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Epigallocatechin-3-gallate-mediated Tollip induction through the 67-kDa laminin receptor negatively regulating TLR4 signaling in endothelial cells

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ABSTRACT

Background: Green tea polyphenol epigallocatechin-3-gallate (EGCG) has the potential to impact a variety of inflammation-related diseases; however, the anti-inflammatory action of EGCG in endothelial cells has not been understood. Recently, we demonstrated that the 67-kDa laminin receptor (67LR) acts as a cell-surface EGCG receptor.
Aim: This research was carried out to clarify the molecular basis for the down-regulation of toll-like receptor 4 (TLR4) signal transduction by EGCG in lipopolysaccharide (LPS)-stimulated endothelial cells.
Results: RNAi-mediated silencing of 67LR resulted in an abrogation of the inhibitory action of EGCG on the LPS-induced activation of downstream signaling pathways. Also, we found that EGCG induced a rapid upregulation of Toll-interacting protein (Tollip), a negative regulator of TLR signaling, through 67LR in endothelial cells. RNAi-mediated silencing of Tollip impaired the TLR4 signaling inhibitory activity of EGCG. Additionally, silencing of Tollip resulted in an abrogation of the inhibitory action of EGCG on the LPS-induced expressions of cell-associated adhesion molecules, such as ICAM-1 and VCAM-1.
Conclusion: Taken together, these novel findings provide new insights into an understanding of negative regulatory mechanisms of the TLR4 signaling pathway and effective therapeutic intervention for the treatment of inflammatory disease.

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Introduction

Natural products and dietary components rich in polyphenols have been regarded as some of the most promising dietary agents for the prevention and treatment of inflammation-related chronic diseases (Scalbert et al., 2005). Several epidemiologic observations have reported that green tea (*Camellia sinensis* L.) has become a popular drink throughout the world, and it is reported to possess significant health promoting effects (Cabrera et al., 2006). Among the numerous polyphenols isolated from green tea, epigallocatechin-3-gallate (EGCG), a major form of tea catechin, has been shown to lower the incidence of inflammation-related diseases in animals and humans (Le Marchand, 2002). EGCG administration has been shown to minimize endothelial damage by reducing the release of pro-inflammatory factors, such as inducible nitric oxide synthase (iNOS)-mediated nitric oxide (NO), interleukin (IL)-6, and tumor necrosis factor (TNF)-α, through inhibition of AP-1 and nuclear factor (NF)-κB pathways in the vascular inflammatory response, which has been especially targeted using EGCG as a treatment modality (Shenouda and Vita, 2007; Aneja et al., 2004). Studies describing the anti-inflammatory action of EGCG have shown that the negative regulation of toll-like receptor

Abbreviations: EGCG, epigallocatechin-3-gallate; 67LR, 67-kDa laminin receptor; iNOS, inducible nitric oxide synthase; Iκ-B, inhibitor of κB; LPS, lipopolysaccharide; NF-κB, nuclear factor-κB; NO, nitric oxide; Tollip, toll-interacting protein; TLR, toll-like receptor; TNF-α, tumor necrosis factor-α; IL, interleukin; MAPK, mitogen-activated protein kinase; IRAK, IL-1 receptor-associated kinase; ICAM, intercellular adhesion molecule; VCAM, vascular cell adhesion molecule.

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(TLR) signaling is through the 67-kDa laminin receptor (67LR), and the EGCG-induced negative regulation ultimately leads to the inhibition of mitogen-activated protein kinases (MAPKs) and NF-κB-mediated pro-inflammatory factors, such as cytokines, iNOS, and COX-2 genes (Byun et al., 2012; Li et al., 2012; Takeda and Akira, 2005). TLRs are pathogen recognition proteins that have important roles in initiating inflammatory action. This action is regulated by several intracellular negative regulators of TLRs, including suppressor of cytokine signaling 1 (SOCS1), Toll-interacting protein (Tollip), and interleukin-1 receptor-associated kinase-M (IRAK-M); and these have become an important topic in research on the innate immune system and mediate through their ability to suppress potently the production of pro-inflammatory mediators induced by the activity of IL-1 receptor-associated kinase (Takeda and Akira, 2005; Liew et al., 2005). Among them, Tollip, a negative regulator of TLRs signaling, also associates directly with TLR4, and plays an inhibitory role in TLR-mediated cell activation induced by lipopolysaccharide (LPS), one of the most powerful activators of TLR4 signaling (Byun et al., 2013). Also, it is well known that negative regulation of TLR signaling induces the inhibition of various pro-inflammatory mediators, such as MAPKs and NF-κB, induced by Tollip expression through 67LR (Fujihara et al., 2003). Recently, we and others have reported that 67LR, a cell-surface EGCG receptor, is involved in the inhibitory effect of EGCG on the TLR4 signaling pathway in macrophages, as well as in vascular endothelial cells (Li et al., 2012; Byun et al., 2010). Despite the report regarding the anti-inflammatory effect of EGCG through 67LR in endothelial cells, there is no evidence yet supporting the role of the intracellular negative regulators of TLRs. In addition, in response to LPS, endothelial cells undergo inflammatory activation, resulting in an increase in cell-associated adhesion molecules, such as intercellular adhesion molecule 1 (ICAM-1) and vascular cell adhesion molecule 1 (VCAM-1), on the surface of the vascular endothelium. This process is one of the key events leading to vascular inflammation, which is prevented by the blocking of MAPKs and NF-κB activation through the EGCG-mediated negative regulation of TLR4 signaling (Li et al., 2012; Ye et al., 2008).

Although some mechanisms for the anti-inflammatory activities of EGCG have been proposed, it is not clear whether EGCG action through the 67LR involves a negative regulator, such as Tollip, for inflammatory responses in vascular endothelial cells. These findings promoted our interest in examining EGCG-mediated Tollip induction through the 67LR in vascular endothelial cells.

Thus, the aim of this work was to elucidate the molecular basis for the down-regulation of TLR4 signal transduction by EGCG in vascular endothelial cells. Here, we show that Tollip induction through the 67LR is essential for mediating the anti-inflammatory action of EGCG in LPS-stimulated endothelial cells.

Materials and methods

Antibodies and reagents

EGCG was purchased from Sigma–Aldrich (St. Louis, MO). LPS from *Escherichia coli* O111:B4 was purchased from Sigma and Invivogen (San Diego, CA). Anti-TLR4 polyclonal Ab, anti-CD14 polyclonal Ab, anti-Tollip monoclonal Ab, VCAM-1 polyclonal Ab, ICAM-1 polyclonal Ab, and anti-67LR (F-18) polyclonal Ab were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-phosphorylated ERK1/2 mAb, anti-phosphorylated JNK mAb, anti-phosphorylated p38 mAb, anti-NF-κB (p65) polyclonal Ab, anti-phosphorylated IκB-α mAb, anti-phosphorylated iNOS mAb, and anti-lamin B polyclonal Ab were obtained from Santa Cruz Biotechnology, Inc. Horseradish peroxidase (HRP)-conjugated anti-mouse IgG Ab and HRP-conjugated anti-rabbit Ab were obtained from Calbiochem (San Diego, CA), and anti-β-actin mAb (AC-15) was purchased from Sigma–Aldrich. IL-6, IL-1β, and TNF-α enzyme-linked immunosorbent assay (ELISA) kits were obtained from BD Biosciences (San Diego, CA).

Cell culture

Mouse MS-1 endothelial cells were purchased from the American Type Culture Collection (Manassas, VA, USA). Cells were maintained in DMEM (GIBCO, Carlsbad, CA, USA) containing 10% fetal bovine serum (FBS, GIBCO), 100 U/ml penicillin, and 100 U/ml streptomycin (complete medium) under humidified conditions at 37 °C and 5% CO_2 in an incubator until they reached 80% confluence.

Construction of 67LR-suppressed cells

Target sequences for short hairpin RNA (shRNA) for 67LR and nonspecific control are as follows: shRNA for 67LR, 5′-GGAGGAATTTCAGGGTGAA-3′; shRNA for nonspecific control, 5′-GCATATGTGCGTACCTAGCAT-3′. The annealed shRNA inserts were cloned into the psiRNA-hH1neo shRNA expression vector (for 67LR shRNA) (InvivoGen, San Diego) according to the manufacturer's protocol.

Construction of Tollip-suppressed cells

Tollip shRNA expression vector was purchased from Santa Cruz Biotechnology. The shRNA plasmids consist of a pool of three to five lentiviral vector plasmids each encoding target-specific 19–25 nt (plus hairpin) shRNAs designed to knockdown gene expression. Each plasmid contains a puromycin resistance gene for the selection of cells stably expressing shRNA. Each vial contains 20 μg of lyophilized shRNA plasmid DNA. The optimal shRNA plasmid DNA:shRNA plasmid transfection reagent ratio is 1 μg:1 μl in 100 μl shRNA plasmid transfection medium. For each transfection, in 6-well tissue culture plates, cells were maintained 2×10^4 cells per well in 2 ml antibiotic-free normal growth medium supplemented with FBS for 18 h, and then aspirate the medium. Next, we added 0.8 ml shRNA plasmid transfection medium to well, and then treated the 200 μl shRNA plasmid DNA/shRNA plasmid transfection reagent complex medium. The cells incubated for 7 h at 37 °C in a CO_2 incubator. We further performed the puromycinneomycin selection for obtaining stably transfected cells.

Measurement of cell proliferation in endothelial cells

To measure cell viability, a WST-1 assay was performed. Endothelial cells were mechanically scraped, seeded in 96-well plates at 5×10^4 cells/ml, and incubated for 24 h. After incubation, the cells were treated with EGCG for 24 h. The tetrazolium salt 2-(4-iodophenyl)-3-(4-nitrophe-nyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, known as WST-1 (Roche, Mannheim), was used to detect the loss of viability. The supernatant medium was replaced by WST-1 and incubated for 2 h. The colored supernatants without particles were transferred into a clean 96-well plate and measured at 450 nm in a multiwell plate reader. The results are given as relative percentage to the untreated control.

ELISA

Supernatants from experimental endothelial cell cultures were collected and stored at −70 °C until use. The levels of IL-6, IL-1β, and TNF-α in the supernatants were determined using cytokine detection ELISA kits (BD Biosciences) according to the manufacturer's instructions, with detection at 450 nm using a microplate reader.

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