development of new blood vessels, in wound healing, and in combating diseases such as arteriosclerosis and ischaemic heart disease. We thus used Matrigel plug assay to assess the role of TRPV1 on angiogenesis in vivo. As shown in Figure 6A and B, the TRPV1 ligand evodiamine promoted vascularization in Matrigel plugs in WT mice. In contrast, evodiamine-induced angiogenesis was markedly reduced in eNOS−/− and TRPV1−/− mice. Moreover, because endothelial dysfunction is an important event in early-stage atherosclerosis, we generated ApoE−/−TRPV1−/− mice to explore the effect of TRPV1-mediated phosphorylation of eNOS, Akt, and CaMKII in a hyperlipidaemia-induced atherosclerotic animal model. ApoE−/−TRPV1−/− mice showed markedly increased atherosclerotic lesions (Figure 6C) and, in parallel, decreased the phosphorylation of eNOS, Akt, and CaMKII in aortas (Figure 6D). These results suggest that TRPV1 may play a crucial role in the pathophysiological function of ECs in vivo.

4. Discussion

In this study, we characterized the molecular mechanisms underlying the role of endothelial TRPV1 in the activation of eNOS in ECs. Exposing ECs to two TRPV1 ligands, evodiamine or capsaicin, promoted NO production, which was a consequence of early (within 10 min) increased eNOS phosphorylation, with no change in eNOS protein level. As well, this increased eNOS phosphorylation was preceded by enhanced phosphorylation of Akt and CaMKII. The initial effects of TRPV1 stimulation in ECs occur very rapidly and include the influx of Ca2+ and phosphorylation of eNOS, Akt, and CaMKII, all of which are evoked within 15–30 min. These initial triggering mechanisms might subsequently promote the production of NO via eNOS phosphorylation, which, in turn, forms nitrite as the stable metabolic product. As shown in Figure 1A, the increase in the level of nitrite following this cascade of reactions occurred at 60 min after ligand stimulation. Because nitrite is a stable metabolic product of NO, it would accumulate in the medium over time. Consequently, the level of nitrite continued to be increased up to 24 h after ligand stimulation. Use of specific inhibitors revealed that the TRPV1 ligand-induced eNOS activation depends on the activation of the PI3K/Akt/CaMKII signalling pathway. Furthermore, all these cascade events were abrogated by blocking TRPV1-mediated Ca2+ entry by a specific antagonist or the removal of extracellular Ca2+, which suggests that Ca2+ was the triggering molecule to initiate these cascade events that led to eNOS activation. In parallel with the eNOS activation, exposing ECs to TRPV1 ligands for only 15 min enhanced the phosphorylation of TRPV1 and markedly increased the physical interaction of TRPV1 with eNOS, Akt, and CaMKII. More importantly, this increased formation of a TRPV1–eNOS complex was abrogated by a specific TRPV1 channel antagonist, inhibitors targeting the
PI3K/Akt/CaMKII pathway, or removal of extracellular Ca\(^{2+}\). Likewise, this enhanced phosphorylation of TRPV1 was prevented by inhibitors for the PI3K/Akt/CaMKII pathway. Therefore, the same Ca\(^{2+}\)-dependent PI3K/Akt/CaMKII signalling was responsible for promoting TRPV1 phosphorylation and formation of a TRPV1–eNOS complex in ECs stimulated with TRPV1 ligands. Consistent with our in vitro findings, WT mouse aortas treated with a TRPV1 ligand showed increased phosphorylation of Akt, CaMKII, and eNOS, but eNOS was not activated by the TRPV1 ligand in TRPV1\(^{-/-}\) aortas. Collectively, our results suggest that TRPV1 activation in ECs may initially trigger Ca\(^{2+}\)-dependent PI3K/Akt/CaMKII signalling, which had three simultaneous consequences: increased phosphorylation of TRPV1, enhanced formation of a TRPV1–eNOS complex, and eNOS activation.

Our finding that Ca\(^{2+}\) was the triggering molecule responsible for all major subsequent events clearly indicates the importance of TRPV1 channel function in the eNOS activation and NO production in ECs. This TRPV1 channel function was promoted by binding the ligand to the channel itself, and presumably the Ca\(^{2+}\) entry is mediated through ligand-gated channels. In terms of function, our
findings are in line with the observation in human cerebral artery ECs that stimulation of TRPV1 by its ligand increases Ca\(^{2+}\) influx, which activates protein kinases G and A, two important enzymes for vascular dilation.\(^6\) In addition to the TRPV1, at least 18 another TRP channel isoforms are expressed in ECs; some are also permeable to Ca\(^{2+}\) and thus have been implicated in the regulation of vascular tone.\(^8\) However, the function of these TRP channels seems to be mediated through receptor-activated channels whose function is promoted when agonists bind to their specific membrane G protein-coupled receptors distinct from the channel protein itself.\(^9\) For example, TRPC1 and C3–C5 may participate in the Ca\(^{2+}\) influx in ECs induced by bradykinin, ATP, or acetylcholine.\(^23\)–\(^26\) all of which are vasodilator agonists. As well, TRPC4- and TRPV4-deficient mice display reduced acetylcholine-induced Ca\(^{2+}\) entry in ECs and decreased endothelium-dependent, NO-mediated vasorelaxation of blood vessels.\(^25\)–\(^27\) However, the activation of another ligand-gated channel, endothelial TRPA1, by its ligand increases Ca\(^{2+}\) influx and produces endothelium-dependent vasodilation independent of the eNOS function.\(^28\) Whether stimulation of these receptor-activated channels in ECs and TRPV1 shares the same molecular mechanisms for eNOS activation is unclear.

Although the importance of TRPV1 in the regulation of several EC functions has been recognized, the signalling pathways underlying these functions are largely unknown. We targeted Akt and CaMKII, two important kinases for eNOS activation, with PI3K and calmodulin, respectively, as their upstream regulators.\(^3\)–\(^4\) Both PI3K/Akt and calmodulin/CaMKII pathways are sensitive to an increase in intracellular Ca\(^{2+}\) and integrate signals from a number of eNOS agonists.\(^29\)–\(^30\) Thus, our findings that both the PI3K/Akt signalling and CaMKII are essential for TRPV1-mediated eNOS activation is not surprising. However, we also demonstrated that PI3K/Akt is the upstream signalling for CaMKII, a relationship that has not been reported in eNOS activation by other agonists in ECs. In fact, the cross-talk between Akt and CaMKII is an important issue that remains to be elucidated.\(^30\)

It is intriguing that, in concert with eNOS activation, TRPV1 activation in ECs also induced a PI3K/Akt/CaMKII-dependent enhancement of both the phosphorylation of TRPV1 and the formation of a TRPV1–eNOS complex. In addition to the kinase-dependent activation mechanisms, physical interaction of eNOS with various proteins such as caveolin-1, calmodulin, and bradykinin B2 receptor is critical in the regulation of eNOS activation.\(^5\)–\(^6\)\(^31\) In addition, bradykinin increases the interaction of eNOS with porin, a voltage-dependent anion/cation channel, which results in a functional enhancement of eNOS activity, which suggests a role of intracellular Ca\(^{2+}\) in mediating this interaction.\(^4\) More importantly, HSPP90 functions as a scaffold for the recruitment of eNOS and Akt leading to eNOS activation.\(^5\) However, TRPV1 can physically interact with several intracellular proteins such as calmodulin and PI3K/Akt, important regulators of eNOS activation, all of which are key regulators for eNOS activation.\(^10\)\(^11\) In neurons, the interaction of TRPV1 with other proteins and the phosphorylation of TRPV1 by protein kinases could further enhance the activity of TRPV1 channel. However, our results suggest that in ECs, apart from its channel function, phosphorylated TRPV1 may function as a scaffold for the recruitment and formation of a complex encompassing eNOS, Akt, and CaMKII, and these protein–protein interactions may also be important for the regulation of eNOS activation.

Our findings regarding the function of TRPV1 are not limited to the cell model. In this study, we showed that the TRPV1 ligand-induced eNOS activation in aortas of WT mice was absent in aortas of TRPV1\(^{-/-}\) mice. In addition, activation of TRPV1 promoted angiogenesis in vivo. Furthermore, under hyperlipidaemia conditions, eNOS activity was lower in aortas from ApoE\(^{-/-}\) TRPV1\(^{-/-}\) mice than in those from ApoE\(^{-/-}\) mice. These findings strongly suggest that similar molecular mechanisms for the regulation of eNOS activity in vitro may occur in vivo. Because the vessels of our knockout mice were devoid of TRPV1, we cannot determine whether the result is owing to a lack of channel function or protein function, or both. In addition, we further demonstrated that TRPV1 activation-induced increase in the phosphorylation of eNOS is CGRP independent (Figure 5B), a result that is in agreement with the previous findings of Poblete et al.\(^19\) However, a report by Rang et al. demonstrated that CGRP mediates the protection of evodiamine against cardiac anaphylaxis.\(^13\) Thus, evodiamine may provide therapeutic function in various organs through dissimilar mechanisms.

In summary, we demonstrate that TRPV1 activation by ligands in ECs may trigger Ca\(^{2+}\)-dependent PI3K/Akt/CaMKII signalling, which leads to enhanced phosphorylation of TRPV1, increased the formation of a TRPV1–eNOS complex, eNOS activation and, ultimately, NO production. In addition to its channel function, TRPV1 may function as a scaffold for the recruitment and interaction with eNOS, Akt, and CaMKII, which may be vital for regulating eNOS activation (Figure 4D). ECs may be exposed to high temperature, protons, and various endogenous TRPV1 ligands that may be formed under pathological conditions, such as tissue ischaemia or inflammation.\(^20\) Because TRPV1 is a polymodal receptor responding to multiple stimuli, it presumably has the advantage of being a sensor to detect the need for eNOS activation. The significance of TRPV1 function has been implicated in cardiovascular diseases.\(^20\)\(^32\)\(^33\) Moreover, TRPV1 may be implicated in hypertension because activation of TRPV1 by capsaicin causes vasorelaxation through an eNOS-dependent mechanism and thereby attenuates hypertension in experimental animals.\(^34\) The molecular mechanisms we reveal may provide important information for developing novel pharmacological targets for treating these eNOS-related cardiovascular diseases.

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