Amplification by (-)-epigallocatechin gallate and chlorogenic acid of TNF-α-stimulated interleukin-6 synthesis in osteoblasts

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Abstract. Polyphenolic compounds in foods and beverages have beneficial effects on human health. (-)-Epigallocatechin gallate (EGCG) and chlorogenic acid (CGA), a major flavonoid in green tea and a major phenolic acid in coffee, respectively, have potent properties, including antioxidative effects. Our previous study demonstrated that p70 S6 kinase acts as a negative regulator in tumor necrosis factor-α (TNF-α)-stimulated interleukin-6 synthesis in osteoblast-like MC3T3-E1 cells. In the present study, the effects of EGCG and CGA on the TNF-α-stimulated interleukin-6 synthesis were investigated in MC3T3-E1 cells. EGCG and CGA significantly enhanced TNF-α-stimulated interleukin-6 release. In addition, the interleukin-6 mRNA expression levels induced by TNF-α were supported by EGCG, as well as CGA. EGCG markedly attenuated the TNF-α-induced phosphorylation of p70 S6 kinase whereas CGA failed to affect the phosphorylation. These results strongly suggest that EGCG and CGA enhance the TNF-α-stimulated interleukin-6 synthesis in osteoblasts, and that the amplifying effect of EGCG, but not CGA, is exerted via inhibiting p70 S6 kinase.

Introduction

Green tea and coffee are the most commonly consumed beverages worldwide. Green tea has been consumed mainly in Eastern countries, while coffee is consumed throughout the world. (-)-Epigallocatechin gallate (EGCG), the most abundant catechin and a major bioactive component in green tea, and chlorogenic acid (CGA), a main phenolic compound in coffee, have beneficial properties for human health, such as antioxidation, anti-inflammation and cancer prevention (1-3).

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Bone metabolism is regulated strictly by two types of functional cells, osteoblasts and osteoclasts, which are responsible for bone formation and resorption, respectively (4). Bone tissue in the skeleton is continuously renewed through a process, known as bone remodeling (5). A decrease in bone mineral density results from an unbalanced bone remodeling process (5). This remodeling process begins with osteoclast bone resorption, followed by osteoblast bone formation (6). Bone remodeling disorder causes metabolic bone disease, including osteoporosis, and the increased risk of fracture. It has been shown that ingestion of green tea prevents age-related bone loss and fractures in elderly individuals (7). Accumulating evidence indicates that catechin suppresses bone resorption and supports osteoblastic bone formation (7,8). Additionally, catechin stimulates alkaline phosphatase activity, a mature osteoblast phenotype, and reduces apoptosis in osteoblasts (7). By contrast, it has been shown that CGA increases mineralization in the tibia, and improves mechanical properties of the femoral diaphysis in rat model (9). In addition, CGA reportedly inhibits osteoclast differentiation and bone resorption by downregulation of receptor activator of nuclear factor κB (RANK) ligand-induced nuclear factor of activated T cell c1 expression (10). However, the exact mechanisms underlying the effects of EGCG or CGA on bone metabolism remain to be elucidated.

Tumor necrosis factor (TNF)-α, a multifunctional cytokine, is firmly established to have an important role in inflammation, infection and cancer (11). With regard to bone metabolism, TNF-α stimulates the activation of RANK in osteoclasts through RANK ligand expression in osteoblasts, which acts as a potent stimulator of osteoclastogenesis (12). It has been reported that TNF-α stimulates the synthesis of interleukin-6 (13). Interleukin-6 is a member of the gp130 cytokine family and has important physiological effects on a wide range of functions, such as promotion of B-cell differentiation, T-cell activation and induction of acute-phase proteins (11,14,15). As for the association between interleukin-6 and bone metabolism, it is well recognized that interleukin-6 stimulates bone resorption and induces osteoclast formation (11,14). By contrast, interleukin-6 has a pivotal role in the process of bone fracture repair (16). Thus, interleukin-6 is currently considered to also be an osteotropic factor and influence bone

formation under the condition of increased bone turnover (17). Our previous studies have shown that interleukin-6 synthesis induced by TNF-α is negatively regulated through the p70 S6 kinase pathway in osteoblast-like MC3T3-E1 cells (18,19).

In the present study, the effects of EGCG or CGA on TNF-α-induced interleukin-6 synthesis in osteoblast-like MC3T3-E1 cells were investigated.

Materials and methods

Materials. EGCG and CGA were obtained from Sigma-Aldrich (St. Louis, MO, USA). TNF-α and mouse interleukin-6 enzyme-linked immunosorbent assay (ELISA) kit were obtained from R&D Systems, Inc. (Minneapolis, MN, USA). Phospho-specific p70 S6 kinase (Thr-389) antibodies (#9205; polyclonal; rabbit anti-mouse; 1:1,000) and p70 S6 kinase antibodies (#9202; polyclonal; rabbit anti-mouse; 1:1,000) were obtained from Cell Signaling Technology, Inc. (Beverly, MA, USA). An ECL western blotting detection system was obtained from GE Healthcare UK Ltd. (Buckinghamshire, UK). Other materials and chemicals were obtained from commercial sources. EGCG was dissolved in dimethyl sulfoxide and CGA was dissolved in ethanol. The maximum concentration of dimethyl sulfoxide or ethanol was 0.1%, which did not affect the assay for interleukin-6 or the western blot analysis.

Cell culture. Cloned osteoblast-like MC3T3-E1 cells derived from newborn mouse calvaria (20) were maintained as previously described (21). Briefly, the cells were cultured in α-minimum essential medium (α-MEM) containing 10% fetal bovine serum (FBS) at 37˚C in a humidified atmosphere of 5% CO_2/95% air. The cells were seeded in 35-mm diameter dishes ($5x10^4$ cells/dish) or 90-mm diameter dishes ($2x10^5$ cells/dish) in α-MEM containing 10% FBS. After 5 days, the medium was exchanged for α-MEM containing 0.3% FBS. The cells were used for experiments after 48 h.

Assay for interleukin-6. The cultured cells were pretreated with various doses of EGCG or CGA for 60 min, and subsequently stimulated by 30 ng/ml of TNF-α or vehicle in 1 ml of α-MEM containing 0.3% FBS for the indicated periods. The conditioned medium was collected at the end of the incubation, and the interleukin-6 concentration was subsequently measured using mouse interleukin-6 ELISA kit according to the manufacturer's protocol.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). The cultured cells were pretreated with 100 μM of EGCG, 50 μM of CGA or vehicle for 60 min, and subsequently stimulated by 30 ng/ml of TNF-α or vehicle in α-MEM containing 0.3% FBS for 3 h. Total RNA was isolated and transcribed into complementary DNA using TRIzol reagent (Invitrogen Corp., Carlsbad, CA, USA) and Omniscript reverse transcriptase kit (Qiagen Inc., Valencia, CA, USA), respectively. RT-qPCR was performed using a LightCycler system in capillaries and Fast Start DNA Master SYBR-Green I provided with the kit (Roche Diagnostics, Basel, Switzerland). Sense and antisense primers for mouse interleukin-6 mRNA were purchased from Takara Bio Inc. (Tokyo, Japan) (primer set ID: MA039013), while mouse glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA primers were synthesized based on the study by Simpson *et al* (22). The amplified products were determined using melting curve analysis and agarose electrophoresis. The interleukin-6 mRNA levels were normalized to those of GAPDH mRNA.

Western blot analysis. The cultured cells were pretreated with various doses of EGCG or CGA for 60 min, and subsequently stimulated by 30 ng/ml of TNF-α or vehicle in α-MEM containing 0.3% FBS for 10 min. The cells were washed twice with phosphate-buffered saline and lysed, homogenized and sonicated in a lysis buffer containing 62.5 mM Tris/HCl (pH 6.8) 2% sodium dodecyl sulfate (SDS), 50 mM dithiothreitol and 10% glycerol. SDS-polyacrylamide gel electrophoresis was performed by the method of Laemmli (23) in 10% polyacrylamide gels. The proteins were fractionated and transferred onto Immun-Blot polyvinyl difluoride (PVDF) membranes (Bio-Rad Laboratories, Hercules, CA, USA). The membranes were blocked with 5% fat-free dry milk in Tris-buffered saline-Tween [TBS-T; 20 mM Tris-HCl (pH 7.6), 137 mM NaCl and 0.1% Tween-20] for 2 h before incubation with primary antibodies. A western blot analysis was performed as previously described (24) using phospho-specific p70 S6 kinase antibodies or p70 S6 kinase antibodies as primary antibodies, with peroxidase-labeled antibodies raised in goat against rabbit immunoglobulin G (no. 074-1506; KPL, Inc., Gaithersburg, MD, USA) used as the secondary antibodies. The primary and secondary antibodies were diluted at 1:1,000 with 5% fat-free dry milk in TBS-T. The peroxidase activity on the PVDF sheet was visualized on X-ray film by means of the ECL Western blotting detection system.

Determination. The absorbance of the enzyme immunosorbent assay samples was measured at 450 nm with the EL 340 Bio Kinetic Reader (Bio-Tek Instruments, Inc., Winooski, VT, USA). A densitometric analysis was performed using a scanner and image analysis software package (ImageJ version 1.47; National Institutes of Health, Bethesda, MD, USA). The phosphorylated protein levels were calculated as follows: The background-subtracted signal intensity of each phosphorylation signal was respectively normalized to the total protein signal and plotted as the fold increase in comparison with that of the control cells treated without stimulation.

Statistical analysis. The data were analyzed by an analysis of variance followed by Bonferroni method for multiple comparisons between pairs, and $P<0.05$ was considered to indicate a statistically significant difference. All the data are presented as the means ± standard error of the mean of triplicate determinations from three independent cell preparations.

Results

Effect of EGCG on the TNF-α-stimulated interleukin-6 release in MC3T3-E1 cells. Our previous study showed that TNF-α stimulates interleukin-6 synthesis in osteoblast-like MC3T3-E1 cells (18). Firstly, the effect of EGCG on the TNF-α-stimulated interleukin-6 release in MC3T3-E1 cells was examined. EGCG significantly amplified the TNF-α-stimulated interleukin-6 release in a time-dependent manner ≤48 h (Fig. 1A). The enhancing effect of EGCG was dose-dependent between 10

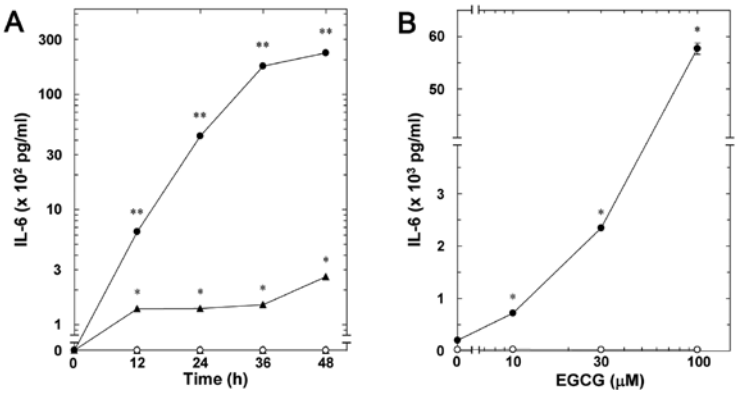

Figure 1. Effect of (-)-epigallocatechin gallate (EGCG) on the tumor necrosis factor-α (TNF-α)-stimulated interleukin-6 release in MC3T3-E1 cells. (A) The cultured cells were pretreated with 100 μM of EGCG (●,○) or vehicle (▲,△) for 60 min, and subsequently stimulated by 30 ng/ml of TNF-α (●,▲) or vehicle (○,△) for the indicated periods. *P<0.05 compared to the value of the control; **P<0.05 compared to the value of TNF-α alone. (B) The cultured cells were pretreated with various doses of EGCG for 60 min, and subsequently stimulated by 30 ng/ml of TNF-α (●) or vehicle (○) for 48 h. *P<0.05 compared to the value of TNF-α alone. The interleukin-6 concentrations in the culture medium were determined using ELISA. Each value represents the mean ± standard error of the mean of triplicate determinations from three independent cell preparations.

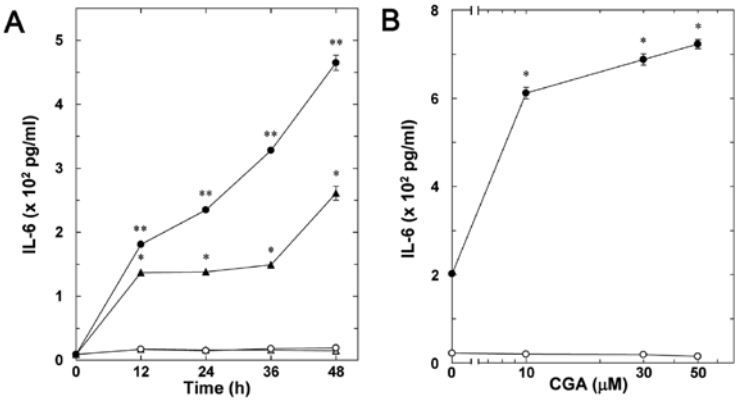

Figure 2. Effect of chlorogenic acid (CGA) on the tumor necrosis factor-α (TNF-α)-stimulated interleukin-6 release in MC3T3-E1 cells. (A) The cultured cells were pretreated with 50 μM of CGA (●,○) or vehicle (▲,△) for 60 min, and subsequently stimulated by 30 ng/ml of TNF-α (●,▲) or vehicle (○,△) for the indicated periods. *P<0.05 compared to the value of the control; **P<0.05 compared to the value of TNF-α alone. (B) The cultured cells were pretreated with various doses of CGA for 60 min, and subsequently stimulated by 30 ng/ml of TNF-α (●) or vehicle (○) for 48 h. *P<0.05 compared to the value of TNF-α alone. The interleukin-6 concentrations in the culture medium were determined using ELISA. Each value represents the mean ± standard error of the mean of triplicate determinations from three independent cell preparations.

and 100 μM (Fig. 1B). The maximum effect of EGCG on the interleukin-6 release was observed at 100 μM.

Effect of CGA on the TNF-α-stimulated interleukin-6 release in MC3T3-E1 cells. The effect of CGA on the TNF-α-stimulated interleukin-6 release in osteoblast-like MC3T3-E1 cells was examined. CGA significantly enhanced the TNF-α-stimulated interleukin-6 release in a time-dependent manner ≤48 h (Fig. 2A). The enhancement by CGA on the release was dose-dependent between 10 and 50 μM (Fig. 2B).

Effects of EGCG or CGA on the TNF-α-induced expression of interleukin-6 mRNA in MC3T3-E1 cells. In order to investigate whether the amplifying effect of EGCG or CGA on the TNF-α-stimulated interleukin-6 release is mediated via transcriptional events in MC3T3-E1 cells, the effects of EGCG or CGA on the TNF-α-induced interleukin-6 mRNA expression were examined. EGCG or CGA, which by itself had little effect on the interleukin-6 mRNA levels, markedly upregulated the TNF-α-induced interleukin-6 mRNA expression levels (Fig. 3).

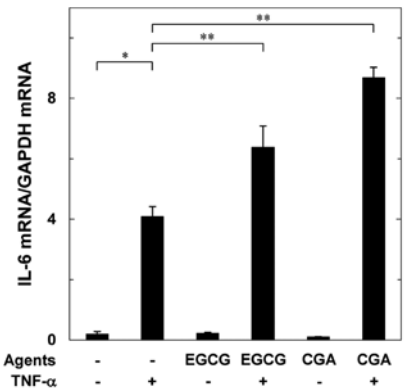

Figure 3. Effects of (-)-epigallocatechin gallate (EGCG) or chlorogenic acid (CGA) on the tumor necrosis factor-α (TNF-α)-induced expression levels of interleukin-6 mRNA in MC3T3-E1 cells. The cultured cells were pretreated with 100 μM of EGCG, 50 μM of CGA or vehicle for 60 min, and subsequently stimulated by 30 ng/ml of TNF-α or vehicle for 3 h. The respective total RNA was subsequently isolated and quantified using RT-qPCR. Each value represents the mean ± standard error of the mean of triplicate determinations from three independent cell preparations. *P<0.05 compared to the value of the control; **P<0.05 compared to the value of TNF-α alone.

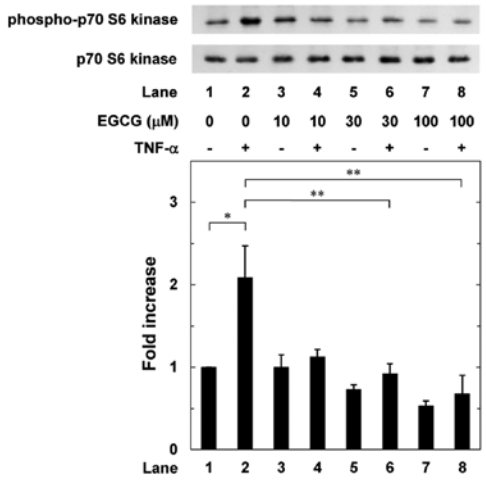

Figure 4. Effect of (-)-epigallocatechin gallate (EGCG) on the tumor necrosis factor-α (TNF-α)-induced phosphorylation of p70 S6 kinase in MC3T3-E1 cells. The cultured cells were pretreated with various doses of EGCG for 60 min, and subsequently stimulated by 30 ng/ml of TNF-α or vehicle for 10 min. The cell extracts were subsequently subjected to SDS-PAGE with subsequent western blot analysis with antibodies against phospho-specific p70 S6 kinase or p70 S6 kinase. The histogram shows quantitative representation of the levels of TNF-α-induced phosphorylation obtained from a laser densitometric analysis of three independent experiments. Each value represents the mean ± standard error of the mean of triplicate determinations. *P<0.05 compared to the value of the control; **P<0.05 compared to the value of TNF-α alone.

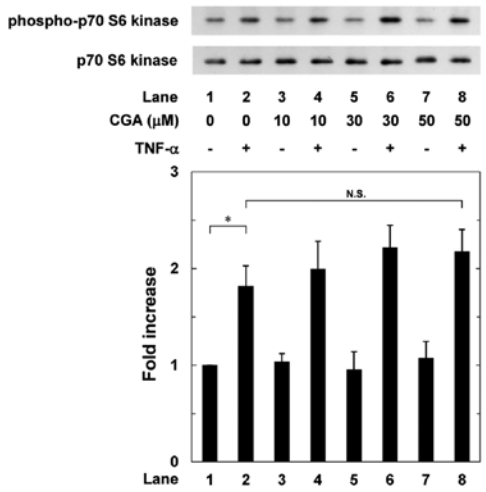

Figure 5. Effect of chlorogenic acid (CGA) on the tumor necrosis factor-α (TNF-α)-induced phosphorylation of p70 S6 kinase in MC3T3-E1 cells. The cultured cells were pretreated with various doses of CGA for 60 min, and subsequently stimulated by 30 ng/ml of TNF-α or vehicle for 10 min. The cell extracts were subjected to SDS-PAGE with subsequent western blot analysis with antibodies against phospho-specific p70 S6 kinase or p70 S6 kinase. The histogram shows quantitative representation of the levels of TNF-α-induced phosphorylation obtained from a laser densitometric analysis of three independent experiments. Each value represents the mean ± standard error of the mean of triplicate determinations. *P<0.05 compared to the value of the control; N.S. designates no significant differences between the indicated pairs.

Effects of EGCG or CGA on the TNF-α-induced phosphorylation of p70 S6 kinase in MC3T3-E1 cells. Our previous study reported that p70 S6 kinase has a suppressive role in the TNF-α-stimulated interleukin-6 synthesis in osteoblast-like MC3T3-E1 cells (19). Therefore, to clarify whether the effects of EGCG or CGA on the TNF-α-stimulated interleukin-6 synthesis are associated with the activation of p70 S6 kinase in MC3T3-E1 cells, the effects of EGCG or CGA on the TNF-α-induced phosphorylation of p70 S6 kinase were further examined. EGCG significantly suppressed the TNF-α-induced phosphorylation of p70 S6 kinase in a dose-dependent manner between 10 and 100 μM (Fig. 4). By contrast, CGA did not affect the TNF-α-induced phosphorylation of p70 S6 kinase between 10 and 50 μM (Fig. 5).

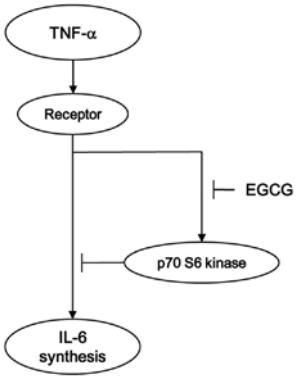

Figure 6. Diagram of the potential mechanism for the amplification by (-)-epigallocatechin gallate (EGCG) of the tumor necrosis factor-α (TNF-α)-induced interleukin-6 synthesis in MC3T3-E1 cells. EGCG enhances the TNF-α-stimulated interleukin-6 synthesis via the suppression of p70 S6 kinase in osteoblast-like MC3T3-E1 cells.

Discussion

In the present study, EGCG, a main flavonoid in green tea, and CGA, a bioactive compound in coffee, were demonstrated to enhance the TNF-α-stimulated interleukin-6 release in osteoblast-like MC3T3-E1 cells. Additionally, the expression levels of interleukin-6 mRNA induced by TNF-α were amplified by EGCG, as well as CGA. Thus, it appears that the enhancing effects of EGCG and CGA on the TNF-α-stimulated interleukin-6 release are mediated through transcriptional levels in MC3T3-E1 cells. Taking the present findings into account, it is most likely that EGCG and CGA in beverages upregulate TNF-α-stimulated synthesis of interleukin-6 in osteoblast-like MC3T3-E1 cells.

Regarding the intracellular signaling of TNF-α-stimulated interleukin-6 synthesis in osteoblasts, our previous study reported that TNF-α stimulates the activation of p70 S6 kinase in osteoblast-like MC3T3-E1 cells, resulting in the limitation of the TNF-α-stimulated interleukin-6 synthesis (19). Based on these results, the precise mechanism behind the enhancement by EGCG and CGA of the TNF-α-stimulated interleukin-6 synthesis in MC3T3-E1 cells was investigated. EGCG markedly suppressed the TNF-α-stimulated phosphorylation of the p70 S6 kinase. However, CGA failed to affect the phosphorylation of p70 S6 kinase. Therefore, it is most likely that EGCG strengthens the TNF-α-stimulated interleukin-6 synthesis through the inhibition of p70 S6 kinase in osteoblast-like MC3T3-E1 cells. By contrast, it appears unlikely that the enhancing effect of CGA on the TNF-α-induced interleukin-6 synthesis is mediated through p70 S6 kinase. In our previous studies, we have reported that p70 S6 kinase negatively regulates the interleukin-6 synthesis stimulated by platelet-derived growth factor (PDGF), a bone remodeling

modulator in osteoblast-like MC3T3-E1 cells (25). Additionally, our previous study showed that EGCG reduces the interleukin-6 synthesis by platelet-derived growth factor without affecting the p70 S6 kinase activation (26). It is generally recognized that platelet-derived growth factor receptor has an intrinsic protein tyrosine kinase activity, whereas TNF-α receptor belongs to the cytokine receptor superfamily, and that the signal transduction mechanisms are quite different from each other (12,27). Thus, it appears likely that different and rather opposite effects of EGCG on p70 S6 kinase activation and interleukin-6 synthesis induced by TNF-α or PDGF-BB in osteoblast-like MC3T3-E1 cells are due to the difference between each receptor-mediated intracellular signaling. Further investigations are required to clarify the details underlying the effects of EGCG and CGA on the interleukin-6 synthesis in osteoblasts. The potential mechanism of EGCG in the TNF-α-stimulated interleukin-6 synthesis in osteoblasts is summarized in Fig. 6.

Polyphenolic compounds in beverages, including green tea and coffee, have beneficial properties for human health. With regard to bone metabolism, accumulating evidence indicates that green tea consumption contributes bone formation, improves bone mineral density and decreases the risk of fracture (7). Additionally, it is currently known that coffee consumption reduces the risk of osteoporosis and osteoporotic fracture in elderly people (28). In the present study, EGCG and CGA amplified the TNF-α-stimulated interleukin-6 synthesis in osteoblast-like MC3T3-E1 cells. In bone metabolism, interleukin-6 has long been recognized to act as a powerful bone resorptive agent and promotes osteoclast formation (29). However, interleukin-6 is currently considered as an osteotropic factor under the condition of increased bone turnover and induces bone formation (17). Bone resorption is the first step of bone remodeling, and subsequently bone formation is initiated (4). To obtain the quantity and quality of bone, adequate bone remodeling processed by osteoclasts and osteoblasts is essential. With regard to bone metabolism as a whole, it is possible that interleukin-6 functions as a bone remodeling mediator. In addition, it has been reported that interleukin-6 has a crucial role in the initiation of bone formation in an early stage of bone fracture repair (16). Therefore, the present findings that EGCG and CGA enhance the TNF-α-stimulated interleukin-6 synthesis in osteoblasts provide a new insight in fracture prevention in elderly people. Further investigations are required to elucidate the exact roles of EGCG and CGA in bone metabolism.

In conclusion, the present results strongly suggest that EGCG and CGA enhance the TNF-α-stimulated interleukin-6 synthesis in osteoblasts and that the enhancement by EGCG, but not CGA, is exerted via inhibiting p70 S6 kinase.

Acknowledgements

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