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Article

Endothelial Nitric Oxide Mediates the Anti-Inflammatory Action of *Torenia Concolor* Lindley Var. *Formosama* Yamazaki

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Abstract: ***Torenia*** *Concolor* Lindley var. *formosama* Yamazaki ethanolic extract (TCEE) is reported to have anti-inflammatory and anti-obesity properties. However, the effects of TCEE and its underlying mechanisms in the activation of endothelial nitric oxide synthase (eNOS) have not yet been investigated. Increasing the endothelium-derived nitric oxide (NO) production has been known to be beneficial against the development of cardiovascular diseases. In this study, we investigated the effect of TCEE on eNOS activation and NO-related endothelial function and inflammation by using *in vitro* system. In endothelial cells (ECs), TCEE increased NO production in a concentration-dependent a manner without affecting the expression of eNOS. In addition, TCEE increased the phosphorylation of eNOS at serine 635 residue (Ser635) and Ser1179, Akt at Ser473, calmodulin kinase II (CaMKII) at threonine residue 286 (Thr286) and AMP-activated protein kinase (AMPK) at Thr172. Moreover, TCEE-induced NO production, and EC proliferation, migration and tube formation were diminished by pretreatment with LY294002 (an Akt inhibitor), KN62 (a CaMKII inhibitor) and compound C (an AMPK inhibitor). Additionally, TCEE attenuated the tumor necrosis factor-α-induced inflammatory response as evidenced by the expression of adhesion molecules in ECs and monocyte adhesion onto ECs. These inflammatory effects of TCEE were abolished by L-NG-nitroarginine methyl ester (a NOS inhibitor). Collectively, our findings suggest that TCEE confers protection from endothelial dysfunction by activating the Akt/CaMKII/AMPK/eNOS/NO signaling pathway.

Keywords: *Torenia Concolor* Lindley var. *formosama* Yamazaki; endothelial nitric oxide synthase; nitric oxide; anti-inflammatory effect

1. Introduction

The endothelium is a monolayered continuous cell sheet lining the luminal surface of vessel walls that not only serves as the cross-bridge of communication between the blood and cells, but also

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actively regulates the functions of surrounding cells through complex signaling pathways [1,2]. Under certain circumstances such as hypercholesterolemia and atherosclerosis, modified LDL impairs the activation of endothelial nitric oxide synthase (eNOS)/nitric oxide (NO) signaling and induces the expression of adhesion molecules such as intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1) in ECs [3,4]. These events have been considered as one of the earliest pathophysiological manifestations of atherosclerosis [5,6]. Several lines of evidence have clearly indicated the crucial role of endothelium-derived NO, whereby it regulates various physiological functions, including vessel relaxation, proliferation and migration of ECs, inhibition of platelet activation and attenuation of inflammatory responses in the vessel wall [7]. Impaired NO production has been considered one of the earliest pathophysiological manifestations for endothelial dysfunction and is highly associated with the prevalence of inflammatory diseases and cardiovascular diseases [8–10]. The regulation of eNOS is tightly regulated not only at the transcriptional level but also by post-translational mechanisms [11,12]. It is well known that eNOS can be activated by several physical and chemical stimuli such as shear stress, estrogen, or bradykinin, and through kinase-dependent signaling pathways including, the PI3K/Akt, calmodulin kinase II (CaMK II) or AMP-activated protein kinase (AMPK) pathways [11,12]. Increasing the activity of eNOS-NO signaling has been considered a therapeutic strategy for the treatment of cardiovascular diseases [13,14]. However, whether TCEE has such beneficial effects on eNOS activation and EC functions, and the molecular mechanisms underlying these effects remain unclear.

During the past decade, considerable efforts have been taken to discover the potential of traditional Chinese medicine in many fields, particularly cancers, inflammatory diseases and cardiovascular diseases [15–20]. *Torenia concolor* Lindley var. *formosana* Yamazaki (TC), a plant native to Taiwan that belongs to the Scrophulariaceae family, has been used in traditional Chinese medicine to treat human diseases including hypertension, stomatitis, hepatitis, pneumonia and gastroenteritis, etc., owing to its detoxification, anti-inflammatory and diuretic effects [21,22]. Experimentally, TC extracts have been reported to have excellent anti-inflammatory effects on macrophages and inhibitory effects on lipid deregulation in adipocytes [21,22]. Moreover, the components of TC such as botulin, betulinic acid and oleanolic acid are reported to exert anti-inflammatory, anti-cancer or anti-hyperglycemic activities [23–25]. Although the protective effects of TC extracts on inflammatory diseases have been examined extensively in *in vitro* and *in vivo* models [21,22,24]; there is little information about the role of TC extracts in endothelial dysfunction and related cardiovascular diseases. Further investigations of the vascular protective effects of TCEE and its underlying molecular mechanisms in eNOS/NO signaling and EC function are warranted.

Given the impact of TCEE on inflammatory and metabolic diseases, the present study aimed to characterize the effects of TCEE on ECs and the molecular mechanisms underlying these effects. First, we investigated effects of TCEE and the related molecular mechanisms underlying the activation of eNOS-NO signaling, and then, we ascertained whether TCEE-mediated enhancement of NO bioavailability contributes to its anti-inflammatory property against inflammatory responses. Our results demonstrated that TECC promotes NO production via the Akt/CaMKII/AMPK/eNOS signaling cascade, and then contributes to proangiogenic and anti-inflammatory responses in ECs.

2. Results

2.1. TCEE Increases NO Production by Increasing the Phosphorylation of eNOS in ECs

To test whether TCEE can activate the eNOS/NO signaling pathway, ECs were treated with the indicated concentrations (0.125, 0.25, 0.5, 1, 2, 4, and 8 μg/ml) of TCEE, and the effects of TCAE on NO production and cell viability were examined. Treating the ECs with 0.125 to 8 μg/ml of TCEE for 24 h increased the NO bioavailability without affecting the cell viability (Fig. 1A-C). However, eNOS protein level was not affected following the treatment of the ECs with 0.125 to 8 μg/ml TCEE for 24 h (Fig. 1D), which suggests that this increase in NO production may be due to the activity of eNOS. Indeed, the increase in NO production was prevented by the pretreatment of the ECs with L-NAME,

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a non-selective inhibitor of NOS (Fig. 1E), which indicates that the NO production was specifically linked to the function of eNOS.

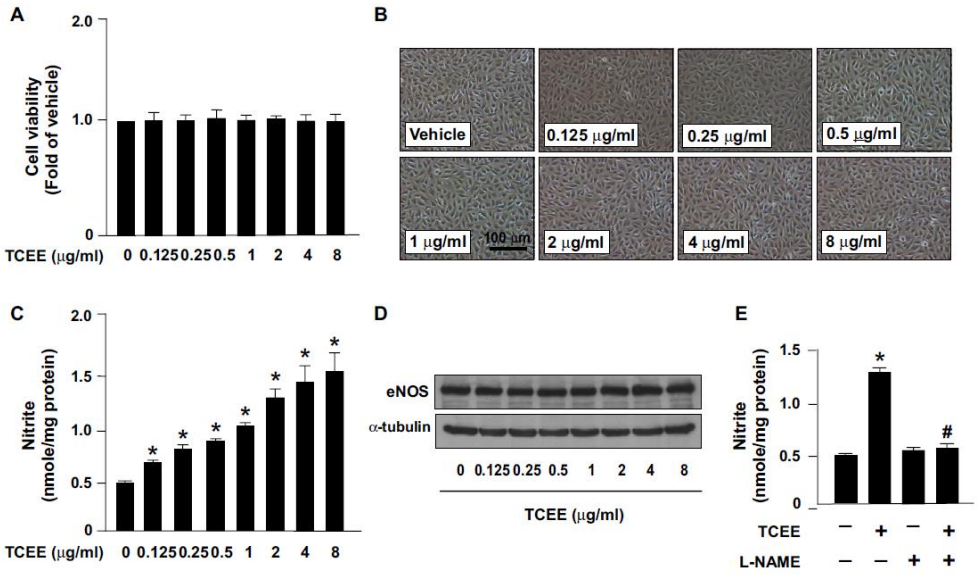

Figure 1. Effects of *Torenia Concolor* Lindley var. *Formosama* Yamazaki ethanol extracts (TCEE) on NO bioavailability and cell viability in ECs. (A) ECs were treated with the indicated concentrations (0.125-8 μg/ml) of TCEE for 24 h. Level of nitrite in culture medium by Griess assay. (B) Microscopic analysis of cells. (C) MTT assay of cell viability. (D) Western blot analysis of total eNOS and α-tubulin. (E) ECs were pretreated with L-NAME (400 μM) for 2 h, and then with TCEE (2 μM) for 24 h. Griess assay of nitrite in the culture medium. Data are mean ± SEM from 5 independent experiments. *P< 0.05 vs. vehicle-treated group; #P< 0.05 vs. TCEE-treated group.

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Next, we examined the effect of TCEE on eNOS phosphorylation at various time points. We found that TCEE induced the phosphorylation of eNOS at Ser635 and Ser1179 in a time-dependent manner without affecting its phosphorylation at Ser617 and Thr497 (Fig. 2A-D). These results suggest that TCEE increases the NO bioavailability by increasing eNOS phosphorylation.

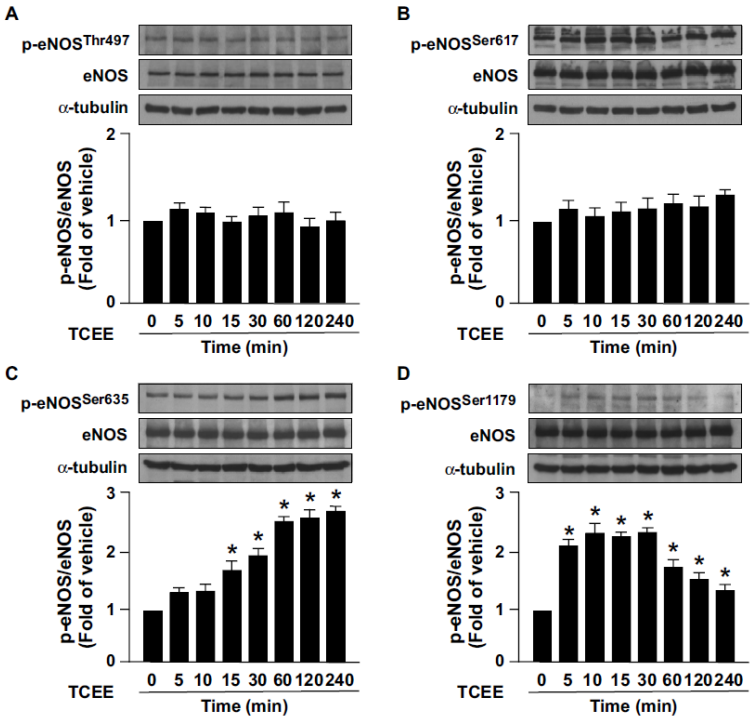

Figure 2. TCEE induces endothelial NO synthase (eNOS) activation in ECs. ECs were treated with TCEE (2 μg/ml) for the indicated times. (A-D) Cells were lysed and subjected to western blot analysis for quantifying the levels of phosphorylated eNOS (at Thr495, Ser617, Ser635 and Ser1177) or total eNOS protein. Data are mean ± SEM from 5 independent experiments. *P< 0.05 vs. vehicle-treated group.

2.2. Activation of the Akt-CaMKII-AMPK Signaling Pathway is Essential for the TCEE-Induced Phosphorylation of eNOS and NO Production

The Akt-CaMKII-AMPK signaling pathway is reported to play an important role in eNOS activation and NO production [12]. Next, we examined whether the Akt-CaMKII-AMPK signaling pathway is involved in TCEE-induced eNOS activation and NO production. TCEE time-dependently increased the level of phosphorylation of Akt, CaMKII and AMPK, which occurred at 15 to 30 min after the treatment (Fig. 3A-C). Inhibition of the Akt-CaMKII-AMPK signaling pathway by LY294002 (an Akt inhibitor), KN62 (a CaMKII inhibitor), or compound C (C.C., an AMPK inhibitor) abrogated the TCEE-induced NO production (Fig. 3D).

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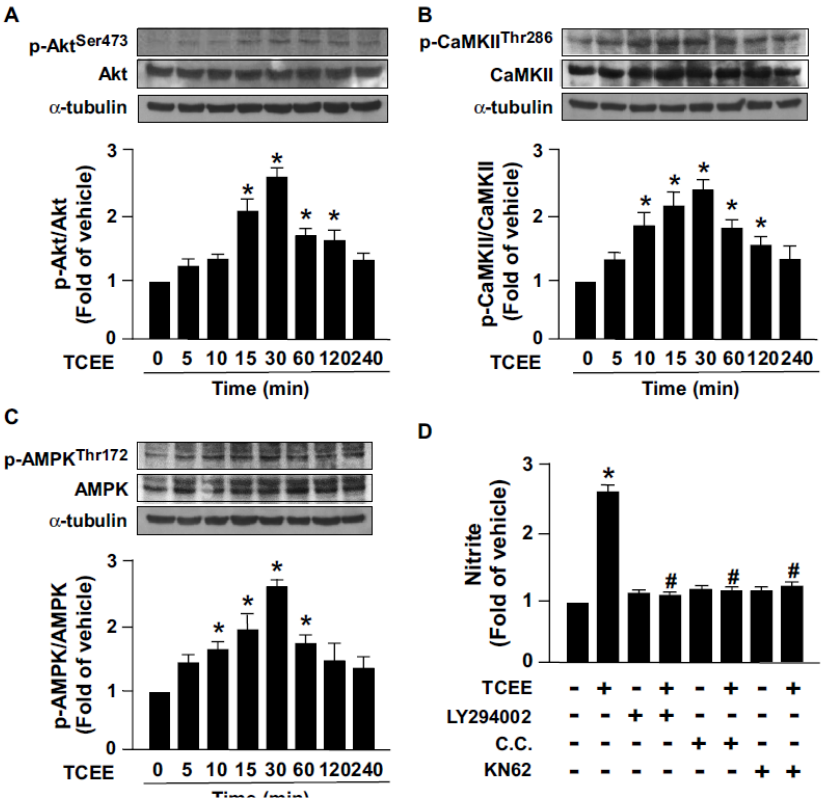

Figure 3. Akt-CaMKII-AMPK signaling is involved in the TCEE-mediated enhancement of NO bioavailability in ECs. (A-C) ECs were treated with TCEE (2 μg/ml) for the indicated times. Western blot analysis of phosphorylated or total of Akt, CaMKII and AMPK and α-tubulin. (D) ECs were pretreated with LY294002 (10 μM), compound C (C.C., 10 μM) or KN62 (10 μM) for 2 h, and then with TCEE (2 μg/ml) for 24 h. Griess assay of nitrite level in the culture medium. Data are mean ± SEM from 5 independent experiments. *P< 0.05 vs. vehicle-treated group; #P< 0.05 vs. TCEE-treated group.

By using these pharmacological inhibitors, the up- and down-stream relationships of the Akt, CaMKII and AMPK following by TCEE treatment were established (Fig. 4A-C). Hence, the activation of the Akt-CaMKII-AMPK signaling cascade is required for TCEE-induced eNOS activation and NO production.

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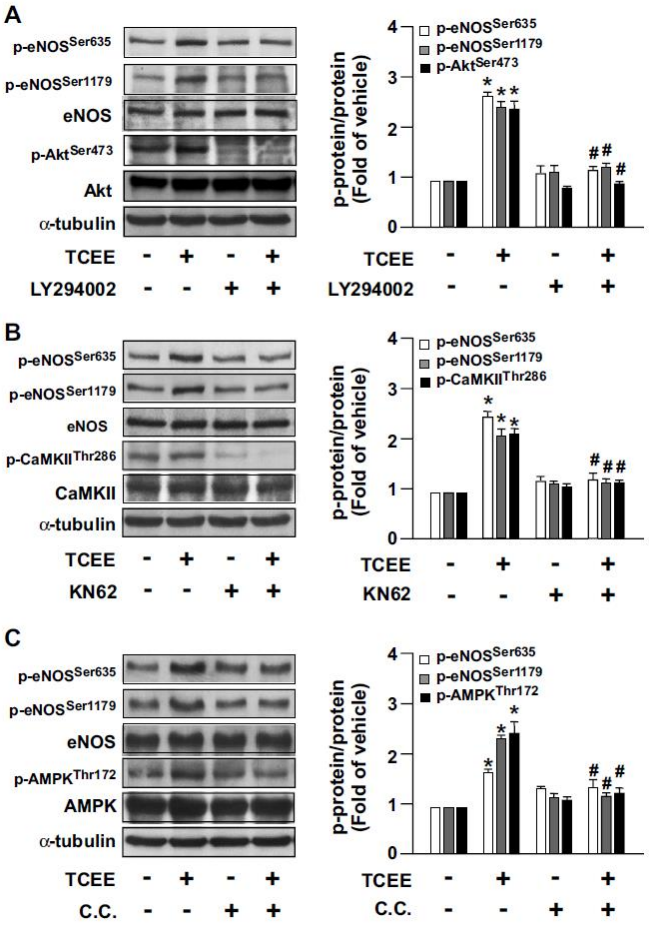

Figure 4. The up- and down-stream relationships of the Akt, CaMKII and AMPK signaling cascade in TCEE-treated ECs. (A-C) ECs were pretreated with LY294002 (10 μM), compound C (C.C., 10 μM) or KN62 (10 μM) for 2 h, and then with TCEE (2 μg/ml) for 30 min. Western blot analysis of phosphorylated or total of Akt, CaMKII, AMPK, eNOS and α-tubulin. Data are mean ± SEM from 5 independent experiments. *P< 0.05 vs. vehicle-treated group; #P< 0.05 vs. TCEE-treated group.

2.3. The Akt-CaMKII-AMPK Signaling Plays a Key Role in the TCEE-Mediated Promotion of EC Proliferation, Migration, and Tube Formation

Under physiological conditions, increased NO levels can promote EC proliferation, migration, and tube formation, and lead to angiogenesis [26]. We then investigated the effects of TCEE on EC proliferation, migration, and tube formation, and whether the Akt-CaMKII-AMPK/eNOS/NO signaling cascade is involved in the proangiogenic effects of TCEE. Our results revealed that treatment with TCEE enhanced EC proliferation, migration, and tube formation, which were abolished by the pretreatment of the ECs with LY294002, KN62, or C.C. (Fig. 5 and 6). These findings suggest that the activation of the Akt-CaMKII-AMPK/eNOS/NO signaling pathway plays a crucial role in the TCEE-mediated benefits associated with the physiological function of ECs.

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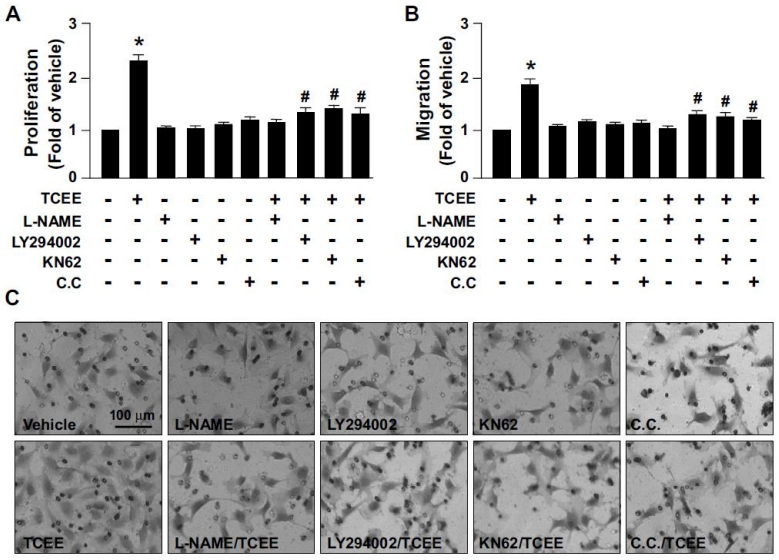

Figure 5. The signaling cascade involved in the TCEE-induced promotion of EC proliferation and migration. ECs were pretreated with LY294002 (10 μM), compound C (C.C., 10 μM) or KN62 (10 μM) for 2 h, then with TCEE (2 μg/ml) for 8 h. (A) MTT assay of EC proliferation. (B and C) EC migration was evaluated by the trans-well assay after TCEE treatment. The number of migrating cells was counted compared to the case for the vehicle-treated cells, and the images were generated by microscopy. Data are mean ± SEM from 5 independent experiments. *P< 0.05 vs. vehicle-treated group; #P< 0.05 vs. TCEE-treated group.

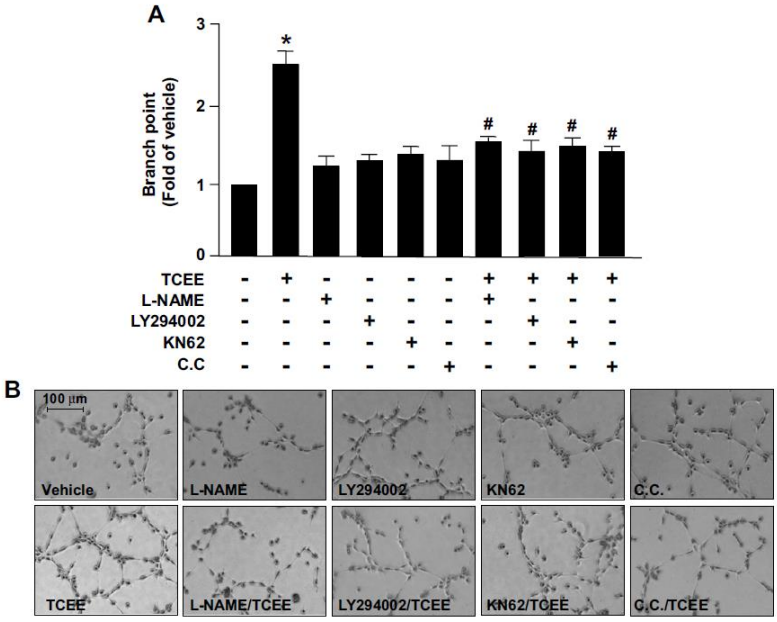

Figure 6. The signaling cascade involved in TCEE-induced promotion of EC tube formation. ECs were pretreated with LY294002 (10 μM), compound C (C.C., 10 μM) or KN62 (10 μM) for 2 h, then with TCEE (2 μg/ml) for 8 h. (A and B) ECs were cultured in the attachment matrix in the indicated treatments. Tube formation was visualized; the bar graphs indicate the branch points as a fold of those in case of the vehicle-treated control in five randomly selected microscopic fields. Data are mean ± SEM from 5 independent experiments. *P< 0.05 vs. vehicle-treated group; #P< 0.05 vs. TCEE-treated group.

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2.4. Activation of eNOS is Essential for the Anti-inflammatory Effects of TCEE

TCEE has been reported to have excellent anti-inflammatory effects [21,22]. Based on the above findings, we thus investigated whether eNOS activation mediates the TCEE-conferred anti-inflammatory effects. We demonstrated that tumor necrosis factor-α (TNF-α) increased the expression of ICAM-1 and VCAM-1, as well as monocyte adhesion onto ECs (Fig. 7A-E). However, these pro-inflammatory responses elicited by TNF-α were abrogated by the NOS inhibitor L-NAME (Figure 7A-E). These findings suggest the importance of eNOS activation for TCEE to elicit its anti-inflammatory property in ECs.

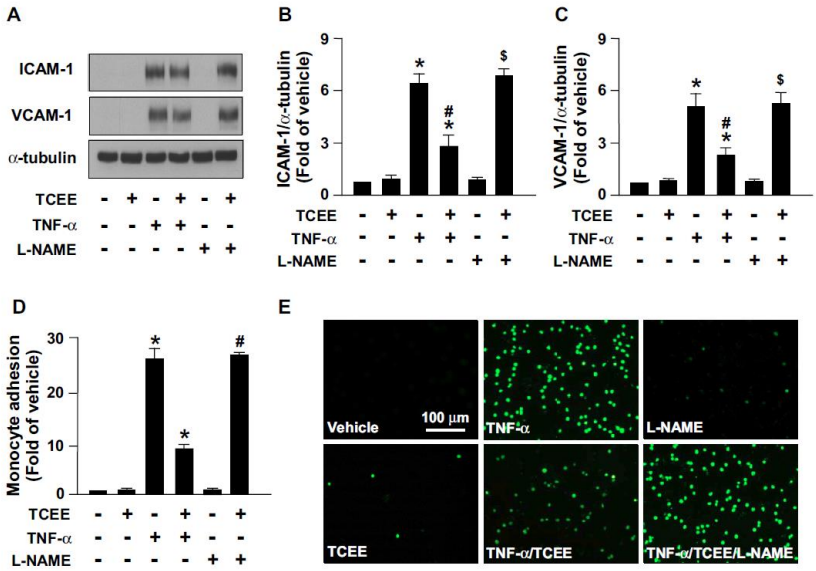

Figure 7. TCEE inhibits the TNFα-induced adhesion of monocytes onto ECs and the protein expression of adhesion molecules in ECs. ECs were pretreated with TNFα (10 ng/ml) or the vehicle for 12 h, followed by the treatment with L-NAME (400 μM) for 1 h and/or TCEE (2 μg/ml) for another 18 h. (A-C) Cellular lysates were subjected to western blot analysis for quantifying the protein expression of ICAM-1, VCAM-1 and α-tubulin. (D and E) BCECF-AM-labeled human monocyte THP-1 cells ($1x10^5$) were added and incubation with ECs for 1 h. The cellular lysates were subjected to fluorometry and photo-micrographed. Data are mean ± SEM from 5 independent experiments. *P< 0.05 vs. vehicle group; #P< 0.05 vs. TCEE-treated group; $P< 0.05 vs. TNF-α+TCEE-treated group.

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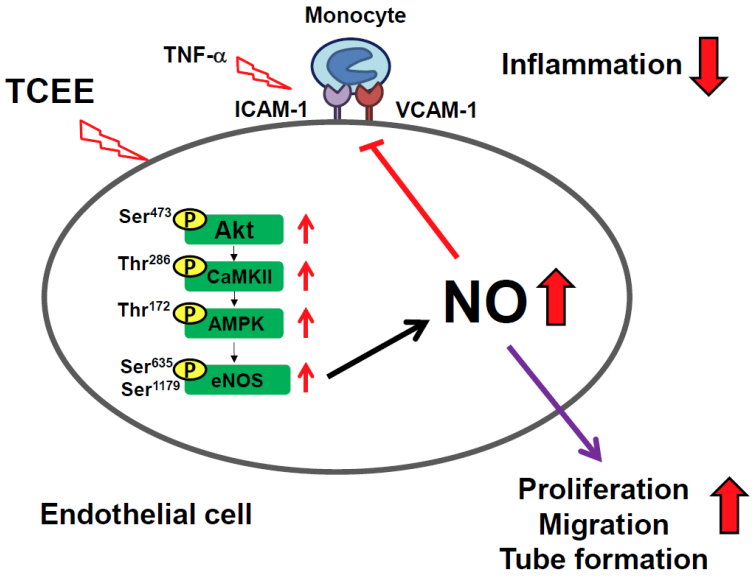

Figure 8. Schematic illustration of the proposed mechanism underlying the TCEE-mediated anti-inflammatory effect in ECs. As shown, challenge with TCEE elicits the activation of the Akt/CaMKII/AMPK/eNOS/NO pathway, which in turn promotes pro-angiogenic activity and inhibits the pro-inflammatory response by TNF-α in ECs.

3. Discussion

TCEE is known to have anti-inflammatory, anti-allergic, and lipid-lowering effects [21,22]; however, its biological impact on cardiovascular diseases remains unclear. In this study, we demonstrated that TCEE increased eNOS phosphorylation, NO production, and angiogenic responses in ECs through the Akt/CaMKII/AMPK signaling pathway. Exposure of ECs to TCEE rapidly increased the phosphorylation of eNOS at Ser635 and Ser1179, and further increased NO production. In addition, the phosphorylation of Akt, CaMKII, and AMPK is involved in TCEE-induced NO production, which is consistent with the results of studies by Ching *et al.* and Guo *et al.*, who found that Akt, CaMKII, and AMPK are the key kinases in eNOS phosphorylation [26,27]. NO is known to be the most important modulator in regulating the physiological functions of ECs such as proliferation, migration, and angiogenesis [28,29]. Indeed, our findings showed that TCEE promoted EC proliferation, migration, and angiogenesis in a NO-dependent manner. Moreover, NO has an anti-inflammatory action and thus, inhibits the activation of circulating leukocytes [30]. Our findings further support this notion by evidencing that TCEE inhibited the TNFα-induced upregulation of the adhesion molecules ICAM-1 and VCAM-1, as well as the monocyte adhesion onto ECs. Collectively, TCEE may exert its anti-inflammatory effects by activating the Akt/CaMKII/AMPK/eNOS/NO signaling pathway in ECs (Fig. 8).

Notably, EC dysfunction is the key event in the early stage of cardiovascular diseases including hypertension and atherosclerosis [31,32]. It is characterized by decreased NO bioavailability and increased expression of adhesion molecules in atherosclerosis [33,34]. Modulating the activation of eNOS-NO signaling has been suggested as a therapeutic strategy for treating or preventing the progression of cardiovascular diseases [4,35]. For instance, Xing et al. and Li. et al. have reported that supplementation with resveratrol or salidroside improves endothelial dysfunction and alleviates atherosclerosis in apolipoprotein E-deficient mice [36,37]. Furthermore, treatment with *Morus alba* extract activates eNOS signaling and maintains blood pressure homeostasis in mice [38]. Our previous study has demonstrated that capsaicin and evodiamin, which are the agonists of transient receptor potential vanilloid 1, retard the progression of atherosclerosis by activating the eNOS/NO signaling pathway [13,15,26,39]. In addition, statins, which are lipid-lowering clinical drugs confer cardiovascular benefits via pleiotropic effects such as increased NO production and anti-inflammatory activities [8,40]. In this study, our data confirmed this notion by evidencing that TCEE

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activates the eNOS-NO signaling pathway and attenuates inflammatory responses; however, the effects of TCEE on atherosclerosis and hypertension, as well as the molecular mechanisms underlying these effects are still unknown. To this end, investigations targeting the key events in the development of atherosclerosis are warranted.

Importantly, previous studies have reported that 11 bioactive components including lupeol, stigmasterol, β-sitosterol, betulin, betulinic acid, oleanolic acid, maslinic acid, alphitolic acid, 3-epimaslinic acid, augustic acid, and β-sitosterol-3-0-β-D-glucoside are found in TC extracts [21,24,25]. Moreover, growing evidence has suggested that these components have protective effects against cardiovascular and inflammatory diseases [11,41]. For example, lupeol is reported to have cardioprotective and anti-inflammatory effects by downregulating the expression of TNF-α, IL-2, IL-4, IL-5, IL-6, IL-11, and prostaglandin E_2 [11,41]. Impaired metabolism of phytosterols including β-sitosterol and stigmasterol causes phytosterolemia, which is closely related to early atherosclerosis [10,42] Supplementation with phytosterols retards the progression of atherosclerosis by reducing the cholesterol absorption [10,42]. Although these studies have pointed out the beneficial effects of many components of TC extracts, in this study, we cannot ascertain which component accounts for the TCEE-mediated enhancement of NO bioavailability and anti-inflammatory effects in ECs. Therefore, further investigation is needed for studying the protective effects of TCEE components and their regulatory mechanisms in cardiovascular diseases.

Moreover, the purification methods by which the TC extracts are isolated may affect their bioactivity [21–25]. Previous studies have reported that TC extracts can be purified using water, ethanol, acetate extract, and *n*-butanol [23–25]. These TC extracts may have a variety of effects in regulating the physiological functions of the cardiovascular system and the development of cardiovascular diseases [22,24]. For instance, all extracts obtained by various purification methods have been shown to have a similar inhibitory effect on lipopolysaccharide-induced NO production in macrophages [22,24]; however, compared with other TC extracts, the TC extract purified using *n*-butanol has the strongest efficacy in inhibiting lipid accumulation via PPARγ activation in adipocytes [22]. Our findings further support this notion by evidencing that all the TC extracts mentioned above have similar efficacies on eNOS-mediated NO production (data not shown). Because the hydrophobic property of TCEE facilitates its absorption into cells, compared to the case for hydrophilic extracts, we used TCEE throughout our study. Endothelium-derived NO plays an important role in regulating the vascular homeostasis, and has a protective role against the deregulation of lipid metabolism and inflammation, which are the hallmarks of cardiovascular and metabolic diseases [43–45]. Our findings further confirmed the beneficial effects of TCEE on vascular biology via the enhancement of NO bioavailability. Collectively, these observations from our study or others suggest that TC extracts may have therapeutic potential for treating inflammatory and metabolic diseases.

In conclusion, this study demonstrates that TCEE has the beneficial effects on angiogenesis and the inhibition of inflammation, by activating the eNOS-NO signaling pathway. Our study provides advanced information about the beneficial effects of TCEE in regulating endothelial function and inflammatory responses. These findings may broaden the biological significance and biomedical implications of TCEE in the treatment of cardiovascular and metabolic diseases.

4. Materials and Methods

4.1. Reagents

Mouse antibody for α-tubulin, LY294002, Griess reagent, MTT assay kit, NG-nitro-L-arginine methyl ester (L-NAME) and phosphatase inhibitor cocktails 1 and 2 were obtained from Sigma-Aldrich (St. Louis, MO, USA). Compound C procured was from Calbiochem (San Diego, CA, USA). Rabbit antibodies for phosphor-eNOS at Ser617, 635 and 1179, phosphor-eNOS at Thr497, phosphor-Akt at Ser473, phosphor-CaMKII at Thr286, phosphor-AMPK at Thr172, intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1) were purchased from Cell Signaling Technology (Beverly, MA, USA). Rabbit antibodies for eNOS, Akt, CaMKII and AMPK

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were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Matrigel was procured from BD Biosciences (San Jose, CA, USA).

4.2. Cell Culture

EA.hy926 endothelial cells and THP-1 cells were obtained from the ATCC (Manassas, VA, USA) and cultured in DMEM or RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin and 100 μg/ml streptomycin (HyClone, Logan, UT, USA). EA.hy926 cells were grown until 100% confluence and then used for further experiments.

4.3. TC Extraction

The TC was ground to a powder, which was extracted with 95% ethanol followed by partition extraction. For the ethanol extraction, TC powder was first soaked in 95% ethanol for 3 days. The ethanol was removed by vacuum concentration to produce a concentrate, which was weighed and then degassed by the nitrogen gas bubbling method to remove the remaining ethanol residue. This process generated an ethanol-free, concentrated extract. One aliquot of the extract was re-dissolved with ddH_2O and placed into a partition-extraction flask into which ethyl acetate and *n*-butanol were sequentially added, in the order of increasing solvent polarity, for partition extraction. The two resulting organic extracts were concentrated by vacuum concentration and referred to as TCEAE, composed of TC and ethyl acetate and TCBUE, composed of TC and *n*-butanol. The concentrated aqueous phase remaining after the extraction was referred to as TCWE, which was composed of TC and water. The other aliquot of the aforementioned ethanol-free, concentrated extract, which was not used in the partition extraction, was set aside and referred to as TCEE. The organic extracts TCEAE, TCBUE and TCEE were re-dissolved in absolute ethanol for the generation of stock solutions, whereas the TCWE extract was mixed with PBS to produce a fourth stock solution. All the stock solutions were stored at -20°C until further use.

4.4. Cell Viability Assay

MTT assay was performed according to the manufacturer's instructions for evaluating the cell viability. First, the cells were incubated with or without TCEE for 24 h; 100 μl of MTT reagent (0.5 mg/ml in medium) was then added to the cells, followed by incubation for 3 h. Cells incubated with the vehicle were considered 100% viable.

4.5. Protein Extraction and Western Blot Analysis

Cells were lysed with the SDS lysis buffer, which contained 1% Triton, 0.1% SDS, 0.2% sodium azide, 0.5% sodium deoxycholate and proteinase inhibitors (1 mmol/L PMSF, 10 mg/ml aprotinin, and 1 mg/ml leupeptin). The lysates were centrifuged at 12000 rpm for 5 min and the resulting supernatants were collected. The extracted protein was quantified by protein assay. The proteins were separated by SDS-PAGE; the protein bands were transferred onto BioTrace PVDF membranes. After being blocked with 5% skim milk, the blots were incubated with the primary antibodies, and then with the secondary antibodies. The protein bands were detected by using an enhanced chemiluminescence kit and quantified by using ImageQuant 5.2 (Healthcare biosciences, Philadelphia, PA, USA).

4.6. Determination of Nitrite Production

Nitric oxide (NO) has an extremely short half-life and is quickly metabolized into nitrite and nitrate. Thus, the level of nitrite was determined by the Griess assay to assess NO production. The cell culture medium was mixed with an equal volume of Griess reagent, and then azo dye production was determined by measuring the absorbance of these samples at 540 nm after 15 min of incubation at room temperature. The level of nitrite was normalized relative to the cellular protein concentration. Sodium nitrite was used as a standard.

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4.7. Cell Proliferation Assay

EA.hy926 cells were cultured on 12-well plates in DMEM containing 10% FBS and subjected to serum starvation for 12 h. After treatment, the cells were labeled BrdU with for 4 h. BrdU incorporation was measured at least in triplicate at each time by use of a cell proliferation ELISA colorimetric kit (Roche, Mannheium, Germany). The absorbance was measured by spectrophotometry at 370 nm and referred at 492 nm.

4.8. Cell Migration Assay

The transwell migration assay was performed using modified chambers inserted into 24-well plates. First, 5×10^6 cells suspended in 300 μl of DMEM were added into the upper chamber. On the other hand, 500 μl of DMEM was added into the lower chamber. After 18 h of incubation, the medium and un-migrated cells in the upper chamber were removed, and the migrated cells in the lower side of the membranes were stained with crystal violet. Images were digitally captured under a Nikon TE2000-U florescence microscope with an image analysis system (QCapture Pro 6.0 (QImaging, BC, Canada).

4.9. Matrigel Angiogenesis Assay

Matrigel was coated onto 3.5-cm dishes and allowed to polymerize for 30 min at 37°C. ECs were seeded onto the Matrigel layer and subjected to the indicated treatments for 9 h. The tube formation ability of the ECs was quantified by counting the number of branch points and the images were photomicrographed under a Nikon TE2000-U florescence microscope with an image analysis system.

4.10. In Vitro Mononuclear-Endothelial Cell Adhesion Assay

Adherence of THP-1 cells onto the ECs was examined under static conditions. For fluorescence staining, THP-1 cells were incubated with BCECF-AM at 37°C for 1 h and then added into the culture medium at a concentration of $1x10^5$ cells/ml; the PHP-1 cells were incubated with the ECs for 1 h. Fluorescence of the cell lysates was measured by fluorometry (Molecular Devices, Sunnyvale, CA, USA) with excitation and emission wavelengths of 485 and 525 nm, respectively. Images were photomicrographed under a Nikon TE2000-U microscope with an image analysis system.

4.11. Statistical Analysis

Results are presented as the mean ± SEM from five independent experiments. Mann-Whitney test was used to compare two independent groups. Kruskal-Wallis test, followed by the Bonferroni post hoc analyses, were used for comparing data from multiple groups. SPSS software v8.0 (SPSS Inc. Chicago, IL, USA) was used for performing the statistical analyses. $P< 0.05$ was considered statistically significant.

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