Available online at www.sciencedirect.com
SCIENCE DIRECT
Biochemical Pharmacology 70 (2005) 363–368
Biochemical Pharmacology
www.elsevier.com/locate/biochempharm

The licorice root derived isoflavan glabridin increases the function of osteoblastic MC3T3-E1 cells

Eun-Mi Choi *

Department of Food and Nutrition, Kyung Hee University, 1, Hoegi-dong, Dongdaemun-gu, Seoul 130-701, Republic of Korea

Received 7 March 2005; accepted 18 April 2005

Abstract

Glabridin, an isoflavan purified from licorice root, exhibits diverse biological activities, including estrogen-like activity. To investigate the bioactivities of glabridin, which act on bone metabolism, the effects of glabridin on the function of mouse osteoblastic cell line (MC3T3-E1) and the production of local factors in osteoblasts were studied. Glabridin (1–10 μM) significantly increased the growth of MC3T3-E1 cells and caused a significant elevation of alkaline phosphatase (ALP) activity, collagen content and osteocalcin secretion in the cells ($P < 0.05$). The effect of glabridin (10 μM) in increasing ALP activity and collagen content was completely prevented by the presence of 10^{-6} M cycloheximide and 10^{-6} M tamoxifen, suggesting that glabridin's effect results from a newly synthesized protein component and might be partly involved in estrogen action. Then, the effects of glabridin on the TNF-α-induced apoptosis and production of prostaglandin E_2 (PGE_2) and nitric oxide (NO) in osteoblasts were examined. Treatment with glabridin (1–10 μM) prevented apoptosis induced by TNF-α (10^{-10} M) in osteoblastic cells. Moreover, glabridin (50 μM) decreased the 10^{-10} M TNF-α-induced production of PGE_2 and NO in osteoblasts. Our data indicate that the enhancement of osteoblast function by glabridin may result in the prevention for osteoporosis and inflammatory bone diseases.
© 2005 Elsevier Inc. All rights reserved.

Keywords: Glabridin; Osteoblastic MC3T3-E1 cells

1. Introduction

Glycyrrhiza glabra, the licorice plant, has been known as a healthy nutrient for more than 3000 years. The licorice roots have long been used as flavorings, sweeteners and as herbal medicine. Among licorice flavonoids, glabridin constituted the major flavonoid in the licorice root extract. Glabridin has been reported to exhibit multiple pharmacological activities, such as cytotoxic activity [1], antimicrobial activity against *Helicobacter pylori* [2], estrogenic and anti-proliferative activity against human breast cancer cells [3], effect on melanogenesis, inflammation [4,5], low-density lipoprotein oxidation [6], inhibition of human cytochrome P450s 3A4, 2B6 and 2C9 activities [7] and protection of mitochondrial functions from oxidative stresses [8]. Several features are common to the structures of glabridin and estradiol. Both have an aromatic ring substituted with a hydroxyl group at para (glabridin) or position 3 (estradiol), with three additional fused rings of a phenanthrenic shape. Both are relatively lipophilic, containing a second hydroxyl group (17β in estradiol and 2′-OH in glabridin). Somjen et al. [9] demonstrated that the phytoestrogen, glabridin, had the same beneficial effects on bone tissues as estradiol or genistein.

Many osteoporotic patients have already lost a substantial amount of bone, and thus, a method to increase bone mass by stimulating new bone formation is needed [10]. In bone formation, osteoblasts are key cell in bone matrix formation and calcification [11]. The mouse osteoblastic cell line, MC3T3-E1, has been shown to differentiate into mature cells following treatment with 17β-estradiol [12]. In the differentiation of MC3T3-E1 cells, the stimulation of cell proliferation and the induction of alkaline phosphatase (ALP) were observed. Recently, it was reported that some naturally occurring compounds induced MC3T3-E1 cell differentiation and their effects on prevention of osteoporosis were studied [13,14]. In the present study, the effects of glabridin on the function of osteoblastic MC3T3-E1 cells were evaluated in vitro. Also, the TNF-α-induced apoptosis and production of PGE_2 and nitric oxide (NO) in osteoblasts were investigated.

* Tel.: +82 2 961 0709; fax: +82 2 968 0260.
E-mail address: cheunmi@hanmail.net.

0006-2952/$ – see front matter © 2005 Elsevier Inc. All rights reserved.
doi:10.1016/j.bcp.2005.04.019

2. Materials and methods

2.1. Reagents

Glabridin isolated from licorice roots was purchased from Wako Pure Chemicals, Industries, Ltd. (Japan). Glabridin was dissolved in dimethylsulfoxide (DMSO) and then diluted with the medium (final DMSO concentration $\leq 0.05\%$ (v/v)). All other reagents were from Sigma Chemical Co. (St. Louis, MO, USA) unless otherwise stated.

2.2. Cell cultures

MC3T3-E1 cells (RCB1126, an osteoblast-like cell line from C57BL/6 mouse calvaria) was obtained from the RIKEN Cell Bank (Tsukuba, Japan). MC3T3-E1 cells were cultured at 37 °C in 5% CO_2 atmosphere in α-modified minimal essential medium (α-MEM; GIBCO). Unless otherwise specified, the medium contained 10% heat-inactivated fetal bovine serum (FBS), 100 U/ml penicillin and 100 μg/ml streptomycin.

2.3. Cell viability: MTT assay

The cells were suspended in medium and plated at a density of 7.0×10^3 cells/well into a 96-well culture dish (Costar, Cambridge, MA). After 24 h, the medium was replaced with phenol red-free media containing 5% charcoal-dextran-treated FBS (CD-FBS) supplemented with glabridin. After 2 days of culture, cell proliferation was measured by MTT assay. This assay is based on the ability of viable cells to convert soluble 3-(4,5-dimethyl-thiazol-2yl)-2,5-diphenyl tetrazolium bromide (MTT) into an insoluble dark blue formazan reaction product. In the bulk cell photometric MTT assay, the bulk conversion of MTT in the well plate was measured photometrically. MTT was dissolved in DPBS at a concentration of 5 g/l and sterilized by passage through a 0.22 mm filter. This stock solution was added (1–10 parts medium) to each well of culture plate, and the plate was incubated at 37 °C for 2 h. DMSO was added to all wells and mixed thoroughly to dissolve the dark blue crystals. After a few minutes at room temperature, to ensure that all the crystals were dissolved, the plates were read on a microplate reader at a wavelength of 570 nm.

2.4. Alkaline phosphatase activity

After the cells were cultured at a density of 10^6 cells into culture dish for 7 days, the medium was replaced with phenol red-free α-MEM containing 5% CD-FBS. Then, the cells were cultured with glabridin in the presence of 10 mM β-glycerophosphate (β-GP); β-GP was added to initiate in vitro mineralization [15]. After 3 days, the medium was removed and the cell monolayer was gently washed twice with PBS. The cells were lysed with 0.2% Triton X-100 and the lysate was centrifuged at $14{,}000 \times g$ for 5 min. The clear supernatant was used for the measurement of ALP activity and protein concentration. ALP activity and protein concentration were determined using an ALP activity assay kit (Wako) and a BCA-protein assay kit (Pierce, Rockford, IL), respectively.

2.5. Collagen contents

After the cells were cultured at a density of 10^6 cells into culture dish for 7 days, the medium was replaced with α-MEM containing 5% CD-FBS. Then, the cells were cultured with glabridin in the presence of 10 mM β-GP for 3 days and cellular collagen content was measured using Sircol Collagen Assay kit (Biocolor Ltd., Northern Ireland). This assay is a quantitative dye-binding method designed for the analysis of collagens extracted from mammalian tissues and cells during in vitro culture. The dye reagent binds specifically to the $[\text{Gly-X-Y}]_n$ helical structure found in mammalian collagens (types I–V).

2.6. Measurement of osteocalcin

After the cells were cultured at a density of 10^6 cells into culture dish for 7 days, the medium was replaced with α-MEM containing 5% CD-FBS. Then, the cells were cultured with glabridin in the presence of 10 mM β-GP for 4 days and osteocalcin content in culture medium was measured using sandwich ELISA assay kit (Biomedical Technologies Inc. USA). Two mouse osteocalcin antibodies are employed, each directed toward an end of the (C- or N-terminal) osteocalcin molecule. The N-terminal antibody is bound to the well, which binds the mouse osteocalcin standard or sample. The biotin-labeled C-terminal mouse osteocalcin antibody completes the sandwich. This sandwich ELISA kit is specific for intact mouse osteocalcin only. Both carboxylated and decarboxylated mouse osteocalcin are recognized.

2.7. Quantitation of apoptosis in MC3T3-E1 cells

TiterTACS kit (R&D System Inc., Minneapolis, MN, USA) was employed to assess apoptosis in osteoblastic cells. This kit provides quantification of apoptosis in cultured cells without direct counting of labeled cells, and detects DNA fragmentation in cells grown as a monolayer. MC3T3-E1 cells were incubated with medium containing 10% FBS for 24 h. Following washing with PBS twice, the cell medium was changed to serum-free medium containing 0.3% BSA (basal), 0.3% BSA and 10^{-10} M TNF-α (control), or 0.3% BSA, 10^{-10} M TNF-α and glabridin. After the incubation for 48 h, cells were harvested, fixed and labeled accord-

ing to the TiterTACS protocol prior to colorimetric analysis.

2.8. PGE_2 immunoassay

After cells were treated with glabridin and 10^{-10} M TNF-α for 48 h, PGE_2 content in the medium was measured with an enzyme immunoassay system (R&D System Inc., Minneapolis, MN, USA) according to the manufacturer's recommendation. This assay is based on the competitive binding technique in which PGE_2 present in a sample competes with a fixed amount of alkaline phosphatase-labeled PGE_2 for sites on a mouse monoclonal antibody. During the incubation, the mouse monoclonal antibody becomes bound to the goat anti-mouse antibody coated onto the microplate. Following a wash to remove excess conjugate and unbound sample, a substrate solution is added to the wells to determine the bound enzyme activity. Immediately following color development, the absorbance is read at 405 nm. The intensity of the color is inversely proportional to the concentration of PGE_2 in the sample.

2.9. Determination of nitrite production

After cells were treated with glabridin and 10^{-10} M TNF-α for 48 h, nitrite production, an indicator of NO synthesis, was measured in the culture supernatant of osteoblasts, as described previously by Kleinerman et al. [16]. Briefly, after mixing 100 μl of Griess reagent (1% sulfanilamide and 0.1% naphthylethylenediamide in 5% phosphoric acid) with 100 μl of culture supernatant, optical density at 540 nm was measured by using a microplate reader. Nitrite concentrations were calculated from the standard curve of sodium nitrite prepared in culture medium.

2.10. Statistics

The results are expressed as mean ± S.E.M. ($n = 6$). Statistical analysis was performed using one-way ANOVA ($P < 0.05$). The analysis was performed using SAS statistical software.

3. Results

3.1. Effect of glabridin on the growth of MC3T3-E1 cells

MC3T3-E1 cells were incubated with glabridin and cell growth was measured. MC3T3-E1 cell growth was promoted by stimulation with glabridin up to approximately two-fold at 10 μM (Fig. 1). Based on this preliminary observation, we evaluated the differentiation-inducing activities of glabridin on MC3T3-E1 cells by assessing for intracellular ALP activity, collagen synthesis and osteocalcin secretion.

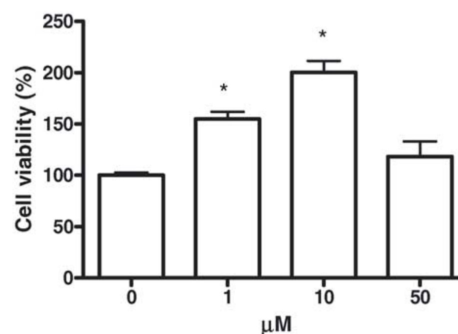

Fig. 1. Effect of glabridin on the growth of MC3T3-E1 cells. Data shown are mean ± S.E.M., expressed as a percentage of control. The control value for MTT assay was 0.247 ± 0.004 OD. $^{*}P < 0.05$ vs. control.

3.2. Effect of glabridin on ALP activity in MC3T3-E1 cells

ALP activity was measured to study the effect of glabridin on the osteoblastic differentiation in MC3T3-E1 cells (Fig. 2). The cultured cells in the presence of glabridin (1–10 μM) caused a significant increase in the ALP activity of osteoblastic cells. However, there was no effect at concentration of 50 μM (Fig. 2A). The effect of glabridin (10 μM) in increasing ALP activity was not seen in the

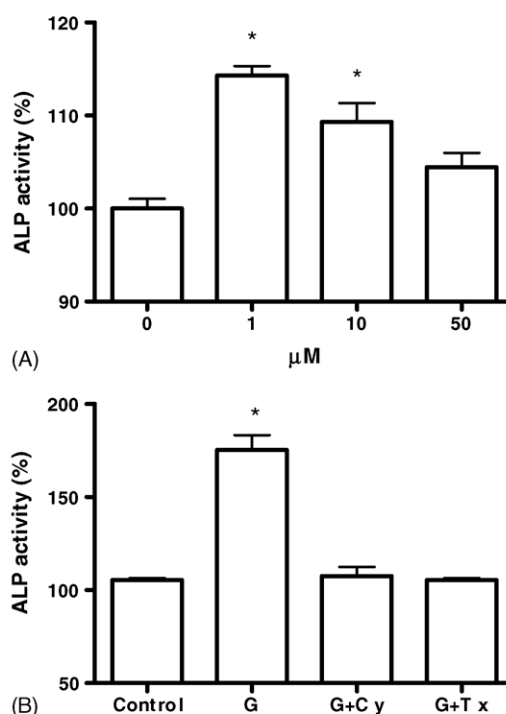

Fig. 2. Effect of glabridin on the alkaline phosphatase activity of MC3T3-E1 cells. MC3T3-E1 cells were cultured in the presence or absence of glabridin (A) and in combination with 10 μM glabridin (G) and 10^{-6} M cycloheximide (Cy) or 10^{-6} M tamoxifen (Tx) (B). Data shown are mean ± S.E.M., expressed as a percentage of control. The control value for ALP activity was 1.880.09 U/mg protein. $^{*}P < 0.05$ vs. control.

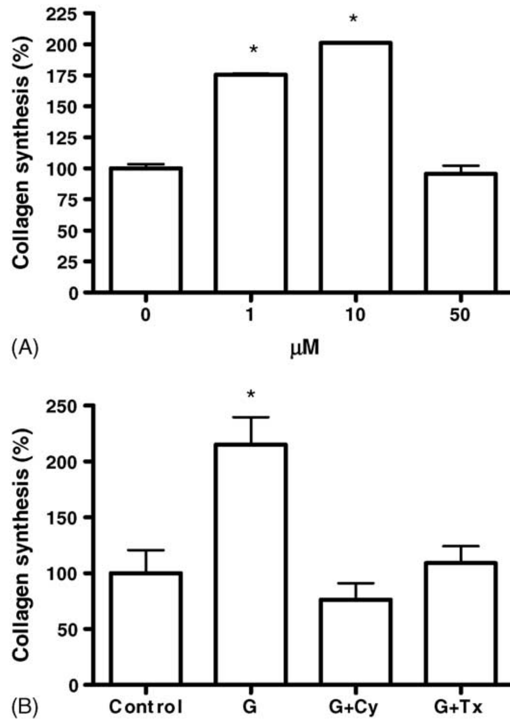

Fig. 3. Effect of glabridin on the collagen synthesis of MC3T3-E1 cells. MC3T3-E1 cells were cultured in the presence or absence of glabridin (A) and in combination with 10 μM glabridin (G) and 10^{-6} M cycloheximide (Cy) or 10^{-6} M tamoxifen (Tx) (B). Data shown are mean ± S.E.M., expressed as a percentage of control. The control value for collagen content was 2.83 ± 0.43 μg/10^7 cells. $^*P < 0.05$ vs. control.

presence of cycloheximide (10^{-6} M) or tamoxifen (10^{-6} M) (Fig. 2B).

3.3. Effect of glabridin on collagen synthesis in MC3T3-E1 cells

The effect of glabridin on collagen synthesis in osteoblastic MC3T3-E1 cells is shown in Fig. 3. The collagen synthesis of MC3T3-E1 cells was significantly increased by the addition of 1–10 μM glabridin (Fig. 3A). The glabridin (10 μM)-induced increase in collagen synthesis was clearly eliminated by the presence of cycloheximide (10^{-6} M) or tamoxifen (10^{-6} M) (Fig. 3B).

3.4. Effect of glabridin on osteocalcin secretion in MC3T3-E1 cells

The MC3T3-E1 cells were treated with various concentrations of glabridin (1–50 μM) and the content of osteocalcin in medium was measured (Fig. 4). The increase in the osteocalcin content was significant at a glabridin concentration of 1–50 μM in MC3T3-E1 cell culture.

3.5. Effect of glabridin for osteoblastic MC3T3-E1 cell apoptosis

Apoptosis is known to be induced by culture with TNF-α. We tested the protective effects of glabridin on induction of MC3T3-E1 cell apoptosis by TNF-α. 10^{-10} M TNF-α treatment showed marked inducing effect on the MC3T3-E1 cell apoptosis, while the apoptotic effect of TNF-α was blocked by addition of 1–10 μM glabridin (Fig. 5).

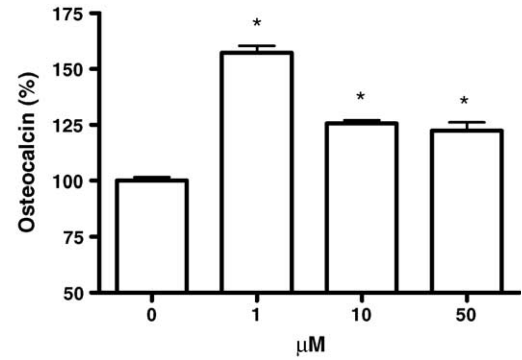

Fig. 4. Effect of glabridin on the osteocalcin secretion of MC3T3-E1 cells. Data shown are mean ± S.E.M., expressed as a percentage of control. The control value for osteocalcin content was 0.11 ± 0.002 ng/10^6 cells. $^*P < 0.05$ vs. control.

3.6. Effect of glabridin on PGE_2 and NO production in MC3T3-E1 cells

PGE_2 is an endogenous promoter of osteoclast formation. As shown in Fig. 6A, in 50 μM glabridin-treated osteoblasts with 10^{-10} M TNF-α, the release of PGE_2 was significantly decreased. Then, the effect of glabridin on the production of NO in osteoblasts was investigated (Fig. 6B). When 10^{-10} M TNF-α was added to cells, the production of NO increased significantly. However, TNF-α-induced NO productions were significantly inhibited by treatment of glabridin (1–50 μM).

4. Discussion

Glabridin is recently known to exhibit variable degrees of estrogen receptor agonism. Tamir et al. [17] reported

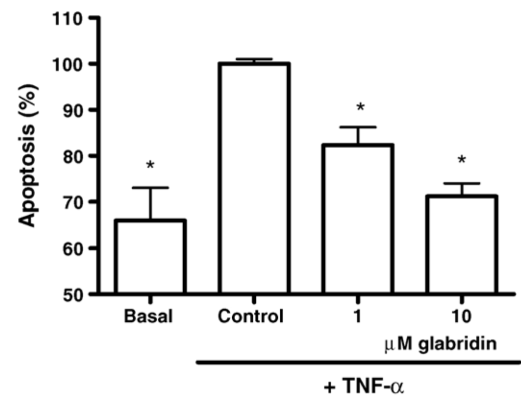

Fig. 5. Effect of glabridin on MC3T3-E1 cell apoptosis induced by TNF-α. Cells were cultured with glabridin in the presence of 10^{-10} M TNF-α. Apoptosis was assessed by cell death ELISA. Data shown are mean ± S.E.M., expressed as a percentage of control. The control value for apoptosis assay was 0.27 ± 0.01 OD. $^*P < 0.05$ vs. control.

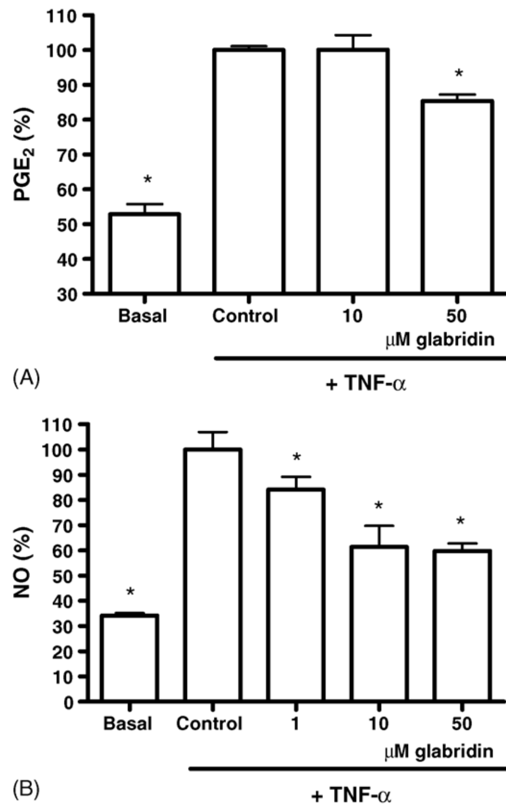

Fig. 6. Effect of glabridin on the TNF-α-induced PGE_2 and NO production of MC3T3-E1 cells. MC3T3-E1 cells were cultured with vehicle or glabridin in the presence of 10^{-10} M TNF-α for 48 h. PGE_2 (A) and nitrite (B) concentration were measured in the conditioned medium. Data shown are mean ± S.E.M., expressed as a percentage of control. Control values of PGE_2 and NO production were 91.25 ± 1.02 pg/ml and 47.06 ± 3.27 mM, respectively/10^5 cells. $^*P < 0.05$ vs. control.

that glabridin is a phytoestrogen, binding to the human estrogen receptor and stimulating creatine kinase activity (an estrogen receptor response marker) in rat uterus, epiphyseal cartilage, diaphyseal bone, aorta and left ventricle of the heart.

In the present study, we examined cell growth, ALP activity, collagen synthesis and osteocalcin secretion to investigate the effects of glabridin on the differentiation of osteoblast-like cells. This study demonstrates that glabridin (1–10 μM) can increase cell growth, ALP activity, collagen synthesis and osteocalcin secretion in osteoblastic MC3T3-E1 cells in vitro. The result supports the stimulatory nature of glabridin toward the function of osteoblastic cells at the low concentrations. In the present study, the effects of glabridin in increasing ALP activity and collagen synthesis in osteoblastic MC3T3-E1 cells were blocked completely by the presence of cycloheximide, an inhibitor of protein synthesis. The anabolic effect of glabridin may be based partly on a newly synthesized protein component. Moreover, the effects of glabridin in elevating cell survival, ALP activity and collagen synthesis in osteoblastic cells were blocked completely by the anti-estrogen tamoxifen. This suggests that the differentiation-promoting effect of glabridin, like that of other phytoestrogens, is estrogen receptor-mediated [18]. These results not only confirm that glabridin acts through the estrogen receptor but also suggest that it has the potential to mimic the beneficial activities of estrogen in bone. Previous reports on the involvement of the two hydroxyl groups of estradiol in binding to the human ER demonstrated that both hydroxyl groups 3 and 17β are required for binding [19]. Tamir et al. [17] indicated that hydroxyl 4′ of glabridin may have the same role as hydroxyl 3 of estradiol. Glabridin lacks the additional hydroxyl group of estradiol at position 17β, but it has an ether oxygen on a parallel position, which could contribute a weaker hydrogen bond to histidine 524 at the ligand-binding domain. This suggests that glabridin may serve as natural estrogen agonists in preventing the symptoms and diseases associated with estrogen deficiency.

Programmed cell death (PCD), or apoptosis, is an important determinant of the life span of cells in regenerating tissues. Regulation of apoptosis is considered an important mechanism for controlling the number of cells available. Failure in the control of programmed cell death may become particularly relevant in pathological conditions, such as degenerative diseases, where apoptosis has been identified as a key factor in disease progression or remission [20]. It was recently shown that osteoblastic cell lines undergo apoptosis in vitro [21] and that estrogen interferes with the apoptotic program of diverse cell systems [22]. In the present study, osteoblastic MC3T3-E1 cells underwent apoptosis following addition of 10^{-10} M TNF-α. Glabridin inhibited the apoptosis of osteoblastic cells induced by TNF-α. This in vitro evidence suggests that glabridin might exert at least part of anti-apoptotic influence on the bone cells. On the other hand, glabridin has been known to have cytotoxic activities and has been identified as an apoptosis inducer of cancer cells. However, the mechanisms through which glabridin may exert the multidirectional action have yet to be clarified.

It is known that some bone-resorbing agents like TNF-α act on osteoblasts and stimulate PGE_2 release from osteoblasts [13]. The released PGE_2 acts on stroma cells and enhances factors that support the differentiation from stem cells to osteoclasts. Furthermore, it is known that the presence of PGE_2 caused a significant decrease in bone ALP activity and a corresponding increase in bone acid phosphatase activity. In the present study, glabridin inhibited PGE_2 production induced by TNF-α in osteoblasts. This result suggests that the inhibitory effect of glabridin on PGE_2 production in osteoblastic cells may lead to the inhibition of differentiation from stem cells to osteoclasts and the stimulation of mineralization.

Nitric oxide is produced by bone cells and organ cultures in response to diverse stimuli, such as proinflammatory cytokines [23]. NO has been shown to modulate osteoclast recruitment and activity [24] and is also thought to play a role in osteoblast function. Cytokine-induced NO production has been shown to inhibit osteoblast growth [25] and to stimulate osteoblast apoptosis [26], probably via oxidative

damage. We showed that glabridin inhibited the TNF-α-induced nitrite production in osteoblastic MC3T3-E1 cells. The result suggests that glabridin may have an important role in the regulation of localized bone destruction associated with inflammatory bone diseases, such as rheumatoid arthritis by inhibition of NO production in osteoblasts. NO reacts with superoxide at a high rate [27] to form peroxynitrite, which is far more reactive and damaging than its precursors. These downstream products of superoxide, including hydrogen peroxide and peroxynitrite, are potent oxidants, which induced oxidative injury of cells and resulted in apoptosis. Aviram [28] reported that licorice-derive glabridin can protect low-density lipoprotein (LDL) against cell-mediated oxidation. Accordingly, glabridin may act as anti-oxidant that can scarvenge the endogenous reactive oxygen species (ROS) and could protect cells from ROS-induced apoptosis effectively.

In conclusion, the estrogenic plant product, glabridin, has a direct stimulatory effect on bone formation in cultured MC3T3-E1 osteoblast cells in vitro. Therefore, glabridin may be a useful tool in the prevention of osteoporosis.

Acknowledgement

This work was supported by the Korea Research Foundation Grant funded by Korean Government (MOEHRD) (R08-2004-000-10006-0).

References

[1] Fukai T, Sakagami H, Toguchi M, Takayama F, Iwakura I, Atsumi T. Cytotoxic activity of low molecular weight polyphenols against human oral tumor cell lines. Anticancer Res 2000;20:2525–36.

[2] Fukai T, Marumo A, Kaitou K, Kanda T, Terada S, Nomura T. Anti-*Helicobacter pylori* flavonoids from licorice extract. Life Sci 2002;71:1449–63.

[3] Tamir S, Eizenberg M, Somjen D, Izrael S, Vaya J. Estrogen-like activity of glabrene and other constituents isolated from licorice root. J Steroid Biochem Mol Biol 2001;78:291–8.

[4] Yokota T, Nishio H, Kubota Y, Mizoguchi M. The inhibitory effect of glabridin from licorice extracts on melanogenesis and inflammation. Pigment Cell Res 1998;11:355–61.

[5] Nerya O, Vaya J, Musa R, Izrael S, Ben-Arie R, Tamir S. Glabrene and isoliquiritigenin as tyrosinase inhibitors from licorice roots. J Agric Food Chem 2003;51:1201–7.

[6] Fuhrman B, Volkova N, Rosenblat M, Aviram M. Lycopene synergistically inhibits LDL oxidation in combination with Vitamin E, glabridin, rosmarinic acid, carnosic acid, or garlic. Antioxid Redox Signal 2000;2:491–506.

[7] Kent UM, Aviram M, Rosenblat M, Hollenberg PF. The licorice root derived isoflavan glabridin inhibits the activities of human cytochrome P450S 3A4, 2B6, and 2C9. Drug Metab Dispos 2002;30:709–15.

[8] Haraguchi H, Yoshida N, Ishikawa H, Tamura Y, Mizutani K, Kinoshita T. Protection of mitochondrial functions against oxidative stresses by isoflavans from *Glycyrrhiza glabra*. J Pharm Pharmacol 2000;52:219–23.

[9] Somjen D, Knoll E, Vaya J, Stern N, Tamir S. Estrogen-like activity of licorice root constituents: glabridin and glabrene, in vascular tissues in vitro and in vivo. J Steroid Biochem Mol Biol 2004;91(3): 147–55.

[10] Mohan S, Kutilek S, Zhang C, Shen HG, Kodama Y, Srivastava AK, et al. Comparison of bone formation responses to parathyroid hormone (1–34), (1–31), and (2–34) in mice. Bone 2000;27:471–8.

[11] Duncan R, Misler S. Voltage-activated and stretch-activated Ba2+ conducting channels in an osteoblast-like cell line (UMR 106). FEBS Lett 1989;251:17–21.

[12] Shinozaki K, Ishikawa H, Tsutsui T. Effect of bisphenol-A and 17β-estradiol on cell proliferation and alkaline phosphatase activity in mouse MC3T3-E1 cells. Shigaku 2000;87:641–9.

[13] Mizutani K, Ikeda K, Kawai Y, Yamori Y. Resveratrol stimulates the proliferation and differentiation of osteoblastic MC3T3-E1 cells. Biochem Biophys Res Commun 1998;253:859–63.

[14] Sugimoto E, Yamaguchi M. Stimulatory effect of daidzein in osteoblastic MC3T3-E1 cells. Biochem Pharmacol 2000;59:471–5.

[15] Kanno S, Anuradha CD, Hirano S. Localization of zinc after in vitro mineralization in osteoblastic cells. Biol Trace Elem Res 2001;83:39–47.

[16] Kleinerman ES, Lachman LB, Knowles RD, Snyderman R, Cianciolo GJ. A synthetic peptide homologous to the envelope proteins of retroviruses inhibits monocyte-mediated killing by inactivating interleukin 1. J immunol 1987;139:2329–37.

[17] Tamir S, Eizenberg M, Somjen D, Stern N, Shelach R, Kaye A, et al. Estrogenic and antiproliferative properties of glabridin from licorice in human breast cancer cells. Cancer Res 2000;60(20):5704–9.

[18] Shao ZM, Wu J, Shen ZZ, Barsky SH. Genistein exerts multiple suppressive effects on human breast carcinoma cells. Cancer Res 1998;58:4851–7.

[19] Wiese TE, Polin LA, Palomino E, Brooks SC. Induction of the estrogen specific mitogenic response of MCF-7 cells by selected analogues of estradiol-7β: a 3D QSAR study. J Med Chem 1997;40:3659–69.

[20] Deshpande R, Khalili H, Pergolizzi RG, Michael SD, Chang MY. Estradiol down-regulates LPS-induced cytokine production and NF-kB activation in murine macrophages. Am J Reprod Immunol 1997;38:46–54.

[21] Hill PA, Tumber A, Meikle MC. Multiple extracellular signals promote osteoblast survival and apoptosis. Endocrinology 1997;138: 3849–58.

[22] Centrella M, McCarthy TL, Kusmik WF, Canalis E. Isoform-specific regulation of platelet-derived growth factor activity and binding in osteoblast-enriched cultures from fetal rat bone. J Clin Invest 1992;89:1076–84.

[23] Lowik CWGM, Nibbering PH, Van der Ruit M, Papapoulos SE. Inducible production of nitric oxide in osteoblast like cells and in fetal bone explants is associated with suppression of osteoclastic bone resorption. J Clin Invest 1994;93:1465–72.

[24] Evans DM, Ralston SH. Nitric oxide and bone. J Bone Miner Res 1996;11:300–5.

[25] Damoulis PD, Hauschka PV. Nitric oxide acts in conjugation with proinflammatory cytokines to promote cell death in osteoblasts. J Bone Miner Res 1997;12:412–22.

[26] Hukkanen M, Hughes FJ, Lee D. Cytokine stimulated expression of inducible nitric oxide synthase by mouse, rat and human osteoblast-like cells and its functional role in osteoblast metabolic activity. Endocrinology 1995;136:5445–53.

[27] Huie RE, Padmaja S. Reaction of NO with O_2. Free Radic Res Commun 1993;18:195–9.

[28] Aviram M. Review of human studies on oxidative damage and antioxidant protection related to cardiovascular diseases. Free Radic Res 2000;33:S85–97.