의학박사 학위논문

Epigallocatechin-3-gallate가 대동맥 내피세포에서 TLR4의 세포외영역 절단을 통해 갖는 LPS에 대한 억제 효과

Epigallocatechin-3-gallate has an inhibitory effect against LPS in aortic endothelial cells by inducing ectodomain shedding of TLR4

울산대학교대학원
의 학 과
백 충 희

Epigallocatechin-3-gallate has an inhibitory effect against LPS in aortic endothelial cells by inducing ectodomain shedding of TLR4

지도교수 양 원 석

이 논문을 의학박사 학위 논문으로 제출함

2018년 12월

울산대학교 대학원
의 학 과
백 충 희

백충희의 의학박사학위 논문을 인준함

심사위원 김 순 배 (인)

심사위원 양 원 석 인

심사위원 장 재 원 인

심사위원 김 효 상 인

심사위원 김 상 현 인

울 산 대 학 교 대 학 원

2018 년 12 월

국문요약

연구배경: Toll-like receptor 4 (TLR4)는 선천성 면역에 중요한 조절자이며, 염증, 자가면역질환, 암, 신경정신병 등에 중요한 역할을 한다. Epigallocatechin-3-gallate (EGCG)는 녹차잎에 가장 풍부한 폴리페놀로 TLR4 의 발현을 감소시키거나 TLR4 신호체계를 막는다고 알려져 있으나 EGCG 가 TLR4 의 활동을 조절하는 기전은 잘 정립되어 있지 않다. 본 연구에서는 사람동맥내피세포에서 EGCG 가 TLR4 의 세포외 영역절단을 일으키는지를 조사하였다.

방법: 배양된 사람동맥내피세포에서 TLR4 의 발현은 웨스턴 블롯을 통해 조사하였다. 세포내 칼슘 레벨은 Fluo-4 AM 을 통해 측정하였고, A disintegrin and metalloprotease 10 (ADAM10)의 역할은 siRNA 와 면역형광법을 통해 알아보았다.

결과: 지질다당류에 의한 ICAM-1 의 발현이 EGCG 에 의해 감소되었다. EGCG 는 용량, 시간 의존적 양상으로 사람동맥내피세포의 TLR4 의 발현을 감소시키고 사람동맥내피세포 배지의 TLR4 의 아미노말단 정도를 증가시켰다. 세포내 칼슘 킬레이터제인 BAPTA-AM 은 EGCG 에 의한 TLR4 의 세포외영역 절단을 막았다. 세포외 칼슘이 없어도 EGCG 는 여전히 세포내 칼슘 레벨을 증가시켰으나 그 효과는 약간 감소하였다. EGCG 처리를 하면, 세포 표면의 ADAM10 의 발현이 증가되었다. BAPTA-AM 은 EGCG 에 의한 ADAM10 의 세포표면으로의 이동을 억제하였으나, 세포외 칼슘의 부재는 ADAM10 의 이동을 막지 않았다. siRNA 로 ADAM10 을 고갈시켰을 때 EGCG 에 의한 TLR4 의 세포외영역 절단이 일어나지 않았고, LPS 에 의한 ICAM-1 의 발현이 EGCG 에 의해 억제되지 않았다.

결론: EGCG 는 사람동맥내피세포에서 세포내 칼슘 농도를 올림으로써 ADAM10 을 통해 TLR4 의 세포외영역 절단을 일으킨다.

중심단어: Toll-like receptor 4, epigallocatechin-3-gallate, A disintegrin and metalloprotease 10, calcium

차 례

그림목차

Introduction

The human immune system includes both innate and adaptive immunity. Innate immune recognition relies on pattern recognition receptors, which are germline-encoded and recognize broad classes of molecular structures common to groups of microorganisms. Toll-like receptors (TLRs) are one of the largest families of pattern recognition receptors that detect microbial components.[1)] TLR4 is expressed on the cell surface.[2)] Lipopolysaccharide (LPS) recognition by TLR4 mediates rapid cytokine production and the recruitment of inflammatory cells to the site of infection.[3)] In addition, TLR4 signaling is involved in sterile inflammation, the regulation of pain, and the restoration of central nervous system homeostasis after injury.[1, 4)] Recent studies have shown that TLR4 is also involved in the pathogenesis of diabetic nephropathy.[5-8)]

Epigallocatechin-3-gallate (EGCG) is the most abundant polyphenol in green tea leaves.[9)] EGCG has been reported to have a variety of anti-allergic, anti-oxidant, and anti-inflammatory properties.[10)] In particular, several studies have shown a cardiovascular benefit of EGCG under both normal and pathological conditions.[9, 11-13)] Cardiovascular disease is the leading cause of death in patients with chronic kidney disease, and TLR4 is known to contribute to the development and perpetuation of endothelial dysfunction in response to uremic toxicity.[14)] In previous studies, EGCG has been shown to decrease the expression of TLR4 and to inhibit TLR4 signaling.[10, 15, 16)] However, the mechanism through which EGCG regulates TLR4 activity is not well-characterized.

A disintegrin and metalloproteases 10 (ADAM10) is known to cleave the ectodomains of cell surface receptors through Ca^{2+}-dependent activation.[17)] EGCG increases cytosolic Ca^{2+} in endothelial cells and vascular smooth muscle cells.[18-20)] Recently, our study suggested that TLR4 may also be a substrate of ADAM10.[21)] Therefore, we hypothesized that EGCG may downregulate TLR4 at the cell surface by causing ectodomain shedding of TLR4 through Ca^{2+}-dependent activation of ADAM10.

This present study investigated whether EGCG causes ectodomain shedding of TLR4 in human aortic endothelial cells (HAECs).

Materials and Methods

Materials

EGCG and 1,2-bis(o-aminophenoxy) ethane-N,N,N',N'-tetraacetic acid-acetoxymethyl ester (BAPTA-AM) were obtained from Sigma-Aldrich (St. Louis, MO, USA). Phosphate-buffered saline (PBS) was used to dissolve EGCG. An antibody raised against a synthetic peptide corresponding to amino acids 100–200 of human TLR4 (sc-52962) and antibodies to human ADAM10, ICAM-1 and actin were from Santa Cruz Biotechnology. Fluo-4 AM (Molecular Probes®), ADAM10 siRNA, and control siRNA (Ambion®) were from ThermoFisher Scientific (Waltham, MA).

Cell culture

HAECs were purchased from Lonza Walkersville (Walkersville, MD, USA), and the cells were cultured in EBM-2 endothelial growth basal medium (Lonza Walkersville). The cells were rested for 16 h in M199 Hank's media (Gibco/ThermoFisher Scientific) containing 2% fetal calf serum (Biological Industries Ltd, Cumbernauld, UK) before each experiment. For treatment, the culture medium was replaced with serum-free M199 Hank's media or Dulbecco's modified Eagle's medium (DMEM) (0 or 1.2 mM Ca^{2+}), as indicated.

Transfection with siRNA

HAECs were seeded in 6-well plates, cultured for 24 hours, and then transfected with siRNA using Lipofectamine (Life Technologies/ThermoFisher Scientific). To generate siRNA-Lipofectamine complexes, 100 pmol siRNA was incubated with 10 µl Lipofectamine diluted in Opti-MEM medium (Life Technologies/ThermoFisher Scientific) for 15 minutes at room temperature. The siRNA-Lipofectamine complexes were then added to cells in fresh serum-free culture medium, and cells were incubated for 6 hours at 37°C in a CO_2 incubator. The medium was replaced with complete growth medium and the cells were incubated for an additional 18 h prior to use in experiments.

Western blot analysis

Treated cells were lysed on ice for 10 minutes in lysis buffer [50 mM Tris-HCl (pH 7.4),

150 mM NaCl, 0.25% sodium deoxycholate, 1% NP-40, and protease and phosphatase inhibitors]. The lysed cells were collected and centrifuged at 4°C for 20 minutes. Proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to Immobilon-P membranes (EMD Millipore, Bedford, MA, USA). The membranes were incubated with primary antibodies and horseradish peroxidase-conjugated secondary antibodies. Bands were visualized by enhanced chemiluminescence (Luminata Forte Western HRP Substrate; EMD Millipore).

To measure secreted TLR4 levels, cell culture supernatants were concentrated using Amicon ultra 10K centrifugal filters (EMD Millipore) and analyzed by Western blotting with a TLR4 antibody.

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

Fluo-4 fluorescence upon binding to Ca^{2+} and is therefore used as an indicator of Ca^{2+} concentration. HAECs in 6-well plates were incubated with Fluo-4 AM (2 μM) for 30 minutes. After washing with Hank's balanced salt solution (HBSS), the cells were placed in HBSS (0 or 1.2 mM Ca^{2+}). The cells were treated with EGCG, and fluorescence images (excitation 494 nm, emission 506 nm) were captured every 20 seconds for 10 minutes using a Zeiss LSM710 laser-scanning confocal microscope (Carl Zeiss, Germany). The fluorescence intensities were measured using ZEN 2011 imaging software (Carl Zeiss, Germany). The change in intracellular Ca^{2+} concentration is represented by the relative fluorescence intensity compared with the initial value.

Immunofluorescence staining

To examine ADAM10 expression by immunofluorescence, the cells were fixed with 4% paraformaldehyde for 10 minutes (without permeabilization) and incubated with 1% bovine serum albumin in PBS for 60 minutes to inhibit nonspecific binding. The cells were then incubated with a goat anti-ADAM10 antibody overnight at 4°C, washed three times with PBS, and incubated again with an AlexaFluor 488-conjugated anti-goat IgG secondary antibody. Immunofluorescence images were captured with a Zeiss LSM710 laser-scanning confocal microscope.

Statistical analysis

Data are presented as the mean ± standard error (SE). Analysis of variance with Scheffe's multiple comparisons test was used to determine statistically significant differences among the groups. A p-value of < 0.05 was considered statistically significant.

Results

LPS-induced ICAM-1 expression is reduced by EGCG

First, the effect of EGCG on LPS-induced intracellular adhesion molecule-1 (ICAM-1) expression in HAECs was evaluated. HAECs were pretreated with EGCG (1, 10, 50, and 100 μM) for 30 minutes. Then, the EGCG-containing culture media was removed and the cells were treated with LPS (100 ng/ml) for 8 hours. As shown in Figure 1, pretreatment of the cells with EGCG for 30 min significantly diminished LPS-induced ICAM-1 expression in a dose-dependent manner, while EGCG had no effect on ICAM-1 expression in the absence of LPS stimulation.

EGCG induces TLR4 ectodomain shedding

To examine whether EGCG causes ectodomain shedding of TLR4, HAECs were incubated with different concentrations of EGCG (0, 1, 10, 50, or 100 μM) for 30 minutes, or with 50 μM EGCG for different times (0, 5, 10, 30, or 60 minutes), and whole cell lysates and conditioned media were collected. TLR4 in the cell lysate was measured by Western blotting using an antibody against TLR4. As shown in Figure 2A, cellular TLR4 expression was decreased by EGCG in a dose-dependent manner. To determine whether this reduction was caused by ectodomain shedding of the receptor, the conditioned medium was subjected to Western blotting using a monoclonal antibody against an epitope in the N-terminus of TLR4. An approximately 48 kDa TLR4-immunoreactive band was identified in the culture supernatant. The decrease in cellular TLR4 after EGCG treatment was accompanied by an increase in the N-terminal fragment of TLR4 in the culture supernatant. EGCG-induced ectodomain shedding of TLR4 was also time-dependent (Figure 2B).

BAPTA-AM prevents EGCG-induced TLR4 ectodomain shedding, but depletion of extracellular Ca^{2+} does not

We investigated whether Ca^{2+} signaling is involved in the EGCG-induced ectodomain shedding of TLR4. HAECs were preincubated with the intracellular Ca^{2+} chelator BAPTA-AM for 30 minutes and then treated with EGCG (50 μM) for 30 minutes, and whole cell lysates were analyzed by Western blotting using an anti-TLR4 antibody. EGCG-induced

ectodomain shedding of TLR4 was inhibited by BAPTA-AM (Figure 3A), indicating that it is Ca^{2+}-dependent. Next, to examine whether extracellular Ca^{2+} influx is essential for EGCG-induced ectodomain shedding of TLR4, the experiment was repeated in Ca^{2+}-free DMEM. As shown in Figure 3B, EGCG still induced ectodomain shedding of TLR4 in the absence of extracellular Ca^{2+}.

EGCG increases cystolic Ca^{2+}

We investigated the effect of EGCG on cytosolic Ca^{2+} levels in HAECs using Fluo-4 AM. EGCG markedly increased intracellular Ca^{2+} levels in the presence of extracellular Ca^{2+} (1.2 mM) (Figure 4A and 4B). However, this effect of EGCG was abolished by BAPTA-AM. Without extracellular Ca^{2+}, EGCG still increased cytosolic Ca^{2+} levels, but the effect was lower than that with extracellular Ca^{2+}. These results indicate that EGCG increases the cystolic Ca^{2+} concentration by inducing both extracellular Ca^{2+} influx and the release of Ca^{2+} from intracellular stores.

ADAM10 contributes to ectodomain shedding of TLR4

Ca^{2+} influx is known to activate ADAM10.[22] To investigate the role of ADAM10 in EGCG-induced TLR4 ectodomain shedding, the localization of ADAM10 was visualized by immunofluorescent staining and confocal microscopy (Figure 5). With EGCG treatment, ADAM10 immunoreactivity at the cell surface increased. BAPTA-AM inhibited EGCG-induced translocation of ADAM10 to the cell surface, but the absence of extracellular Ca^{2+} did not block ADAM10 translocation.

To determine the role of ADAM10 in EGCG-induced ectodomain shedding of TLR4, HAECs were transfected with control siRNA or ADAM10 siRNA and treated with EGCG. Depletion of ADAM10 prevented EGCG-induced ectodomain shedding of TLR4 (Figure 6A). We next investigated whether depletion of ADAM10 reverses the inhibitory effect of EGCG on TLR4-induced ICAM-1 expression. As shown in Figure 6B, EGCG did not inhibit TLR4-induced ICAM-1 expression when ADAM10 was depleted by siRNA.

Discussion

In the present study, the mechanism underlying the anti-TLR4 effects of EGCG in HAECs was investigated. We show that EGCG induces ectodomain shedding of TLR4 and thereby diminishes the responsiveness of HAECs to LPS. EGCG increased the cytosolic Ca^{2+} concentration in HAECs, both by increasing extracellular Ca^{2+} influx and by inducing the release of Ca^{2+} from intracellular stores. This increase in intracellular Ca^{2+} induced the translocation of ADAM10 to the plasma membrane, where it likely catalyzes the cleavage of the TLR4 ectodomain.

TLRs are important pattern recognition receptors involved in innate immunity.[2)] They are type I transmembrane receptors composed of extracellular leucine-rich repeat motifs and a cytoplasmic Toll/interleukin-1 receptor homology domain.[23)] TLRs can recognize common molecular structures expressed by certain groups of microorganisms.[24)] LPS is a component of the outer leaflet of the outer membrane of Gram-negative bacteria and is recognized by TLR4 and other molecules, including myeloid differentiation-2 (MD-2).[2)] Stimulation of human vascular endothelial cells with LPS induces NF-κB activation and enhances the expression of adhesion molecules, including ICAM-1 and vascular cell adhesion molecule-1 (VCAM-1).[25)] Adhesion molecules in turn play a vital role in the process of leukocyte transmigration by mediating interactions between immune cells and endothelial cells.[26)] In the present study, EGCG pretreatment attenuated LPS-induced ICAM-1 expression by increasing TLR4 ectodomain shedding.

EGCG is the most potent anti-oxidant of the green tea polyphenols.[27)] In previous studies, EGCG inhibited the activation of the transcription factors NF-κB and AP-1, which are induced by many pro-inflammatory stimuli, including UV irradiation, LPS, and the cytokine IL-β.[27-29)] EGCG has also been shown to inhibit TLR4 signaling.[23, 30)] Several studies were performed to elucidate the mechanisms of inhibition of TLR4 by EGCG. EGCG was found to inhibit both TLR4-mediated MyD88- and Toll/interleukin-1 receptor (TIR) domain-containing adaptor-inducing interferon-β (TRIF)-dependent signaling and subsequent inflammatory target gene expression.[27)] It also reduced LPS-induced activation of TLR4 downstream signaling pathways in macrophages, endothelial cells, and dendritic cells through the 67 kDa laminin receptor (67LR).[10, 15, 31)] Our data elucidated another mechanism through which EGCG inhibits TLR4. In the present study, EGCG reduced TLR4 signaling through ADAM10-mediated ectodomain shedding of TLR4 in HAECs.

This mechanism could also apply to the regulation of other transmembrane receptors. In our previous study,[32] EGCG induced ectodomain shedding of TNFR1 and thereby attenuated the effect of TNF-α in vascular endothelial cells.

ADAMs are membrane-bound enzymes responsible for the shedding or cleavage of various cell surface molecules, including adhesion molecules, cytokines/chemokines, and growth factors.[33] Besides the metalloproteinase domain, ADAMs also contain a disintegrin domain that binds to various integrins, although this function is not well understood.[34] The mammalian genome consists of 40 ADAM family members, but only 12 ADAM genes, of which ADAM10 is one, encode proteolytically active proteases.[35] In the present study, when HAECs were transfected with control siRNA or ADAM10 siRNA, depletion of ADAM10 prevented ectodomain shedding of TLR4. In addition, EGCG induced the translocation of ADAM10 from the cytoplasm to the plasma membrane, where TLR4 is expressed. These findings suggest that TLR4 may be a substrate of ADAM10 and implicate ADAM10 in EGCG-induced ectodomain cleavage of TLR4.

Calcium influx is known to be necessary for the activation of ADAM10.[36] ADAM10 is synthesized as an inactive precursor form (~100 KDa). By proprotein convertase-mediated cleavage, it is converted to an active form (~60 kDa).[37] Calcium influx was reported to increase the amount of the active form of ADAM10.[36] However, in our previous study, extracellular calcium influx induced by 1,25-dihydroxyvitamin D_3 ($1,25D_3$) resulted in ADAM10-dependent ectodomain shedding of TNFR1 without increasing the maturation of inactive ADAM10.[38] Instead, extracellular calcium influx triggered the translocation of ADAM10 to the cell surface. In the present study, ADAM10 expression at the cell surface increased with EGCG treatment. However, BAPTA-AM, an intracellular Ca^{2+} chelator, inhibited the translocation of ADAM10 to the cell surface. While $1,25D_3$ did not cause ectodomain shedding of cell surface receptors in the absence of extracellular Ca^{2+} [39], EGCG-induced ectodomain shedding of TLR4 still occurred in the absence of extracellular Ca^{2+}. The driving force for Ca^{2+} influx induced by $1,25D_3$ may depend on the extracellular-intracellular calcium concentration gradient[38] because intracellular calcium concentrations are much lower than extracellular calcium concentrations. In addition to increasing the influx of extracellular Ca^{2+} through channels sensitive to nonspecific Ca^{2+} channel blockers [40], there is evidence suggesting that EGCG can affect Na^+/Ca^{2+} exchange[41] and intracellular Ca^{2+} storage.[40, 42] Both extracellular Ca^{2+}

influx and the intracellular release of stored Ca^{2+} may contribute to the elevation of cytosolic Ca^{2+} by EGCG.[32] Therefore, unlike $1,25D_3$, EGCG could be able to induce ectodomain shedding of TLR4 in the absence of extracellular Ca^{2+}.

Atherosclerosis is considered to be an inflammatory disease. Recent studies have suggested that TLR4-mediated inflammation is involved in the development of atherosclerosis.[23] A potential role for the TLRs in cancer development has been suggested, based on the widespread association of TLR polymorphisms with cancer risk.[43, 44] In addition, some evidence suggests that TLR4 is related to the development of neuropsychiatric diseases.[45] In addition to the roles of TLR2 and TLR4 in the development of inflammatory and autoimmune kidney disease, activation of these receptors has also been reported to promote renal inflammation, leukocyte infiltration, and progressive fibrosis in non-immune kidney diseases such as ischemia-reperfusion injury.[46] In this regard, our data suggest that EGCG may have beneficial effects in several diseases by reducing TLR4 expression on endothelial cells through ectodomain shedding.

Conclusion

Our results demonstrate that EGCG induces ADAM10-mediated ectodomain shedding of TLR4. EGCG may thus reduce TLR4-mediated effects such as inflammation in many clinical settings.

References

1. Peri F, Piazza M. Therapeutic targeting of innate immunity with Toll-like receptor 4 (TLR4) antagonists. Biotechnol Adv 2012;30(1):251-60.
2. Miyake K. Innate immune sensing of pathogens and danger signals by cell surface Toll-like receptors. Semin Immunol 2007;19(1):3-10.
3. Hayashi F, Means TK, Luster AD. Toll-like receptors stimulate human neutrophil function. Blood 2003;102(7):2660-9.
4. Garcia Bueno B, Caso JR, Madrigal JL, Leza JC. Innate immune receptor Toll-like receptor 4 signalling in neuropsychiatric diseases. Neurosci Biobehav Rev 2016;64:134-47.
5. Cha JJ, Hyun YY, Lee MH, Kim JE, Nam DH, Song HK, et al. Renal protective effects of toll-like receptor 4 signaling blockade in type 2 diabetic mice. Endocrinology 2013;154(6):2144-55.
6. Lin M, Yiu WH, Li RX, Wu HJ, Wong DW, Chan LY, et al. The TLR4 antagonist CRX-526 protects against advanced diabetic nephropathy. Kidney Int 2013;83(5):887-900.
7. Lin M, Yiu WH, Wu HJ, Chan LY, Leung JC, Au WS, et al. Toll-like receptor 4 promotes tubular inflammation in diabetic nephropathy. J Am Soc Nephrol 2012;23(1):86-102.
8. Yang WS, Kim JS, Han NJ, Lee MJ, Park SK. Toll-like receptor 4/spleen tyrosine kinase complex in high glucose signal transduction of proximal tubular epithelial cells. Cell Physiol Biochem 2015;35(6):2309-19.
9. Chowdhury A, Sarkar J, Chakraborti T, Pramanik PK, Chakraborti S. Protective role of epigallocatechin-3-gallate in health and disease: A perspective. Biomed Pharmacother 2016;78:50-9.
10. Hong Byun E, Fujimura Y, Yamada K, Tachibana H. TLR4 signaling inhibitory pathway induced by green tea polyphenol epigallocatechin-3-gallate through 67-kDa laminin receptor. J Immunol 2010;185(1):33-45.
11. Hotta Y, Huang L, Muto T, Yajima M, Miyazeki K, Ishikawa N, et al. Positive inotropic effect of purified green tea catechin derivative in guinea pig hearts: the measurements of cellular Ca2+ and nitric oxide release. Eur J Pharmacol

2006;552(1-3):123-30.

12. Ramesh E, Elanchezhian R, Sakthivel M, Jayakumar T, Senthil Kumar RS, Geraldine P, et al. Epigallocatechin gallate improves serum lipid profile and erythrocyte and cardiac tissue antioxidant parameters in Wistar rats fed an atherogenic diet. Fundam Clin Pharmacol 2008;22(3):275-84.
13. Ramesh E, Geraldine P, Thomas PA. Regulatory effect of epigallocatechin gallate on the expression of C-reactive protein and other inflammatory markers in an experimental model of atherosclerosis. Chem Biol Interact 2010;183(1):125-32.
14. Martin-Rodriguez S, Caballo C, Gutierrez G, Vera M, Cruzado JM, Cases A, et al. TLR4 and NALP3 inflammasome in the development of endothelial dysfunction in uraemia. Eur J Clin Invest 2015;45(2):160-9.
15. Byun EB, Mi S, Kim JH, Song DS, Lee BS, Park JN, et al. Epigallocatechin-3-gallate-mediated Tollip induction through the 67-kDa laminin receptor negatively regulating TLR4 signaling in endothelial cells. Immunobiology 2014;219(11):866-72.
16. Seong KJ, Lee HG, Kook MS, Ko HM, Jung JY, Kim WJ. Epigallocatechin-3-gallate rescues LPS-impaired adult hippocampal neurogenesis through suppressing the TLR4-NF-kappaB signaling pathway in mice. Korean J Physiol Pharmacol 2016;20(1):41-51.
17. van der Vorst EP, Keijbeck AA, de Winther MP, Donners MM. A disintegrin and metalloproteases: molecular scissors in angiogenesis, inflammation and atherosclerosis. Atherosclerosis 2012;224(2):302-8.
18. Campos-Toimil M, Orallo F. Effects of (-)-epigallocatechin-3-gallate in Ca2+ -permeable non-selective cation channels and voltage-operated Ca2+ channels in vascular smooth muscle cells. Life Sci 2007;80(23):2147-53.
19. Guo BC, Wei J, Su KH, Chiang AN, Zhao JF, Chen HY, et al. Transient receptor potential vanilloid type 1 is vital for (-)-epigallocatechin-3-gallate mediated activation of endothelial nitric oxide synthase. Mol Nutr Food Res 2015;59(4):646-57.
20. Kim HS, Montana V, Jang HJ, Parpura V, Kim JA. Epigallocatechin gallate (EGCG) stimulates autophagy in vascular endothelial cells: a potential role for reducing lipid accumulation. J Biol Chem 2013;288(31):22693-705.

21. Yang WS, Kim JJ, Han NJ, Lee EK, Park SK. 1,25-Dihydroxyvitamin D3 Attenuates the Effects of Lipopolysaccharide by Causing ADAM10-Dependent Ectodomain Shedding of Toll-Like Receptor 4. Cell Physiol Biochem 2017;41(5):2104-16.
22. Saftig P, Reiss K. The "A Disintegrin And Metalloproteases" ADAM10 and ADAM17: novel drug targets with therapeutic potential? Eur J Cell Biol 2011;90(6-7):527-35.
23. Zhao L, Lee JY, Hwang DH. Inhibition of pattern recognition receptor-mediated inflammation by bioactive phytochemicals. Nutr Rev 2011;69(6):310-20.
24. Kaisho T, Akira S. Toll-like receptor function and signaling. J Allergy Clin Immunol 2006;117(5):979-87; quiz 88.
25. Ming X, Ding M, Zhai B, Xiao L, Piao T, Liu M. Biochanin A inhibits lipopolysaccharide-induced inflammation in human umbilical vein endothelial cells. Life Sci 2015;136:36-41.
26. Li J, Ye L, Wang X, Liu J, Wang Y, Zhou Y, et al. (-)-Epigallocatechin gallate inhibits endotoxin-induced expression of inflammatory cytokines in human cerebral microvascular endothelial cells. J Neuroinflammation 2012;9:161.
27. Youn HS, Lee JY, Saitoh SI, Miyake K, Kang KW, Choi YJ, et al. Suppression of MyD88- and TRIF-dependent signaling pathways of Toll-like receptor by (-)-epigallocatechin-3-gallate, a polyphenol component of green tea. Biochem Pharmacol 2006;72(7):850-9.
28. Barthelman M, Bair WB, 3rd, Stickland KK, Chen W, Timmermann BN, Valcic S, et al. (-)-Epigallocatechin-3-gallate inhibition of ultraviolet B-induced AP-1 activity. Carcinogenesis 1998;19(12):2201-4.
29. Wheeler DS, Catravas JD, Odoms K, Denenberg A, Malhotra V, Wong HR. Epigallocatechin-3-gallate, a green tea-derived polyphenol, inhibits IL-1 beta-dependent proinflammatory signal transduction in cultured respiratory epithelial cells. J Nutr 2004;134(5):1039-44.
30. Xu MJ, Liu BJ, Wang CL, Wang GH, Tian Y, Wang SH, et al. Epigallocatechin-3-gallate inhibits TLR4 signaling through the 67-kDa laminin receptor and effectively alleviates acute lung injury induced by H9N2 swine influenza virus. Int Immunopharmacol 2017;52:24-33.

31. Byun EB, Choi HG, Sung NY, Byun EH. Green tea polyphenol epigallocatechin-3-gallate inhibits TLR4 signaling through the 67-kDa laminin receptor on lipopolysaccharide-stimulated dendritic cells. Biochem Biophys Res Commun 2012;426(4):480-5.
32. Yang WS, Moon SY, Lee MJ, Park SK. Epigallocatechin-3-Gallate Attenuates the Effects of TNF-a in Vascular Endothelial Cells by Causing Ectodomain Shedding of TNF Receptor 1. Cellular Physiology and Biochemistry 2016;38:1963-1974.
33. van der Vorst EPC, Weber C, Donners M. A Disintegrin and Metalloproteases (ADAMs) in Cardiovascular, Metabolic and Inflammatory Diseases: Aspects for Theranostic Approaches. Thromb Haemost 2018;118(7):1167-75.
34. Bridges LC, Bowditch RD. ADAM-Integrin Interactions: potential integrin regulated ectodomain shedding activity. Curr Pharm Des 2005;11(7):837-47.
35. Reiss K, Saftig P. The "a disintegrin and metalloprotease" (ADAM) family of sheddases: physiological and cellular functions. Semin Cell Dev Biol 2009;20(2):126-37.
36. Nagano O, Murakami D, Hartmann D, De Strooper B, Saftig P, Iwatsubo T, et al. Cell-matrix interaction via CD44 is independently regulated by different metalloproteinases activated in response to extracellular Ca(2+) influx and PKC activation. J Cell Biol 2004;165(6):893-902.
37. Kasina S, Scherle PA, Hall CL, Macoska JA. ADAM-mediated amphiregulin shedding and EGFR transactivation. Cell Prolif 2009;42(6):799-812.
38. Yang WS, Kim HW, Lee JM, Han NJ, Lee MJ, Park SK. 1,25-dihydroxyvitamin D3 causes ADAM10-dependent ectodomain shedding of tumor necrosis factor receptor 1 in vascular smooth muscle cells. Mol Pharmacol 2015;87(3):533-42.
39. Yang WS, Yu H, Kim JJ, Lee MJ, Park SK. Vitamin D-induced ectodomain shedding of TNF receptor 1 as a nongenomic action: D3 vs D2 derivatives. J Steroid Biochem Mol Biol 2016;155(Pt A):18-25.
40. Kim HJ, Yum KS, Sung JH, Rhie DJ, Kim MJ, Min DS, et al. Epigallocatechin-3-gallate increases intracellular [Ca2+] in U87 cells mainly by influx of extracellular Ca2+ and partly by release of intracellular stores. Naunyn Schmiedebergs Arch Pharmacol 2004;369(2):260-7.
41. Lorenz M, Hellige N, Rieder P, Kinkel HT, Trimpert C, Staudt A, et al. Positive

inotropic effects of epigallocatechin-3-gallate (EGCG) involve activation of Na+/H+ and Na+/Ca2+ exchangers. Eur J Heart Fail 2008;10(5):439-45.

42. Kargacin ME, Emmett TL, Kargacin GJ. Epigallocatechin-3-gallate has dual, independent effects on the cardiac sarcoplasmic reticulum/endoplasmic reticulum Ca2+ ATPase. J Muscle Res Cell Motil 2011;32(2):89-98.

43. El-Omar EM, Ng MT, Hold GL. Polymorphisms in Toll-like receptor genes and risk of cancer. Oncogene 2008;27(2):244-52.

44. Forrest MS, Skibola CF, Lightfoot TJ, Bracci PM, Willett EV, Smith MT, et al. Polymorphisms in innate immunity genes and risk of non-Hodgkin lymphoma. Br J Haematol 2006;134(2):180-3.

45. Garcia Bueno B, Caso JR, Madrigal JL, Leza JC. Innate immune receptor Toll-like receptor 4 signalling in neuropsychiatric diseases. Neurosci Biobehav Rev 2016;64:134-47.

46. Lin M, Tang SC. Toll-like receptors: sensing and reacting to diabetic injury in the kidney. Nephrol Dial Transplant 2014;29(4):746-54.

Figure 1. LPS-induced ICAM-1 expression was inhibited by EGCG. HAECs were preincubated with EGCG for 30 min. Then, the EGCG-containing culture media was removed and the cells were treated with LPS (100 ng/ml) for 8 h (n = 7). Whole cell lysates were analyzed by Western blotting using anti-ICAM-1 and anti-actin antibodies (*p < 0.05 vs. the untreated control; **p < 0.05 vs. LPS).

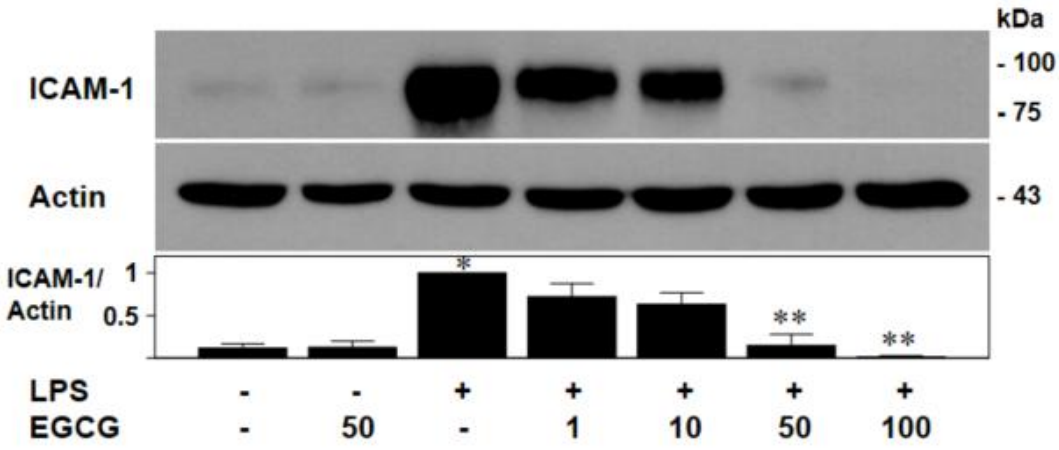


Figure 2. EGCG induces ectodomain shedding of TLR4. (A) HAECs were incubated with the indicated concentrations of EGCG for 30 minutes. (B) HAECs were incubated with EGCG (50 μM) for the indicated times. Cell lysates and culture supernatants were analyzed by Western blotting using a polyclonal anti-TLR4 antibody (cell lysates) or a monoclonal antibody against the N-terminal region of human TLR4 (culture media). (n = 5, *p < 0.05 vs. the untreated control).

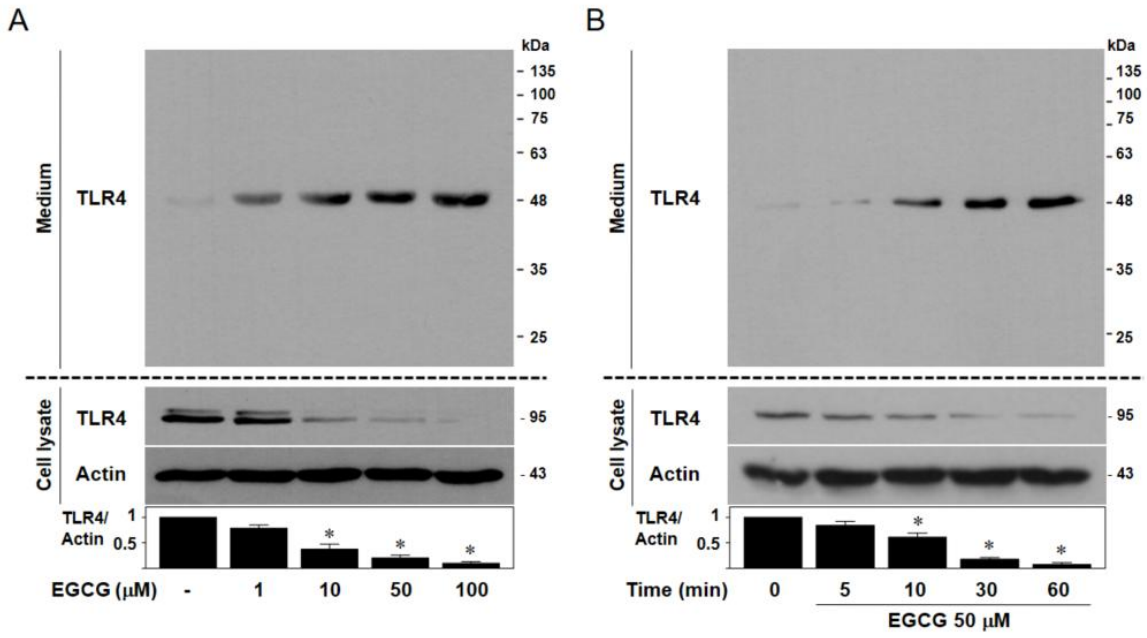


Figure 3. BAPTA-AM prevents EGCG-induced TLR4 ectodomain shedding, but the absence of extracellular Ca^{2+} does not. (A) HAECs were preincubated with BAPTA-AM (an intracellular Ca^{2+} chelator) and then treated with EGCG for 30 minutes (n = 3). (B) HAECs were incubated with EGCG (50 μM) for 30 minutes in Ca^{2+}-free DMEM or DMEM containing 1.2 mM Ca^{2+} (n = 4). Cell lysates and culture media were analyzed by Western blotting using a polyclonal anti-TLR4 antibody (cell lysates) or a monoclonal antibody against the N-terminal region of human TLR4 (culture media). *p < 0.05 vs. the untreated control; **p < 0.05 vs. EGCG.

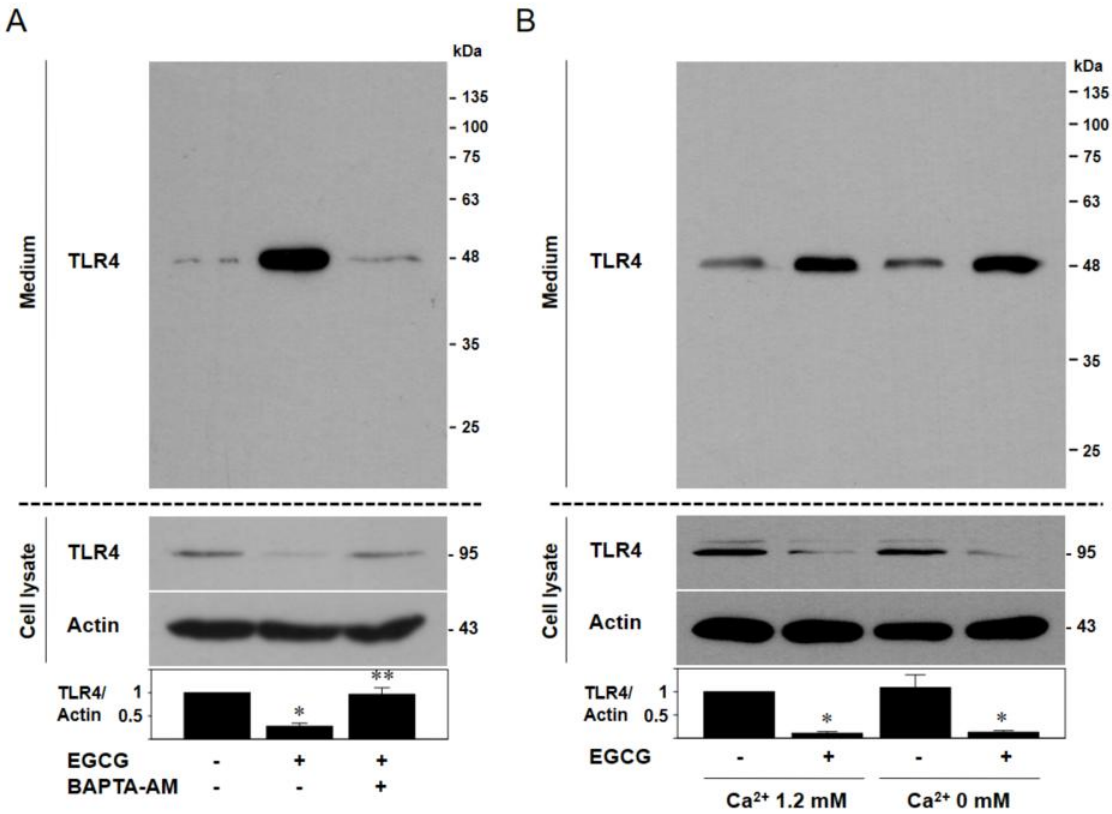


Figure 4. EGCG increases cystolic Ca^{2+} by increasing both extracellular Ca^{2+} influx and the release of stored intracellular Ca^{2+}. HAECs were preincubated with BAPTA-AM (10 μM) for 30 minutes, loaded with Fluo-4 AM (2 μM), and resuspended in Ca^{2+}-free HBSS or HBSS containing 1.2 mM Ca^{2+}. The cells were then treated with EGCG (50 μM) and the fluorescence intensity was recorded every 20 seconds for 10 minutes. (A) Representative images before and after EGCG treatment. (B) The addition of EGCG is indicated by an arrow (n = 137 cells in the EGCG group, 102 cells in the EGCG + BAPTA group, and 117 cells in the Ca^{2+}-free EGCG group from three independent experiments).

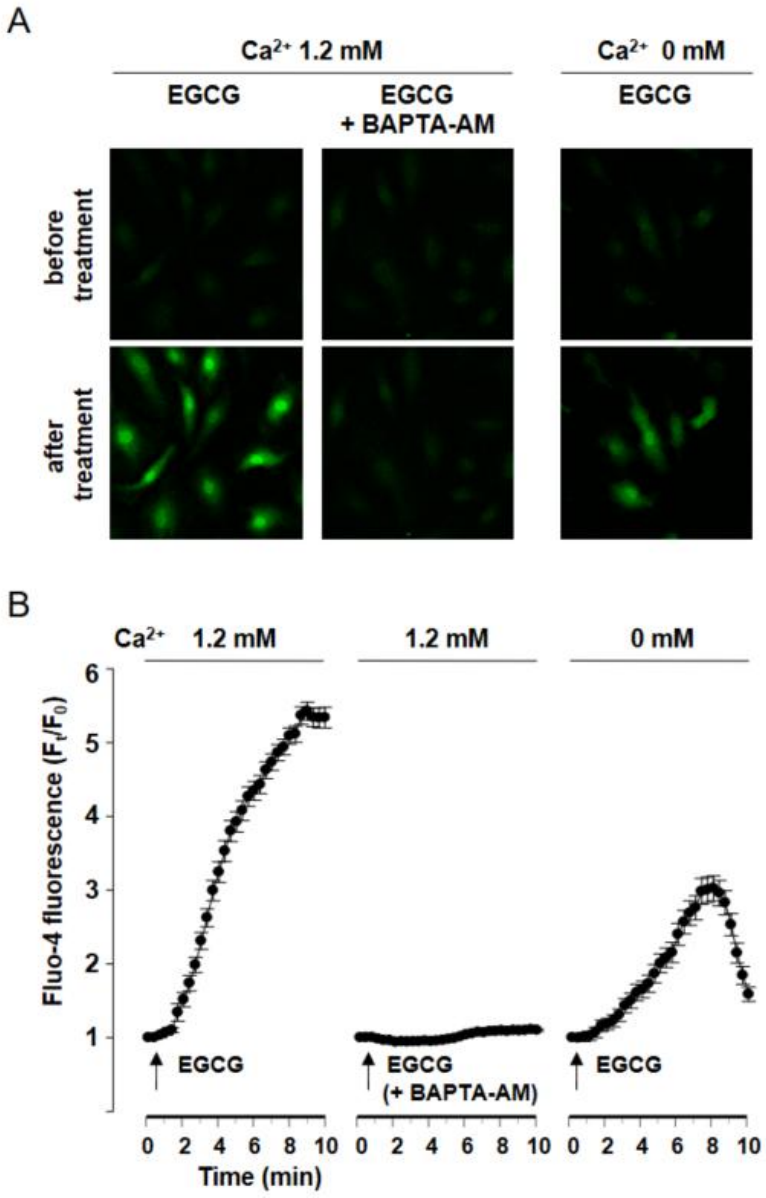


Figure 5. EGCG induces Ca^{2+}-dependent ADAM10 translocation to the plasma membrane. HAECs in Ca^{2+}-free DMEM or DMEM containing 1.2 mM Ca^{2+} were preincubated with BAPTA-AM (10 μM) for 30 minutes, and then treated with EGCG (50 μM) for 10 minutes. The cells were fixed with 4% paraformaldehyde for 10 min (without permeabilization) and immunostained for ADAM10, and staining was visualized by confocal microscopy. Cell nuclei were stained with 4′,6-diamidino-2-phenylindole (DAPI). Representative images from three independent experiments are shown.

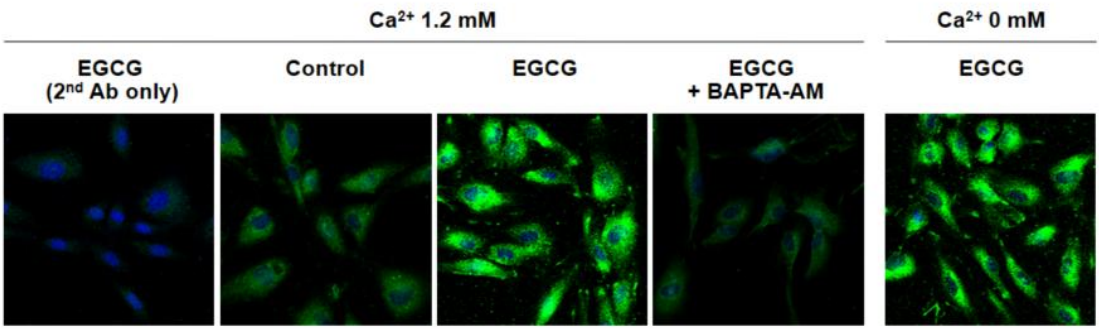
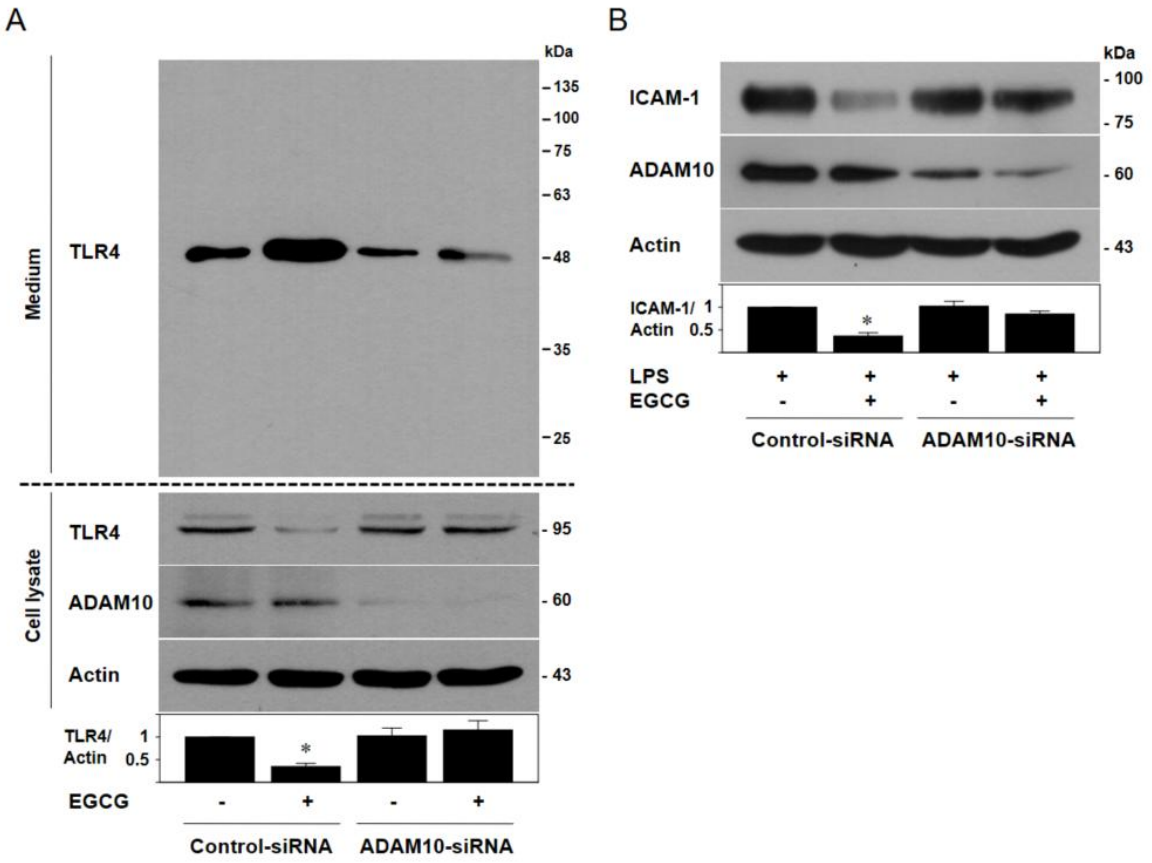


Figure 6. ADAM10 contributes to ectodomain shedding of TLR4. (A) Depletion of ADAM10 inhibits EGCG-induced ectodomain shedding of TLR4. HAECs were transfected with control siRNA or ADAM10 siRNA and then incubated with EGCG (50 μM) for 30 minutes in M199 Hank's media (1.26 mM Ca^{2+}). Whole cell lysates and conditioned media were analyzed by Western blotting using anti-TLR4, anti-ADAM10, and anti-actin antibodies (n = 4). (B) Depletion of ADAM10 abolishes the inhibitory effect of EGCG against LPS. HAECs were transfected with control siRNA or ADAM10 siRNA, incubated with or without EGCG (50 μM) for 30 minutes in M199 Hank's media (1.26 mM Ca^{2+}), and further treated with LPS for 8 hours. Whole cell lysates were analyzed by Western blotting using anti-ICAM-1, anti-ADAM10, and anti-actin antibodies (n = 4). *p < 0.05 vs. the untreated control.



Abstract

Background: Toll-like receptor 4 (TLR4) is an important regulator of innate immunity, with roles in inflammation, autoimmune disease, cancer, and neuropsychiatric disease. Epigallocatechin-3-gallate (EGCG), the most abundant polyphenol in green tea leaves, has been shown to decrease the expression of TLR4 and inhibit TLR4 signaling, but the mechanism through which EGCG regulates TLR4 activity is not well-characterized. In this study, we explored whether EGCG causes ectodomain shedding of TLR4 in human aortic endothelial cells (HAECs).

Methods: TLR4 expression in cultured HAECs was assessed by Western blotting. Intracellular Ca^{2+} levels were measured using Fluo-4 AM, and the role of A disintegrin and metalloprotease 10 (ADAM10) was characterized using siRNA and immunofluorescence.

Results: Lipopolysaccharide (LPS)-induced ICAM-1 expression was attenuated by EGCG pretreatment. EGCG decreased HAEC expression of TLR4 and increased the levels of the N-terminus of TLR4 in HAEC culture media in a dose- and time-dependent manner. BAPTA-AM, an intracellular Ca^{2+} chelator, prevented EGCG-induced ectodomain shedding of TLR4. In the absence of extracellular Ca^{2+}, EGCG still increased cytosolic Ca^{2+} levels, but the effect was reduced. With EGCG treatment, ADAM10 expression at the cell surface increased. BAPTA-AM inhibited EGCG-induced translocation of ADAM10 to the cell surface, but the absence of extracellular Ca^{2+} did not block ADAM10 translocation. ADAM10 depletion with siRNA prevented EGCG-induced ectodomain shedding of TLR4, and LPS-induced ICAM-1 expression was not inhibited by EGCG in the absence of ADAM10.

Conclusion: EGCG induces ADAM10-mediated ectodomain shedding of TLR4 in HAECs by increasing the cytosolic Ca^{2+} concentration.