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The Structural Characteristics of Green Tea Polyphenols on Lipopolysaccharide-Stimulated RAW Cells

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Abstract

The inflammatory response of macrophages is involved in pathogenesis of lifestyle-related diseases. Green tea consumption reduces the incidence of lifestyle-related diseases. This study investigated the anti-inflammatory effect of polyphenols of green tea including gallic acid, (+)-catechin, (-)-catechin, (-)-epicatechin and (-)-epigallocatechin-3-gallate (EGCG) *in vitro*. The macrophage cell line RAW264 cells were pre-treated with different concentrations of polyphenols (gallic acid, (+)-catechin, (-)-catechin, (-)-epicatechin and EGCG) for 4 h, and were stimulated with LPS for 45 min, 2 h and 24 h. After 24 h LPS challenge, cell lysates and supernatants were harvested. The protein concentration of whole cell lysate was used for determination of cell growth/viability by the BCA assay. The production of TNF-α and IL-6 was measured by ELISA. The total expression and phosphorylation of p38 MAPK was detected by Western blotting. Our results showed that the total protein content of cells was decreased after LPS challenge, while this effect was attenuated when cells were pre-treated with 10 μM gallic acid and EGCG. Pre-treatment with 1 and 10 μM EGCG and (-)-catechin significantly decreased the production of TNF-α and IL-6. Furthermore, pre-treatment with 10 μM gallic acid significantly reduced the production of TNF-α and IL-6. Pre-treatment with 10 μM (+)-catechin, (-)-catechin, (-)-epicatechin, and EGCG enhanced the expression and phosphorylation of p38 MAPK after stimulation with LPS for 45 min and 2 h. By contrast, pre-treatment with gallic acid did not affect the production and phosphorylation of p38 MAPK. These results demonstrated that polyphenols with pyrogallol-type structures in green tea attenuate the activation of macrophages.

Keywords: Green tea polyphenols; EGCG; Inflammation; Macrophages

Abbreviations: D-PBS: Dulbecco's Phosphate-buffered Saline; EGCG: Epigallocatechin-3-gallate; ELISA: Enzyme-linked Immunosorbent Assay; ERK: Extracellular Signal–regulated Kinases; IL-1β: Interleukin-1β; JNK: c-Jun N-terminal

Kinase; LPS: Lipopolysaccharide; NF-κB: Nuclear Factor-κB; NO: Nitric Oxide; p38 MAPK: p38 Mitogen-activated Protein Kinases; RIPA buffer: Radio Immune Precipitation Assay buffer; TLR4: Toll-like Receptor 4; TNF-α: Tumor Necrosis Factor-α

Introduction

Inflammation is involved in pathogenesis of lifestyle-related diseases such as metabolic syndrome, type 2 diabetes, atherosclerosis and cancer [1-3]. On the inflammation of lifestyle-related diseases, the infiltration of macrophages and inflammatory response of macrophages are the main source of inflammation. Especially, the several pro-inflammatory cytokines, TNF-α, IL-1β and IL-6 are secreted from the tissue-infiltrating macrophages, and cause chronic low-grade inflammation. Activation of Toll-like receptor 4 (TLR4) signaling pathway plays a crucial role in the production of pro-inflammatory cytokines by macrophages [4]. TLR4 is the receptor for lipopolysaccharide (LPS), a major component of the cell well of Gram-negative bacteria. LPS induces the activation of TLR4 and mitogen-activated protein kinase (MAPK) pathways in macrophages, and the secretion of several pro-inflammatory cytokines and inflammatory mediators. MAPK pathways mediate the intracellular signals and regulate the production of pro-inflammatory and anti-inflammatory cytokines in response to stimulation of LPS [5,6]. MAPK pathways include ERK1/2, p38 MAPK, and JNK. Especially, p38 MAPK pathway is a critical role in downstream of TLR4-mediated activation [7]. Activation of TLR4 in macrophages is associated with the innate immune response to infection. Activation of the TLR4 signaling pathway is also involved in inflammation of lifestyle-related diseases [8-10]. For this reason, inhibition of TLR4-induced inflammatory signaling pathway plays an important role in the prevention of lifestyle-related diseases.

Diet and lifestyle are major factors that cause diseases. Thus, dietary supplements are important for preventing lifestyle-related diseases. Several studies reported that polyphenols of green tea have health benefits in preventing lifestyle-related diseases [11-13]. The polyphenols of green tea include (-)-epigallocatechin-3-gallate (EGCG), (-)-epicatechingallate (ECG), (-)-epigallocatechin (EGC), (+)-gallocatechingallate (GCG), (-)-epicatechin, (+)-gallocatechin (GC) and (+)-catechin. The (-)-catechin is the negative form of catechin. Furthermore, gallic acid is a component of polyphenols in green tea. Catechin consists of a flavan-3-ol structure that contains two or more aromatic rings. Among major green tea polypheonls, EGC, ECG, and EGCG are polyphenols without pyrogallol-type structures. In contrast, (+)-catechin, (-)-catechin and (-)-epicatechin are polyphenols without pyrogallol-type structure. Moreover, gallic acid is a galloyl moiety. The pyrogallol-type polyphenols enhance the phagocytic activity of macrophages [14], and bind with a cell-surface 67-kDa laminin receptor [15,16].

Among the polyphenols of green tea, ECGC is the most abundant polyphenol, and has been studied well. EGCG exerts anti-inflammatory effects in LPS-stimulated macrophages such as decreasing the production of the pro-inflammatory cytokines TNF-α and IL-6 and inflammatory mediator NO, and suppressing the activation of MAPKs (ERK1/2, p38 MAPK, and JNK) and nuclear factor-κB (NF-κB) signaling pathways [17-19]. However, the other polyphenols of green tea have not been fully investigated, especially, the anti-inflammatory effects of gallic acid and polyphenol without pyrogallol-type structure ((+)-catechin, (-)-catechin, (-)-epicatechin). Thus, in the present study, we investigated the anti-inflammatory effects of polyphenols of green, including gallic acid, (+)-catechin, (-)-catechin, epicatechin and EGCG in LPS-stimulated RAW264 cells. The hypothesis of this study is that polyphenols with pyrogallol-type structure are more effective than polyphenols without pyrogallol-type structure for anti-inflammatory effect in on LPS-stimulated RAW264 cells.

Materials and Methods

Reagents

The pure compound gallic acid (monohydrate) and (-)-catechin were obtained from Wako Chemicals (Osaka, Japan). The pure compounds (+)-catechin (hydrate), (-)-epicatechin and EGCG were obtained from Sigma-Aldrich (St. Louis, USA). The p38 MAPK, phospho-p38 MAPK and β-actin antibodies were obtained from Cell Signaling Technology, Inc. (Beverly, USA).

Cells and Cell Culture

Mouse macrophage cell line RAW264 cells were obtained from European Collection of Cell Culture (ECACC, Salisbury, UK) and were cultured in DMEM supplemented with 10% (v/v) FBS (HyClone, Logan, USA) in a humidified atmosphere of 5% CO_2 at 37 ˚C and passaged every 2-3 days to maintain growth.

Polyphenol Treatment and LPS Challenge

Cells were seeded on per well of 6-well plate at a

concentration of 1.45 × 10^6 cells, and were allowed to acclimate for 24 h. After 24 h, cells were pre-treated with different concentrations (1 and 10 μM) of polyphenols (gallic acid, (+)-catechin, (-)-catechin, (-)-epicatechin and EGCG) or D-PBS for 4 h. Polyphenol were diluted in D-PBS and used at the indicated concentrations. After pre-treatment with polyphenols, cells were washed with D-PBS before LPS stimulation. The cells were treated with LPS (50 ng/ml) (Sigma-Aldrich, St. Louis, USA) for 24 h. LPS were diluted in D-PBS and used at the indicated concentrations. The supernatant and whole cell lysate were harvested and stored frozen at -80˚C until analysis.

Total Protein Analysis

The total protein of the cells was used to detect the effects of green tea polyphenols on cell growth/viability [20]. Cells were lysed in 100 μl RIPA buffer (Thermo Scientific, Rockford, USA). The whole cell lysate was used for the determination of protein concentration using the micro-bicinchoninic acid (BCA) assay (Thermo Scientific, Rockford, USA), according to the manufacturer's instruction.

Cytokine Measurement

The production of TNF-α, IL-1β, and IL-6 were measured by enzyme-linked immunosorbent assay (ELISA). The cell supernatant (100 μl) was used for the determination of cytokine concentration by ELISA (R&D Systems, Minneapolis, USA) according to the manufacturer's protocol. TNF-α concentration was measured using a TNF-α DuoSet ELISA kit (range: 31.2 - 2,000 pg/ml) (R&D Systems, Minneapolis, USA). IL-1β concentration was measured using an IL-1β DuoSet ELISA kit (range: 15.6 - 1,000 pg/ml) (R&D Systems, Minneapolis, USA). IL-6 concentration was measured using an IL-6 DuoSet ELISA kit (range: 15.6 - 1,000 pg/ml) (R&D Systems, Minneapolis, USA).

Western Blotting

Cells were lysed in 100 μl RIPA buffer (Thermo Scientific, Rockford, USA), and the cell lysates were centrifuged at 16,000 g for 20 min at 4˚C, and the supernatant was collected as whole-cell extracts. For Western blotting, equal amount of cellular proteins (whole-cell extracts: 10 μg/lane) were separated by 10% SDS-PAGEs, and transferred to PVDF membranes. The protein expression levels were analyzed using antibodies against phospho-p38 MAPK, and p38 MAPK (Cell Signaling, Beverly, USA). Epitopes on proteins recognized specifically by antibodies were visualized by using ECL Prime Western Blotting Detection Reagent (GE Healthcare, Little Chalfont, U.K.). The band intensities were quantified using ImageJ software (NIH, Bethesda, USA).

Statistical Analysis

All results are expressed as means ± standard error of the mean (SEM). Each value is the mean of three independent experiments. Statistical analysis was performed using SPSS V22.0 (IBM, Tokyo, Japan). The *p* values were determined by two-way ANOVA, and repeated measures with Bonferroni post-hoc tests. The *p* values of <0.05 were considered statistically significant.

Results and Discussion

Gallic Acid and EGCG Exhibit protection of Cell Growth/viability on LPS-stimulated RAW264 Cells

RAW264 cells were pre-treated with or without various concentrations of polyphenols of green tea for 4 h, and stimulated with 50 ng/ml LPS for 24 h. As shown in Figure 1, the total protein of cells was decreased when stimulated with LPS for 24 h. In addition, pre-treatment of 10 μM gallic acid and EGCG significantly prevented this decrease in total protein of LPS-stimulated RAW264 cells ($p<0.05$) (Figure 2). Pre-treatment with 10 μM (-)-catechin also produced the similar effect, but it was not significant ($p=0.07$). Pre-treatment of 10 μM EGCG was significantly more effective than pre-treatment with 10 μM (+)-catechin for preserving cell viability ($p<0.05$) (Figure 2).

Gallic acid, (-)-catechin and EGCG Inhibit the Release of LPS-induced TNF-α and IL-6 in RAW264 Cells

Both pre-treatment with 1 and 10 μM EGCG reduced the production of TNF-α by LPS-stimulated RAW264 cells significantly as shown in Figure 3A ($p<0.05$). Pre-treatment with 1 and 10 μM (-)-catechin also significantly suppressed the production of TNF-α ($p<0.05$). Furthermore, pre-treatment with 10 μM gallic acid significantly suppressed the production of TNF-α ($p<0.05$). At the concentration of 10 μM, production of TNF-α was greater in response to treatment with (+)-catechin and epicatechin compared with (-)-catechin ($p<0.05$). Pre-treatment with 1 and 10 μM EGCG and (-)-catechin suppressed the production of IL-6 by LPS-stimulated RAW264 cells ($p<0.05$) (Figure 3B). Pre-treatment with 10 μMgallic acid also suppressed IL-6 production ($p<0.05$) (Figure 3B). The concentration of IL-1β following stimulation with LPS was too low to detect (data not shown).

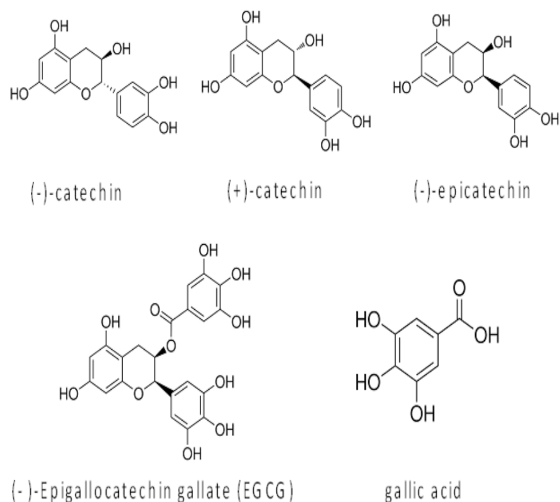

Figure 1: Chemical structure of polyphenols in green tea.

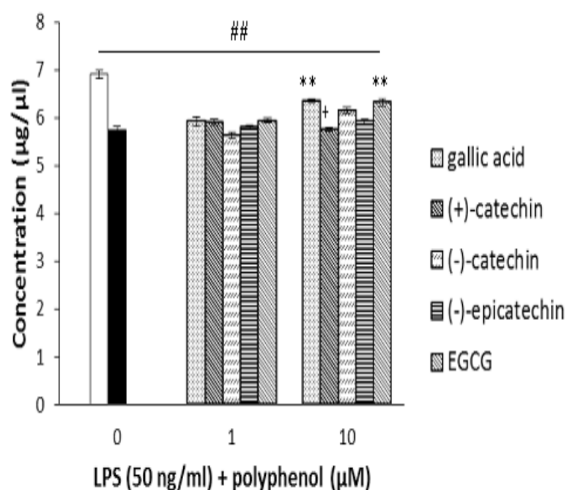

Figure 2: Thegallic acid and EGCG prevented from decreasing total protein of LPS-stimulated RAW264 cells. Cells were pre-treated with polyphenol of green tea for 4h, and stimulated with LPS (50ng/ml) for 24 h. The open bar: without pre-treatment with polyphenols and LPS challenge. The close bar: without pre-treatment with polyphenols. Each column represents the mean ± SEM from three independent experiments. #: *p*<0.05, ##: *p*<0.01 versus the negative control. *: *p*<0.01, **: *p*<0.01 versus the pre-treatment without polyphenol of green tea. +: *p*<0.05 versus the same concentration of EGCG.

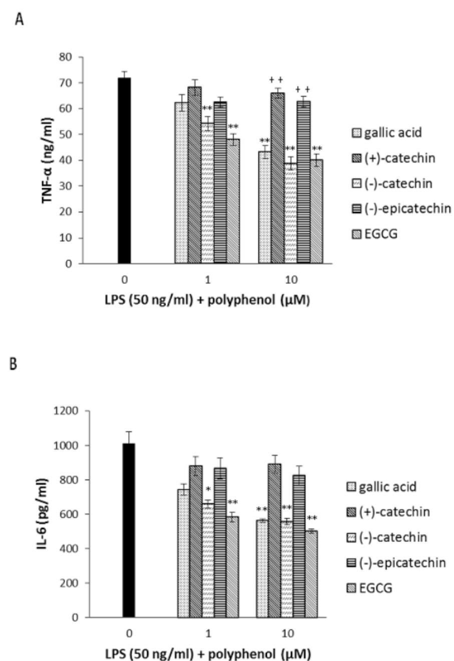

Figure 3: The gallic acid, (-)-catechin and EGCG suppressed LPS-induced pro-inflammatory cytokine production. The Effect of polyphenol of green tea on LPS-induced TNF-α (A) and IL-6 (B) production in RAWcells. The close bar: without pretreatment with polyphenols. Each column represents the mean ± SEM from three independent experiments. *: *p*<0.05, **: *p*<0.01 versus the pre-treatment without polyphenol of green tea. +: *p*<0.05, ++: *p*<0.01 versus the same concentration of EGCG.

The Polyphenols with a Flavan-3-ol Structure Regulated Production and Pof p-38 MAPK

RAW264 cells were pre-treated with or without 10 µM of polyphenols of green tea for 4 h, and stimulated with 50 ng/ml LPS for 45 min and 2 h. LPS-induced production and phosphorylation of p38 MAPK was increased at 45 min (early stage), but after LPS challenge 2 h (late stage) was decreased compared with LPS challenge for 45 min. As shown in Figure 4, pre-treatment with polyphenols that have a flavan-3-ol structure (i.e., (+)-catechin, (-)-catechin, (-)-epicatechin and EGCG) enhanced total expression and phosphorylation of p38 MAPK after LPS challenge 45 min. By contrast, total expression and phosphorylation of p38 MAPK were suppressed after LPS challenge for 2 h. After LPS challenge for both 45 min and 2 h, gallic acid neither enhanced nor suppressed production and phosphorylation of p38 MAPK.

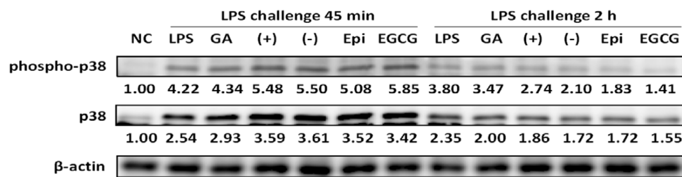

Figure 4: The polyphenol with flavan-3-ol structure enhanced production and phosphorylation of p38 MAPK after LPS challenge 45 min, while suppressed production and phosphorylation of p38 MAPK after LPS challenge 2 h. NC, negative control; LPS, stimulated with LPS (50 ng/ml) and without pre-treatment of polyphenol; GA, pre-treatment with gallic acid 4 h; (+), pre-treatment with (+)-catechin 4 h; (-), pre-treatment with (-)-catchin 4 h; Epi, pre-treatment with (-)-epicatechin 4 h; EGCG, pre-treatment with EGCG 4 h.

The Structural Characteristics of Green Tea Polyphenols on LPS-induced Inflammation by Macrophages

LPS stimulation induces cell death and apoptosis of macrophages [21-23]. Our results indicated that the cell growth/viability was inhibited 24 h after LPS challenge. Pre-treatment with EGCG and gallic acid increased cell growth/viability after LPS challenge. Interestingly, cell growth/viability of LPS-stimulated RAW264 cells was not affected with pre-treatment of (+)-catechin, (-)-catechin and (-)-epicatechin. Xaus et al. demonstrated TNF-α plays an important role in LPS-induced apoptosis on macrophages [23]. Our results showed that pretreatment with (-)-catechin inhibited LPS-induced TNF-α production, but did not increase cell growth/viability after LPS challenge. Taken together, these results suggest that polyphenols with a pyrogollol-type structure may help to protect the cell growth/viability of LPS-stimulated RAW264 cells. Ahn et al. demonstrated that EGCG inhibited proliferative responses of LPS-stimulated dendritic cells [24]. This finding suggests that EGCG suppressed the effect of LPS challenge on immune cells, and our results demonstrated that the pyrogallol-type structure of some polyphenols might protect against the effects of LPS challenge in macrophages.

In a previous study, Hong Byun et al. demonstrated that EGCG inhibited the production of TNF-α and IL-6 by LPS-stimulated mouse peritoneal macrophages [17]. Our results showed that polyphenols of green tea with pyrogallol-type structure, such as EGCG and gallic acid have anti-inflammatory effect on LPS-stimulated RAW264 cells. Concerning anti-inflammatory effect of gallic acid, only pre-treatment with 10 μM gallic acid affected the production of TNF-α and IL-6 of LPS-stimulated RAW264 cells. Furthermore, among the polyphenols in green tea without pyrogallol-type structure, only (-)-catechin suppressed the production of TNF-α and IL-6 of LPS-stimulated RAW264 cells. In the previous studies of catechin, Singh et al. demonstrated that catechin attenuated the production of TNF-α by LPS-stimulated human monocytic cell line THP-1 cells [19], but Youn et al. found that catechin did not suppress NF-κB activation of LPS-stimulated RAW264 cells [25]. NF-κB activation is major upstream signaling for the secretion of TNF-α. These previous studies might use the form of catechin, such as (-)-catechin, (+)-catechin, and (±)-Catechinhydrate. Our results demonstrated that (-)-catechin exerted anti-inflammatory effect on LPS-stimulated RAW264 cells, but (+)-catechin did not suppress pro-inflammatory cytokine production on LPS-stimulated RAW264 cells.

The Effects of Green Tea Polyphenols on LPS-induced p38 MAPK

MAPK pathways enhance the activation of NF-κB, the core of production of pro-inflammatory cytokines [5]. In the LPS-induced MAPK pathway, the total expression and phosphorylation of p38 MAPK increased in early stage, whereas it was diminished in late stage [26,27]. Our results demonstrated that polyphenols with a flavan-3-ol structure enhanced the production and phosphorylation of p38 MAPK in early stage (after 45 min LPS challenge). This result suggests that polyphenols with flavan-3-ol structure accelerate the response toward LPS on p38 MAPK pathway. In the present study, the polyphenols with flavan-3-ol attenuated the production and phosphorylation of p38 MAPK in late stage (after 2 h LPS challenge). On the other hand, gallic acid neither enhanced nor attenuated the expression and phosphorylation of p38 MAPK, which suggests that the anti-inflammatory effect of gallic acid on LPS-stimulated RAW264 cells is regulated through other pathways.

The anti-inflammatory effects of EGCG are regulated through a 67-kDa laminin receptor [17]. The binding activity to this receptor is higher for EGCG (pyrogallol-type) compared with ECG (catechol-type) [15]. Interestingly, (+)-catechin, (-)-epicatechin and gallic acid cannot bind to 67-kDa laminin receptor. Thus, the anti-inflammatory effects of gallic acid and (-)-catechin might be mediated through other receptors or signaling pathways. The results of the present study demonstrate that both gallic

acid and (-)-catechin could inhibit the release of LPS-induced TNF-α and IL-6 in RAW264 cells, but (-)-catechin did not exert protection of cell growth/viability on LPS-stimulated RAW264 cells. These findings suggest that the pyrogallol-type structure improves cell growth/viability in LPS-stimulated RAW264 cells, and the anti-inflammatory effects of (-)-catechin is not controlled through pyrogallol-induced signaling pathways. Furthermore, the polyphenols with flavan-3-ol structure regulated LPS-induced production and phosphorylation of p38 MAPK. In contrast, the anti-inflammatory effect of gallic acid on LPS-stimulated RAW264 cells might be regulated through other pathways.

Conclusion

Our results suggest that pyrogollol-type polyphenols such as gallic acids and EGCG might protect the cell growth/viability against the effects of LPS stimulation in macrophages, and EGCG had the most powerful anti-inflammatory effects compared with other green tea polyphenols. Furthermore, the polyphenols with flavan-3-ol regulated the production and phosphorylation of p38 MAPK in LPS-stimulated macrophages.

Acknowledgments

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