FEBS Letters 585 (2011) 814–820

journal homepage: www.FEBSLetters.org

Green tea polyphenol epigallocatechin-3-gallate inhibits TLR2 signaling induced by peptidoglycan through the polyphenol sensing molecule 67-kDa laminin receptor

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ARTICLE INFO

Article history:
Received 6 February 2011
Accepted 8 February 2011
Available online 12 February 2011

Edited by Barry Halliwell

Keywords:
Epigallocatechin-3-gallate
67-kDa laminin receptor
Peptidoglycan
Toll-like receptor 2
Toll-interacting protein

ABSTRACT

Here we show the molecular basis for the inhibition of peptidoglycan (PGN)-induced TLR2 signaling by a major green tea polyphenol epigallocatechin-3-gallate (EGCG). Recently, we identified the 67-kDa laminin receptor (67LR) as the cell-surface EGCG receptor. Anti-67LR antibody treatment or silencing of 67LR resulted in abrogation of the inhibitory action of EGCG on PGN-induced production of pro-inflammatory mediators and activation of mitogen-activated protein kinases. Silencing of Toll-interacting protein (Tollip), a negative regulator of TLR signaling impaired the TLR2 signaling inhibitory activity of EGCG, suggesting that TLR2 response could be inhibited by EGCG via 67LR and Tollip.

1. Introduction

Toll-like receptors (TLRs) are a family of membrane proteins that trigger innate immune responses and are pathogen recognition proteins that have important roles in detecting microbes [1]. Among several kinds of TLRs, TLR2 is preferentially involved in the inflammatory response to lipoteichoic acid, lipopeptides, and glycans from a variety of microbes [1]. Peptidoglycan (PGN), a major component of the cell wall of gram-positive bacteria, is one of the most powerful activators of TLR2 signaling [2,3]. PGN induces most of the clinical manifestations of bacterial infections, including inflammation, fever and septic shock [4]. Most of these effects are due to the activation of macrophages and production of pro-inflammatory cytokines, such as tumor necrosis factor-α (TNF-α) and interleukin (IL)-6 [5,6].

Green tea (*Camellia sinensis* L.) is one of the most popular beverages in the world, and it has been reported that daily consumption of green tea is associated with many important health benefits, such as a reduced risk of atherosclerosis and cancer [7,8]. The beneficial properties of green tea are attributable to its abundant polyphenolic compounds, known as catechins. The green tea polyphenols include catechin, (−)-epicatechin (EC), (−)-epigallocatechin (EGC), (−)-epicatechin-3-gallate (ECG), and (−)-epigallocatechin-3-gallate (EGCG). Of these, EGCG is a predominant catechin and has a variety of biological and pharmacological properties including cancer-preventive, anti-allergic, anti-oxidative stress and anti-inflammatory activities [8–11].

Activation of TLR2 in response to PGN induces activation of the mitogen-activated protein kinase (MAPK) pathway, nuclear factor-κB (NF-κB) and induction of pro-inflammatory cytokines [12,13]. Recently, it has been reported that EGCG inhibited MAPK phosphorylation and subsequently suppressed both IκBα-dependent and -independent signal transduction pathways for the activation of NF-κB, leading to the expression of cytokines and chemokines induced by caries-related bacteria and TLR2 ligand [14]. Although some mechanisms for the anti-inflammatory activities of EGCG have been proposed, the molecular mechanism for this inhibitory action of EGCG on TLR2 signaling is has not yet been established.

The 67-kDa laminin receptor (67LR) is a non-integrin cell-surface receptor for laminin with high affinity [15]. Its role as a laminin receptor makes it an important molecule in cell

Abbreviations: EGCG, epigallocatechin-3-gallate; 67LR, 67-kDa laminin receptor; TLRs, toll-like receptors; Tollip, toll-interacting protein; TNF-α, tumor necrosis factor-α; IL, interleukin; NO, nitric oxide; PGN, peptidoglycan; shRNA, short hairpin RNA; COX-2, cyclooxygenase-2; MAPK, mitogen-activated protein kinase; NF-κB, nuclear factor-κB; SOCS1, suppressor of cytokine signaling 1; IRAK, IL-1 receptor-associated kinase; MyD88, myeloid differentiation protein; TRAF6, tumor necrosis factor receptor-associated factor 6

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0014-5793/$36.00
doi:10.1016/j.febslet.2011.02.010

adhesion to the basement membrane and the metastasis of tumor cells [16]. An increase in the expression of 67LR as compared with the corresponding normal tissue has been found in a variety of tumor cells [17,18]. Furthermore, the protein interacts specifically with the major Dengue virus serotypes [19]. Recently, we identified 67LR as a cell-surface EGCG receptor that mediates the anti-cancer action of physiologically achievable concentrations (0.1–1 μM) of EGCG [20]. Others showed that RNAi-mediated silencing of 67LR results in abrogation of EGCG-induced apoptosis in multiple myeloma cells [21]. Furthermore, this receptor has also been shown to be responsible for the inhibitory action of EGCG on degranulation in basophils [22,23]. In macrophages, EGCG inhibits LPS-induced TLR4 signaling through 67LR [24]. However, it is not clear whether EGCG action through 67LR involves the negative regulatory effect of EGCG on TLR2 ligand PGN-induced inflammatory responses. In this study, we tried to illuminate the molecular basis for the downregulation of TLR2 signal transduction by EGCG. Here we show that 67LR and Tollip are indispensable for mediating anti-inflammatory action of EGCG on TLR2 signaling. Our finding provides a new insight into the understanding of negative regulatory mechanisms for the TLR2 signaling pathway.

2. Materials and methods

2.1. Reagents

EGCG, PGN, anti-β-actin polyclonal antibody, and horseradish peroxidase (HRP)-conjugated anti-rabbit antibody were purchased from Sigma (St. Louis, MO). Anti-67LR monoclonal antibody (MLuC5) was purchased from NeoMarkers (Fremont, CA). Anti-TLR2 polyclonal antibody, anti-MyD88 polyclonal antibody, anti-CD14 polyclonal antibody, anti-Tollip monoclonal antibody, anti-phosphorylated ERK1/2 monoclonal antibody, anti-ERK1/2 polyclonal antibody, anti-phosphorylated JNK monoclonal antibody, anti-JNK polyclonal antibody, anti-phosphorylated p38 monoclonal antibody, anti-p38 polyclonal antibody and horseradish HRP-conjugated anti-goat donkey IgG antibody were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA), and HRP-conjugated anti-mouse IgG antibody, HRP-conjugated streptavidin and anti-mouse IgM antibody were obtained from Zymed Laboratories (San Francisco, CA). HRP-conjugated anti-rabbit antibody was purchased from ICN Pharmaceuticals (Aurora, OH). An anti-mouse TNF-α antibody was obtained from Endogen (Woburn, MA). Biotinylated anti-mouse TNF-α antibody was obtained from Biosource (Camarillo, CA). IL-6 ELISA kit was obtained from eBioscience (San Diego, CA).

2.2. Isolation of peritoneal macrophages

Thioglycollate-elicited peritoneal macrophages were obtained from specific pathogen-free male BALB/c mice at 6–8 weeks of age by injection of 1 ml of sterile 3% thioglycollate solution (Difco, Detroit, MI) for 4 days before lavage with 10 ml of phosphate buffered saline. The peritoneal macrophages were washed once with RPMI-1640 (without phenol red) supplemented with 10% endotoxin-free heat-inactivated fetal bovine serum (Intergen, Purchase, NY), 100 U/ml penicillin, and 100 U/ml streptomycin. The cells were resuspended in RPMI-1640 at a density of 2×10^6 cells/ml. The cells were plated and incubated for 4 h at 37 °C in a humidified incubator containing 5% CO_2 to allow macrophage adherence.

2.3. Cell culture

RAW264.7 cells were cultured in DMEM supplemented with antibiotics (100 U/ml penicillin and 100 U/ml streptomycin), and 10% (v/v) fetal bovine serum (Intergen, Purchase, NY). The cells were maintained at 37 °C in a humidified incubator containing 5% CO_2. In all experiments, cells were allowed to acclimate for 24 h before any treatments.

2.4. Anti-67LR antibody treatment

Thioglycollate-elicited peritoneal macrophages were seeded on the plates and incubated at 37 °C in 5% CO_2 for 24 h before any treatments. The cells were incubated with either anti-67LR antibody (20 μg/ml) or control mouse IgM (20 μg/ml) at 37 °C in 5% CO_2 for 1 h before the addition of EGCG or LPS.

2.5. 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.

2.6. Construction of Tollip-suppressed cells

Tollip shRNA expression vector was purchased from Santa Cruz Biotechnology. shRNA plasmids consist of a pool of three to five lentiviral vector plasmids each encoding target-specific 19–25 nt (plus hairpin) shRNAs.

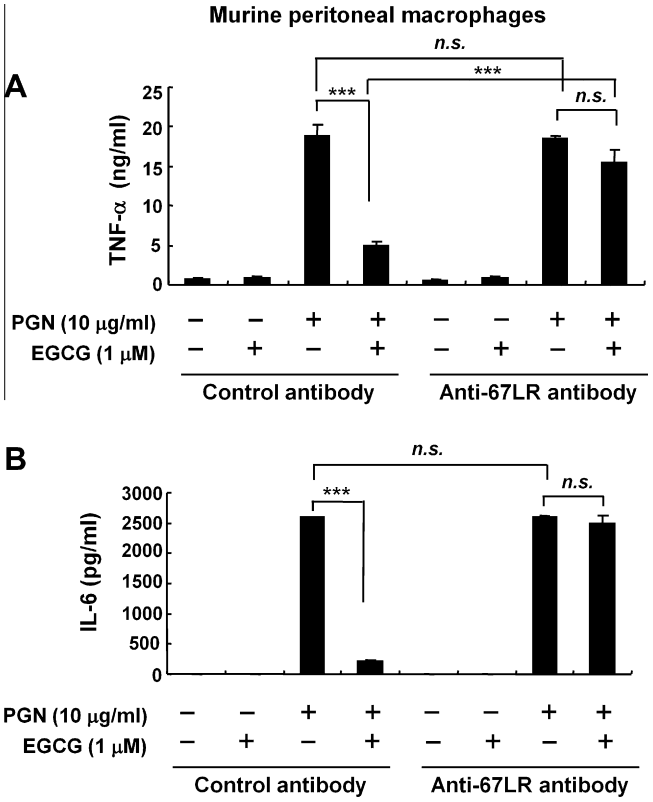

Fig. 1. Anti-inflammatory action of EGCG is mediated through the 67LR. Thioglycollate-elicited murine peritoneal macrophages were incubated with either anti-67LR antibody or control antibody for 1 h, and the cells were pretreated with EGCG (1 μM) for 1 h before exposure to PGN for 24 h. The amount of TNF-α (A) and IL-6 (B) in culture medium was measured by ELISA. All data were expressed as the means ± S.D. ($n = 3$). Statistical significance was analyzed by Student's t-test. The value of $^{***}P < 0.001$ was considered to be statistically significant. The value of n.s. was defined as no significant effect.