Original Paper

Epigallocatechin-3-Gallate Attenuates the Effects of TNF- α in Vascular Endothelial Cells by Causing Ectodomain Shedding of TNF Receptor 1

Won Seok Yang^a Soo Young Moon^b Mee Jeong Lee^c Su-Kil Park^a

^aDivision of Nephrology, Department of Internal Medicine, Asan Medical Center, College of Medicine, University of Ulsan, Seoul, ^bAsan Institute for Life Sciences, Seoul, ^cDepartment of Pediatrics, College of Medicine, Dankook University, Cheonan, Korea

Key Words

A disintegrin and metalloprotease 10 • Calcium influx • Cyclopiazonic acid • Epigallocatechin-3-gallate • Thapsigargin • Tumor necrosis factor receptor 1

Abstract

Background/Aims: We investigated the mechanism underlying anti-tumor necrosis factor- α (TNF- α) effects of epigallocatechin-3-gallate (EGCG) in human aortic endothelial cells. **Methods:** Tumor necrosis factor receptor 1 (TNFR1) was assessed by Western blot analysis. Cytosolic Ca²⁺ was measured using Fluo-4 AM. A disintegrin and metalloprotease 10 (ADAM10) was localized by immunofluorescence staining. **Results:** EGCG caused ectodomain shedding of TNFR1 within 30 min and attenuated TNF- α -induced endothelin-1 (ET-1) expression. EGCG-induced TNFR1 ectodomain shedding was prevented by BAPTA-AM (intracellular Ca²⁺ chelator), but not by the absence of extracellular Ca²⁺. In physiologic extracellular Ca²⁺ concentration, EGCG markedly increased cytosolic Ca²⁺. Even in the absence of extracellular Ca²⁺, EGCG raised cytosolic Ca²⁺, though less potently. siRNA depletion of ADAM10 prevented EGCG-induced ectodomain shedding of TNFR1 and also diminished the inhibitory effect of EGCG on TNF- α -induced ET-1 expression. EGCG caused translocation of ADAM10 to the plasma membrane, and this effect was prevented by BAPTA-AM. Besides extracellular Ca²⁺ influx, release of intracellular stored Ca²⁺ caused ADAM10-dependent ectodomain shedding of TNFR1. **Conclusion:** EGCG decreases the responsiveness of cells to TNF- α by causing ADAM10-dependent ectodomain shedding of TNFR1. This effect was attributed to its property to increase cytosolic Ca²⁺ through both extracellular Ca²⁺ influx and release of stored Ca²⁺.

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Su-Kil Park, M.D.

Department of Internal Medicine, Asan Medical Center, University of Ulsan, 88 Olympic-Ro 43-Gil, SongPa-Gu, Seoul 138-736, (Korea)
Tel. +82-2-3010-3263, Fax +82-2-3010-6963, E-Mail skpark@amc.seoul.kr

Introduction

Epigallocatechin-3-gallate (EGCG) is a catechin that is abundant in green tea [1]. In animal and human studies, it had beneficial effects on autoimmune disease [2], atherosclerosis [3, 4] and cancer [5, 6]. *In vitro* studies have revealed that EGCG exerts diverse biological effects on the cells through regulation of cellular signaling as well as anti-oxidative action [7].

Tumor necrosis factor- α (TNF- α) is a pro-inflammatory cytokine that has a pivotal role in the development of atherosclerosis [8] and cancer [9] as well as immune-mediated disease [10]. One of the biologic actions of EGCG is to attenuate the effects of TNF- α . EGCG was shown to inhibit TNF- α -induced productions of fractalkine, chemokine (C-C motif) ligand 2 and plasminogen activator inhibitor-1 in human umbilical vein endothelial cells [11-13]. It also inhibited TNF- α -induced expression of vascular cell adhesion molecule-1 in human aortic endothelial cells (HAECs) and thereby prevented TNF- α -stimulated monocyte adhesion [14]. Besides, EGCG protected against TNF- α -mediated lung inflammation in mice [15]. It is well-known that TNF- α stimulates the generation of reactive oxygen species (ROS) and activates transcriptional factors including nuclear factor- κ B (NF- κ B) to increase the transcription of target genes [16]. As the mechanisms of the anti-TNF- α effects, EGCG was shown to suppress the generation of ROS or inhibit NF- κ B [11-15].

To exert the cellular effects, TNF- α should bind to the receptors on the cell surface. Two different receptors, tumor necrosis factor receptor 1 (TNFR1) and 2, mediate the signaling of TNF- α [17]. Recently, we found that extracellular Ca^{2+} influx, induced by vitamin D₃ or an L-type calcium channel agonist Bay K8644, causes a disintegrin and metalloprotease (ADAM) 10-mediated ectodomain shedding of TNFR1 in human aortic smooth muscle cells [18, 19]. The shedding of TNFR1 ectodomain results in decreased responsiveness of the cells to TNF- α by reducing the number of receptors on the cell surface. In addition, the released ectodomain portion of TNFR1 may bind to TNF- α in the extracellular space and neutralize its activity [20]. Although the mechanisms are not completely elucidated, EGCG also increases cytosolic Ca^{2+} in vascular smooth muscle cells and endothelial cells by extracellular Ca^{2+} influx or release from endoplasmic reticulum (ER) storage Ca^{2+} [21-23]. Thus, we hypothesized that EGCG may have anti-TNF- α effects by causing ectodomain shedding of TNFR1 through Ca^{2+} -dependent activation of ADAM10.

Endothelin-1 (ET-1) is a potent vasoconstrictive peptide produced by vascular endothelial cells [24]. Its effects on vascular smooth muscle cells are implicated in diverse vascular diseases such as hypertension and atherosclerosis [24]. In our previous study [25], TNF- α was shown to stimulate ET-1 production in HAECs.

In the present study, therefore, we investigated whether EGCG causes ectodomain shedding of TNFR1 in HAECs and thereby attenuates the stimulatory effect of TNF- α on ET-1 expression.

Materials and Methods

Materials

EGCG, thapsigargin, cyclopiazonic acid, calcium chloride, 4', 6-diamidino-2-phenylindole dihydrochloride (DAPI) and 1,2-bis(o-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid)-acetoxymethyl ester (BAPTA-AM) were purchased from Sigma-Aldrich (St. Louis, MO, USA). EGCG was dissolved in phosphate buffered saline (PBS). TNF- α was from R&D Systems (Abingdon, UK). Antibodies to human ADAM10 and actin were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Antibodies to human TNFR1 (ab19139; raised against synthetic peptides corresponding to amino acids 29-43 of human TNFR1 in the N-terminus) and ET-1 were from Abcam, Inc. (Cambridge, MA, USA). ADAM10-siRNA, control-siRNA (Ambion®) and Fluo-4 AM (Molecular probes®) were from Life Technologies (Paisley, UK).

Cell culture

HAECs were obtained from Lonza Walkersville, Inc. (Walkersville, MD, USA). The cells were cultured in EBM-2 endothelial growth basal medium (Lonza Walkersville, Inc.). Before each experiment, the cells were

rested for 24 h in RPMI-1640 (Life Technologies) containing 2 % fetal calf serum (Biological Industries Ltd., Cumbernauld, UK) and then the culture medium was replaced with serum-free RPMI-1640 (Ca^{2+} adjusted to 1.2 mM by calcium chloride supplementation) or Dulbecco's modified Eagle's medium (DMEM) (Ca^{2+} 0 or 1.2 mM), as appropriately.

Transfection of siRNA

siRNA transfection was performed on the cells seeded in a 6-well plate and cultured for 24 h, using Lipofectamine® Reagent (Life Technologies). In brief, 100 pmol siRNAs were incubated with 10 μl of lipofectamine reagent diluted in Opti-MEM® medium (Life Technologies) for 15 minutes at room temperature, by which siRNA-lipofectamine complexes are formed. To perform transfection, siRNA-lipofectamine complexes were added to the cells in fresh serum-free culture medium. After 6 h incubation at 37°C in a CO_2 incubator, the medium was replaced with complete growth medium containing 10 % fetal calf serum, and the cells were further incubated for 18 h. Thereafter, the cells were subjected to the experiments.

Western blot analysis

Equal amounts of the cell lysates were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to an Immobilon-P membrane (EMD Millipore Co., Bedford, MA, USA). The membrane was probed with a primary antibody. Bands were visualized using horseradish peroxidase conjugated secondary antibody and the enhanced chemiluminescence agent (Luminata™ Forte Western HRP Substrate; EMD Millipore Co.).

To detect TNFR1 in cell culture supernatants, the conditioned media were harvested and concentrated using Amicon® ultra 10K centrifugal filter device (EMD Millipore Co.). Thereafter, the samples were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis and immunoblot analysis using an antibody against N-terminus of TNFR1.

Measurement of cytosolic Ca^{2+} by confocal microscopy

HAECs cultured in a 6-well plate were loaded with Fluo-4 AM (2 μM). After gentle washing twice with Hank's balanced salt solution (HBSS), the cells were treated with EGCG, thapsigargin or cyclopiazonic acid in 1.2 mM Ca^{2+} -containing HBSS or Ca^{2+} -free HBSS. The Fluo-4 in the cells was excited by wavelength at 494 nm and fluorescence images were captured at 506 nm in a 20 sec interval for 15 min using Zeiss LSM710 laser-scanning confocal microscope (Carl Zeiss, Germany). The fluorescence intensities were quantified using ZEN 2011 imaging Software (Carl Zeiss, Germany). The change in intracellular Ca^{2+} concentration was represented by relative fluorescence intensity compared with the initial value.

Immunofluorescence staining

To investigate the localization of ADAM10, the cells were washed with PBS, fixed with 4% paraformaldehyde for 10 min, permeabilized with 0.4% Triton X-100 in PBS for 5 min, and incubated with 1% BSA in PBS for 60 min to block nonspecific binding. Thereafter, the cells were incubated with goat anti-ADAM10 antibody overnight at 4°C, washed 3 times with PBS and then incubated with FITC-conjugated anti-goat IgG secondary antibody. The immunoreactivity for ADAM10 was captured using a confocal microscope.

Statistical analysis

Data are presented as means $\pm$ SE (standard error), with n representing the number of different experiments. An analysis of variance with Scheffe's multiple-comparison test was used to determine statistically significant differences among the groups. A p value of <0.05 was considered statistically significant.

Results

EGCG attenuates TNF- α -induced ET-1 expression

We first examined the effect of EGCG on TNF- α -induced ET-1 expression in HAECs. HAECs were pretreated with different concentrations of EGCG for 30 min. Thereafter, the culture media containing EGCG were discarded and replaced with fresh media without EGCG,

Fig. 1. EGCG attenuates TNF- α -induced ET-1 expression. HAECs were incubated with different concentrations of EGCG for 30 min in RPMI-1640 containing 1.2 mM Ca²⁺. Thereafter, the media containing EGCG were discarded and replaced with fresh RPMI-1640 without EGCG, and the cells were stimulated with TNF- α (10 ng/ml) for 24 h. Whole cell lysates were analyzed by Western blotting using anti-ET-1 and anti-actin antibodies. (n = 3, *p < 0.05 compared with control; **p < 0.05 as compared with TNF- α).

Fig. 2. EGCG causes ectodomain shedding of TNFR1. HAECs in RPMI-1640 containing 1.2 mM Ca²⁺ were incubated with different concentrations of EGCG for 30 min (A) (n = 4), incubated with 50 μM of EGCG for 0, 2.5, 5, 10 or 30 min (B) (n = 4), or incubated with 20 μM of EGCG for 0, 10 min, 30 min, 8 h or 24 h (C) (n = 3). Whole cell lysates and conditioned media were analyzed by Western blotting using an antibody against the N-terminus of TNFR1. (*p < 0.05 compared with control).

and the cells were further incubated with TNF- α (10 ng/ml) for 24 h. TNF- α increased ET-1 expression, while pretreatment of the cells with EGCG attenuated the stimulatory effect of TNF- α in a dose-dependent manner (Fig. 1).

EGCG causes ectodomain shedding of TNFR1

To test whether EGCG causes ectodomain shedding of TNFR1, we incubated HAECs with EGCG at the doses of 0, 1, 10, 50 or 100 μM for 30 min or at the dose of 50 μM for 0,

Fig. 3. EGCG-induced TNFR1 ectodomain shedding is dependent on the elevation of cytosolic Ca²⁺. (A, B) EGCG-induced TNFR1 ectodomain shedding is prevented by BAPTA-AM, but not by the absence of extracellular Ca²⁺. (A) HAECs were preincubated with or without BAPTA-AM (10 μ M) for 30 min, and then further treated with EGCG (50 μ M) for 30 min in RPMI-1640 containing 1.2 mM Ca²⁺ (n = 3). (B) HAECs were incubated with or without EGCG (50 μ M) for 30 min in DMEM medium containing no Ca²⁺ or 1.2 mM Ca²⁺ (n = 6). Whole cell lysates were analyzed by Western blotting using anti-TNFR1 antibody. (*p < 0.05 compared with control; **p < 0.05 compared with EGCG). (C) EGCG increases cytosolic Ca²⁺ by both extracellular Ca²⁺ influx and release of intracellular storage Ca²⁺. HAECs were preincubated with or without BAPTA-AM (10 μ M) for 30 min, and loaded with Fluo-4 AM (2 μ M), and placed in 1.2 mM Ca²⁺-containing HBSS or Ca²⁺-free HBSS. The cells were treated with EGCG (50 μ M), and intracellular Ca²⁺ fluorescence intensities were recorded every 20 sec for 15 min. Addition of EGCG is indicated by an arrow. (means $\pm$ SE of n = 123 ~ 166 cells in each group from three independent experiments).

2.5, 5, 10 or 30 min, and obtained whole cell lysates and the conditioned media. TNFR1 was measured by Western blot analysis. As shown in Fig. 2A, 2B, EGCG decreased TNFR1 in the cell lysates. TNFR1 released in the culture medium was detected by the antibody recognizing N-terminus of TNFR1, and the band was located at ~ 25 kDa, indicating an N-terminal fragment of TNFR1. In contrast to the cellular TNFR1, TNFR1 in the culture medium was increased by EGCG. These effects of EGCG on TNFR1 were dose-, and time-dependent. Within 30 min, EGCG (50 μ M) markedly decreased cellular TNFR1.

To assess the prolonged effect of EGCG, we treated the cells with a lower dose of EGCG (20 μ M) for up to 24 h. Cellular TNFR1 decreased significantly within 30 min after EGCG treatment, but recovered to the control level at 24 h (Fig. 2C).

EGCG-induced TNFR1 ectodomain shedding is prevented by BAPTA-AM, but not by the absence of extracellular Ca²⁺

Next, we explored whether Ca²⁺ signaling is implicated in the ectodomain shedding of TNFR1 in the cells treated with EGCG. HAECs were preincubated with or without BAPTA-

Fig. 4. Ca^{2+} -dependent ADAM10 activation is implicated in the ectodomain shedding of TNFR1. (A) EGCG induces Ca^{2+} -dependent ADAM10 translocation to the plasma membrane. HAECs in DMEM medium containing no Ca^{2+} or 1.2 mM Ca^{2+} were preincubated with or without BAPTA-AM (10 μM) for 30 min, and then further treated with EGCG (50 μM) for 10 min. After then, the cells were fixed with paraformaldehyde, permeabilized with Triton-X, immunostained for ADAM10, and visualized with confocal microscopy. Cell nuclei were stained with DAPI. Representative images from three independent experiments are shown. Notice the ADAM10 immunoreactivity at the surface of the cells treated with EGCG. (B, C) Depletion of ADAM10 abolishes EGCG-induced ectodomain shedding of TNFR1 and the inhibitory effect of EGCG. (B) HAECs were transfected with control-siRNA or ADAM10-siRNA, and then incubated with EGCG (50 μM) for 30 min in RPMI-1640 containing 1.2 mM Ca^{2+} . Whole-cell lysates were analyzed by Western blotting using anti-TNFR1, anti-ADAM10 and anti-actin antibodies. (n = 3, *p < 0.05 compared with control). (C) HAECs were transfected with control-siRNA or ADAM10-siRNA, and then incubated with or without EGCG (50 μM) for 30 min in RPMI-1640 containing 1.2 mM Ca^{2+} . Thereafter the culture media containing EGCG were discarded and replaced with fresh RPMI-1640 without EGCG, and the cells were stimulated with TNF-α (10 ng/ml) for 24 h. Whole cell lysates were analyzed by Western blotting using an anti-ET-1 antibody. (n = 3, *p < 0.05 compared with control).

AM, an intracellular Ca^{2+} chelator, for 30 min and then further treated with EGCG for 30 min. Thereafter, whole cell lysates were analyzed by Western blotting using anti-TNFR1 antibody. As shown in Fig. 3A, the ectodomain shedding of TNFR1 by EGCG was abrogated by BAPTA-AM.

To determine whether extracellular Ca^{2+} influx is essential for EGCG-induced ectodomain shedding of TNFR1, we tested it in Ca^{2+} -free DMEM culture medium. As shown in Fig. 3B, EGCG induced ectodomain shedding of TNFR1 even in the absence of extracellular Ca^{2+} .

EGCG increases cytosolic Ca^{2+} by both extracellular Ca^{2+} influx and release of stored Ca^{2+}

Next, we investigated the effect of EGCG on cytosolic Ca^{2+} level in HAECs, using Fluo-4 AM, a calcium fluorescent indicator (Fig. 3C). In the presence of extracellular Ca^{2+} (1.2 mM), EGCG greatly increased cytosolic Ca^{2+} concentration. EGCG-induced elevation of cytosolic Ca^{2+} was abolished by BAPTA-AM, a cell-permeant chelator for Ca^{2+} .

Fig. 5. Cytosolic release of stored Ca^{2+} causes ectodomain shedding of TNFR1. (A) Thapsigargin and cyclopiazonic acid increase cytosolic Ca^{2+} by release of stored Ca^{2+} . HAECs were loaded with Fluo-4 AM (2 μM), and treated with DMSO (vehicle), thapsigargin (0.2 μM) or cyclopiazonic acid (2 μM). The experiments were performed in Ca^{2+} -free HBSS. Time-response traces of intracellular Ca^{2+} fluorescence intensity are shown. Addition of DMSO, thapsigargin or cyclopiazonic acid is indicated by an arrow. (means $\pm$ SE of $n = 121 \sim 151$ cells in each group from three independent experiments). (B) Thapsigargin and cyclopiazonic acid cause ectodomain shedding of TNFR1, which is inhibited by intracellular Ca^{2+} chelator. HAECs were incubated with or without BAPTA-AM (10 μM) for 30 min. Thereafter, the culture medium was changed to Ca^{2+} -free DMEM, and the cells were further treated with thapsigargin (0.2 μM) or cyclopiazonic acid (2 μM) for 30 min. Whole cell lysates and conditioned media were analyzed by Western blotting using anti-TNFR1 antibody. ($n = 3$, * $p < 0.05$ compared with control; ** $p < 0.05$ compared with thapsigargin; # $p < 0.05$ compared with control; ## $p < 0.05$ compared with cyclopiazonic acid).

In the absence of extracellular Ca^{2+} , EGCG still increased cytosolic Ca^{2+} , but the potency was lower than that in the presence of extracellular Ca^{2+} .

ADAM10 mediates EGCG-induced ectodomain shedding of TNFR1

Because calcium influx is known to activate ADAM10 [26], we investigated the role of ADAM10 in EGCG-induced TNFR1 ectodomain cleavage. By immunofluorescent staining and confocal microscopy, the location of ADAM10 was visualized (Fig. 4A). ADAM10 was detected predominantly in the cytoplasm of untreated cells. On treatment with EGCG, ADAM10 immunoreactivity increased at the cell surface. EGCG-induced translocation of ADAM10 to the cell surface was inhibited by BAPTA-AM but not by the absence of extracellular Ca^{2+} .

To determine whether ADAM10 is involved in EGCG-induced ectodomain shedding of TNFR1, we transfected HAECs with control-siRNA or ADAM10-siRNA, and treated them with EGCG. Depletion of ADAM10 prevented the ectodomain cleavage of TNFR1 induced by EGCG (Fig. 4B).

We next investigated whether depletion of ADAM10 reverses the inhibitory effect of EGCG on TNF- α -induced ET-1 expression. As shown in Fig. 4C, EGCG could not inhibit TNF- α -induced ET-1 expression when ADAM10 was depleted by siRNA transfection.

Cytosolic release of stored Ca^{2+} causes ADAM10-mediated ectodomain shedding of TNFR1

Because EGCG elevated cytosolic Ca^{2+} even in the absence of extracellular Ca^{2+} and caused ectodomain shedding of TNFR1, we tested whether cytosolic release of stored Ca^{2+} , using thapsigargin and cyclopiazonic acid, causes ADAM10-mediated ectodomain shedding of TNFR1. Thapsigargin and cyclopiazonic acid elevate cytosolic Ca^{2+} by inhibiting Ca^{2+} -ATPase that transports cytosolic Ca^{2+} into ER [27].

Fig. 6. Cytosolic release of stored Ca^{2+} activates ADAM10 to cause ectodomain shedding of TNFR1. (A) Thapsigargin and cyclopiazonic acid induce ADAM10 translocation to the plasma membrane. HAECs were incubated with or without BAPTA-AM (10 μM) for 30 min. In Ca^{2+} -free DMEM medium, the cells were further treated with thapsigargin (0.2 μM) or cyclopiazonic acid (2 μM) for 10 min. After then, the cells were fixed with paraformaldehyde, permeabilized with Triton-X, immunostained for ADAM10, and visualized with confocal microscopy. Cell nuclei were stained with DAPI. Representative images from three independent experiments with similar results are shown. Notice the ADAM10 immunoreactivity at the surface of the cells treated with thapsigargin or cyclopiazonic acid. (B) Depletion of ADAM10 abolishes thapsigargin- and cyclopiazonic acid-induced ectodomain shedding of TNFR1. HAECs were transfected with control-siRNA or ADAM10-siRNA. In Ca^{2+} -free DMEM medium, the cells were incubated with thapsigargin (0.2 μM) or cyclopiazonic acid (2 μM) for 30 min. Whole cell lysates were analyzed by Western blotting using anti-TNFR1, anti-ADAM10 and anti-actin antibodies. ($n = 4$, * $p < 0.05$ compared with control).

As shown in Fig. 5A, both thapsigargin and cyclopiazonic acid acutely increased the cytosolic Ca^{2+} in the absence of extracellular Ca^{2+} . As with EGCG, both thapsigargin and cyclopiazonic acid caused ectodomain shedding of TNFR1, and this effect was inhibited by BAPTA-AM (Fig. 5B).

Next, we examined the involvement of ADAM10 in thapsigargin- and cyclopiazonic acid-induced ectodomain shedding of TNFR1. As shown in Fig. 6A, both thapsigargin and cyclopiazonic acid induced plasma membrane translocation of ADAM10, while BAPTA-AM suppressed this effect. On the other hand, depletion of ADAM10 by siRNA transfection prevented thapsigargin- and cyclopiazonic acid-induced ectodomain shedding of TNFR1 (Fig. 6B).

Discussion

In the present study, we explored the mechanism underlying anti-TNF- α effects of EGCG in HAECs and our data demonstrated that EGCG rapidly causes ectodomain shedding of TNFR1 and thereby attenuates the cellular effect of TNF- α . ADAM10 was implicated in the cleavage of TNFR1 ectodomain. This effect of EGCG was attributed to its property to elevate

cytosolic Ca^{2+} through both extracellular Ca^{2+} influx and release of stored Ca^{2+} . The elevated cytosolic Ca^{2+} triggered the translocation of ADAM10 to the plasma membrane where it may catalyze the cleavage of TNFR1.

TNF- α exerts its biologic effect on the cells through its receptors expressed on the cell surface. EGCG has been shown to suppress the effects of TNF- α in a variety of cell types including vascular endothelial cells [11-15], but it has not been properly investigated whether EGCG has a regulatory effect on TNF receptors. So far, there were two studies that measured the effect of EGCG on the expression of TNFR1 [13, 28], in which bovine coronary artery and human umbilical vein endothelial cells were treated with or without EGCG (40 μM) for 15 h or 27 h, and TNFR1 in the cells treated with EGCG was not different from that of control cells. In contrast, our data showed that EGCG markedly reduces cellular TNFR1 within 30 min in a dose-dependent manner at the concentrations between 1 ~ 100 μM . The reduction of cellular TNFR1 was accompanied by an increase of N-terminal fragment of TNFR1 in cell culture supernatant, indicating that the extracellular domain of TNFR1 is cleaved and shedded into extracellular space. The different findings observed in these studies can be explained by the temporal changes in the cellular level of TNFR1 after EGCG treatment. EGCG rapidly caused ectodomain shedding of TNFR1, but the decreased cellular TNFR1 was recovered to the control level at 24 h, and it may be because the cleaved TNFR1 was replaced later by newly synthesized TNFR1.

BAPTA-AM is permeable to the plasma membrane due to the AM group. Once it is within the cells, the AM group is removed by esterase in the cytoplasm, and then BAPTA is able to chelate Ca^{2+} . In the present study, EGCG increased cytosolic Ca^{2+} , and EGCG-induced TNFR1 ectodomain shedding was dependent on its ability to increase cytosolic Ca^{2+} because this effect was abolished by BAPTA-AM. Normally, there is a large concentration gradient of Ca^{2+} across the cell membrane. In extracellular fluid, Ca^{2+} exists in the range of 1.10-1.35 mM [29], while cytosolic Ca^{2+} is maintained at much lower concentration (about 100 nM) in the resting state [30]. This concentration gradient is formed by various calcium pumps. In contrast to low Ca^{2+} in the cytoplasm, ER contains high concentration of Ca^{2+} by the action of the sarco- and endoplasmic reticulum Ca^{2+} -ATPase (SERCA) that transports Ca^{2+} against a concentration gradient, and serves as a storage site of calcium [27]. In response to various stimuli, cytosolic Ca^{2+} may increase by Ca^{2+} influx from extracellular fluid or by Ca^{2+} release from intracellular storage, and acts as a signaling molecule [31]. EGCG has been shown to increase cytosolic Ca^{2+} in the previous studies. In rat aortic smooth muscle cells [21], EGCG caused extracellular Ca^{2+} influx via voltage-operated Ca^{2+} channels and Ca^{2+} -permeable non-selective cation channels. In bovine aortic endothelial cells, EGCG was shown to increase cytosolic Ca^{2+} by release of ER storage Ca^{2+} [22] or extracellular Ca^{2+} influx [23]. Thus, EGCG seems to regulate cytosolic Ca^{2+} by several different mechanisms. Our data in HAECs were consistent with the previous studies. In the presence of extracellular Ca^{2+} , EGCG markedly increased cytosolic Ca^{2+} . In the absence of extracellular Ca^{2+} , EGCG still raised cytosolic Ca^{2+} . However, the magnitude of increase was less than that in the presence of extracellular Ca^{2+} . Therefore, both extracellular Ca^{2+} influx and release of storage Ca^{2+} were considered to contribute to EGCG-induced elevation of cytosolic Ca^{2+} .

The expressions of cell surface receptors and signaling molecules are regulated by ADAMs that cleave the extracellular portion of the transmembrane proteins. So far, 12 proteolytically active ADAMs have been identified [32]. In the present study, EGCG caused translocation of ADAM10 from the cytoplasm to the plasma membrane where TNFR1 is expressed. On the other hand, depletion of ADAM10 by siRNA transfection abolished EGCG-induced TNFR1 ectodomain shedding and also diminished the inhibitory effect of EGCG on TNF- α -induced ET-1 production. Thus, ADAM10 was implicated in EGCG-induced ectodomain cleavage of TNFR1.

Extracellular Ca^{2+} influx, induced by vitamin D_3 , Bay K8644 or ionomycin, has been shown to activate ADAM10 in our own and other previous studies [18, 19, 33], but it has not yet been verified whether release of intracellular stored Ca^{2+} also activates ADAM10. In the present study, even in the absence of extracellular Ca^{2+} , EGCG increased cytosolic Ca^{2+}

and caused plasma membrane translocation of ADAM10 and induced ectodomain shedding of TNFR1, suggesting that release of storage Ca^{2+} also activates ADAM10. ER is the major Ca^{2+} store in the cells [34]. The SERCA inhibitors, thapsigargin and cyclopiazonic acid, are known to block the ability of the cell to pump Ca^{2+} into ER and acutely raise cytosolic Ca^{2+} by releasing ER stored Ca^{2+} [35, 36]. In the present study, both thapsigargin and cyclopiazonic acid raised cytosolic Ca^{2+} in the absence of extracellular Ca^{2+} and caused plasma membrane translocation of ADAM10 and ectodomain shedding of TNFR1, while these effects on ADAM10 and TNFR1 were abolished by BAPTA-AM. On the other hand, depletion of ADAM10 prevented SERCA inhibitor-induced ectodomain shedding of TNFR1. Taken together with our previous studies [18, 19], these findings demonstrate that elevation of cytosolic Ca^{2+} is the trigger factor for ADAM10 activation whether it is caused by influx of extracellular Ca^{2+} or release of intracellular stored Ca^{2+} .

Vascular inflammation is implicated in the pathogenesis of hypertension [37]. Proinflammatory cytokines, including $\text{TNF-}\alpha$, cause endothelial dysfunction leading to reduced vasodilatory responses [38]. In addition, $\text{TNF-}\alpha$ stimulates endothelial cells to produce ET-1, a potent vasoconstrictor [25]. Chronic inflammation is also a cardinal feature of atherosclerotic vascular disease [39, 40], in which $\text{TNF-}\alpha$ plays a critical role in the development of the early lesions of atherosclerosis [8]. In animal studies, EGCG was shown to be effective in preventing endothelial dysfunction, hypertension and atherosclerosis [3, 41]. In this regard, our data suggest that EGCG may have the beneficial effects on cardiovascular diseases in part by reducing the responsiveness of endothelial cells to $\text{TNF-}\alpha$ through ectodomain shedding of TNFR1.

In summary, EGCG elevates cytosolic Ca^{2+} in HAECs by both extracellular Ca^{2+} influx and release of stored Ca^{2+} . Elevated cytosolic Ca^{2+} triggers ADAM10-mediated ectodomain shedding of TNFR1 and thereby reduces the response of the cells to $\text{TNF-}\alpha$. By inducing ectodomain shedding of TNFR1, EGCG may downregulate the inflammatory response of vascular endothelial cells.

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Disclosure Statement

The authors declare no potential conflict of interest.

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