(-)-epigallocatechin Gallate Attenuates The Induction Of Hsp27 Stimulated By Sphingosine 1-phosphate Via Suppression Of Phosphatidylinositol 3-kinase/akt Pathway In Osteoblasts

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Introduction: Heat shock proteins (HSPs) are stimulated in cells in response to the biological stress. Though the functions of the low-molecular-weight HSPs, such as HSP27, are still little known. In osteoblasts, down-regulation of proliferation is accompanied by a transient increase of the HSP27 mRNA expression. In addition, heat-stimulated induction of HSP27 is reportedly facilitated by estrogen. These findings lead us to speculate that HSP27 takes part in the coordination of osteoblast functions. However, the exact roles of HSP27 in osteoblasts remain to be clarified.

Sphingosine 1-phosphate produced by osteoclasts plays a crucial role in the recruitment of osteoprogenitors to sites of bone resorption. As for osteoblasts, sphingosine 1-phosphate prevents apoptosis via phosphatidylinositol 3 kinase (PI3-kinase) in primary calvaria rat osteoblasts. We have shown that sphingosine 1-phosphate stimulates the induction of HSP27 via p38 mitogen-activated protein (MAP) kinase and PI3-kinase/Akt in osteoblast-like MC3T3-E1 cells.

Catechin is one of the major flavonoids, which shows antioxidative, antiproliferative and proapoptotic effects. In bone metabolism, catechin suppresses bone resorption. As for osteoblasts, catechin stimulates alkaline phosphatase activity, a mature osteoblast phenotype and reduces the production of bone-resorptive cytokines, such as tumor necrosis factor-α (TNF-α) and interleukin-6 (IL-6) in osteoblast-like MC3T3-E1 cells. On the other hand, we have reported that (-)-epigallocatechin gallate (EGCG) enhances vascular endothelial growth factor (VEGF) synthesis stimulated by prostaglandin F2α via upregulating stress-activated protein kinase/c-Jun N-terminal kinase (SAPK/JNK) activation in MC3T3-E1 cells. In addition, we have recently reported that EGCG inhibits prostaglandin D2-stimulated HSP27 induction via suppression of the p44/p42 MAP kinase pathway in these cells. But so far, the precise mechanism underlying the effects of EGCG on bone metabolism has not been clarified.

In the present study, we investigated the effect of EGCG on the induction of HSP27 stimulated by sphingosine 1-phosphate in osteoblast-like MC3T3-E1 cells. We here show that EGCG suppresses the induction of HSP27 stimulated by sphingosine 1-phosphate via inhibition of PI3-kinase/Akt pathway but not p38 MAP kinase pathway in these cells.

Methods: Materials. Sphingosine 1-phosphate was purchased from Sigma Chemical Co. (St. Louis, MO). EGCG was obtained from Calbiochem-Novabiochem (La Jolla, CA). Phospho-specific p38 MAP kinase antibodies, p38 MAP kinase antibodies, phospho-specific MAP kinase-activated protein kinase-2 (MAPKAP-2) antibodies, MAPKAP-2 antibodies, phospho-specific Akt antibodies, Akt antibodies, phospho-specific glycogen synthase kinase-3β (GSK-3β) antibodies and GSK-3β antibodies were purchased from Cell Signaling Technology, Inc. (Beverly, MA). HSP27 antibodies for Western blotting analysis were obtained from R&D Systems, Inc. (Minneapolis, MN). Glyceraldehyde 3 phosphate dehydrogenase (GAPDH) antibodies and HSP27 antibodies for immunofluorescence microscopy studies
were obtained from Santa Cruz biotechnology, Inc. (Santa Cruz, CA). Alexa Fluor 488® conjugated goat anti-rabbit antibodies and Alexa Fluor 555® phalloidin were obtained from Invitrogen Corporation, Inc. (Carlsbad, CA). An ECL Western blotting detection system was obtained from Amersham Japan (Tokyo, Japan). Other materials and chemicals were obtained from commercial sources. Sphingosine 1-phosphate was dissolved in dimethyl sulfoxide. The maximum concentration of dimethyl sulfoxide was 0.1%, which did not affect all results.

Cell culture. Cloned osteoblast-like MC3T3-E1 cells derived from newborn mouse calvaria were maintained as previously described. Briefly, the cells were cultured in α-minimum essential medium (α-MEM) containing 10% fetal calf serum (FCS) at 37ºC in a humidified atmosphere of 5% CO2/95% air. The cells were seeded into 90-mm diameter dishes (25 x 10⁴/dish) for Western blot analysis or 35-mm diameter glass-bottom dishes (3 x 10⁴/dish) for immunofluorescence microscopy study. After 5 days, the medium was exchanged to α-MEM containing 0.3% FCS. The cells were then used for experiments after 48 h. When indicated, the cells were pretreated with EGCG at the indicated concentrations.

Western blot analysis. The cultured cells were stimulated by sphingosine 1-phosphate in serum-free α-MEM for the indicated periods. The cells were washed twice with phosphate-buffered saline and then lysed, homogenized, sonicated and immediately boiled in a lysis buffer containing 62.5 mM Tris/Cl, pH 6.8, 2% sodium dodecyl sulfate (SDS), 50 mM dithiothreitol, and 10% glycerol. The sample was used for the analysis by Western blotting. SDS-polyacrylamide gel electrophoresis (PAGE) was performed by the method of Laemmli in 10% polyacrylamide gel. Western blot analysis was performed as described previously, using HSP27 antibodies, GAPDH antibodies, phospho-specific p38 MAP kinase antibodies, p38 MAP kinase antibodies, phospho-specific MAPKAP-2 antibodies, MAPKAP-2 antibodies, phospho-specific Akt antibodies, Akt antibodies, phospho-specific GSK-3β antibodies or GSK-3β antibodies with peroxidase-labeled antibodies raised in goat anti-rabbit IgG which were used as second antibodies. Peroxidase activity on PVDF membranes was visualized on X-ray film by means of the ECL Western blotting detection system and was quantitated using NIH image software. All of Western blot analyses were repeated at least three times in independent experiments.

Immunofluorescence microscopy study. The cultured cells were pretreated with EGCG at the indicated concentrations for 1 h and then exposed to sphingosine 1-phosphate (30 μM) for 9 h. They were then fixed with 3% paraformaldehyde for 10 min on ice and exposed to 0.1% Triton X-100 for 10 min to permeabilize the cell membrane, and they were then exposed to HSP27 antibodies (1:100 dilution) in the presence of 1% BSA for 1 h, followed by exposure to Alexa Fluor 488® conjugated calf anti-goat IgG antibodies (1:500) for 1 h. Finally, they were exposed to Alexa Fluor 555® phalloidin and DAPI for 20 min. The cells were examined by fluorescent microscopy, BIOREVO (BZ-9000) (Keyence, Tokyo, Japan) as manufacturer’s protocol.

Statistical Analysis. The data were analyzed by ANOVA followed by Bonferroni method for multiple comparisons between pairs, and a p<0.05 was considered significant. All data are presented as the mean ± SEM of triplicate determinations.

Results: EGCG significantly reduced the induction of HSP27 stimulated by sphingosine 1-phosphate in a dose-dependent manner between 10 and 30 μM. Immunofluorescence microscopy studies revealed that sphingosine 1-phosphate certainly stimulated the induction of HSP27 in the cytosol of these cells, and that EGCG clearly suppressed its induction. However, sphingosine 1-phosphate-stimulated phosphorylation of p38 MAP kinase or that of MAPKAP-2 was not affected by EGCG. By contrast, EGCG
markedly suppressed the phosphorylations of both Akt and glycogen synthase kinase-3β stimulated by sphingosine 1-phosphate.

**Discussion:** In the present study, we first demonstrated that EGCG significantly suppressed the induction of HSP27 stimulated by sphingosine 1-phosphate in osteoblast-like MC3T3-E1 cells. Moreover, in immunofluorescence microscopy studies, we also showed that EGCG suppressed the induction of HSP27 stimulated by sphingosine 1-phosphate in a dose-dependent manner.

**Significance:** These results strongly suggest that EGCG suppresses the induction of HSP27 stimulated by sphingosine 1-phosphate via attenuation of not p38 MAP kinase pathway but phosphatidylinositol 3-kinase/Akt pathway in osteoblasts.

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