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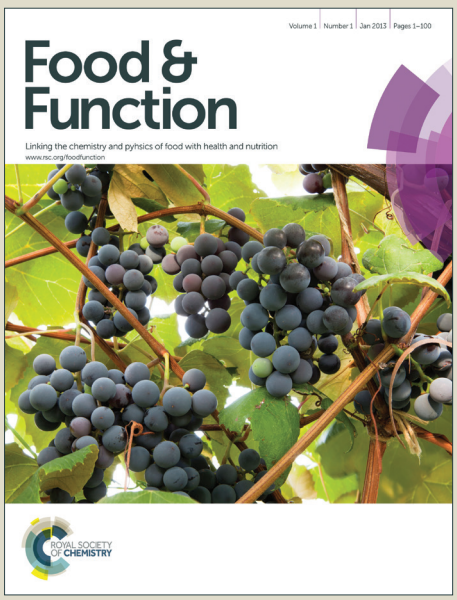

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Protective effect of liquiritigenin against methylglyoxal cytotoxicity in osteoblastic MC3T3-E1 cells

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Abstract

Methylglyoxal (MG), a reactive dicarbonyl compound, is a metabolic byproduct of glycolysis and elevated MG levels are related to contribute to diabetic complications. Glycation reactions of MG with amino acids can induce oxidative stress, leading to subsequent cytotoxicity. In the present study, the effect of liquiritigenin on MG-induced cytotoxicity was investigated using osteoblastic MC3T3-E1 cells. Pretreatment of MC3T3-E1 cells with liquiritigenin prevented the MG-induced cell death and production of protein adduct, intracellular reactive oxygen species, mitochondrial superoxide, cardiolipin peroxidation, and TNF-α in osteoblastic MC3T3-E1 cells. In addition, liquiritigenin increased the activity of glyoxalase I inhibited by MG. These findings suggest that liquiritigenin provide a protective action against MG-induced cell damage by reducing oxidative stress and by increasing MG detoxification. Pretreatment with liquiritigenin prior to MG exposure reduced MG-induced mitochondrial dysfunction by preventing mitochondrial membrane potential dissipation and adenosine triphosphate loss. Additionally, the nitric oxide and PGC-1α levels were significantly increased by liquiritigenin, suggesting that liquiritigenin may induce mitochondrial biogenesis. Our findings indicate that liquiritigenin might exert its therapeutic effects via enhancement of glyoxalase I activity and mitochondrial function, and anti-oxidant and anti-inflammatory activities. Taken together, liquiritigenin has potential as a preventive agent against the development of diabetic osteopathy related to MG-induced oxidative stress in diabetes.

Keywords: liquiritigenin, methylglyoxal, osteoblasts, cytotoxicity, mitochondrial function

1 Introduction

Methylglyoxal (MG), a highly reactive dicarbonyl species of α-oxoaldehyde produced mainly from cellular glycolytic intermediates, is a potent glycating agent. MG–protein adducts are generated by irreversible nonenzymatic modification of free amino groups of proteins, and carbonyl stress results from an imbalance between reactive carbonyl species levels, efficiency of scavenger and detoxification pathways, and accumulation of MG–protein adducts.[1] Moreover, the crosslinking reaction during MG amino acid glycation has been shown to yield the superoxide radical anion.[2] Consequently, protein damage by MG can be mediated not only via carbonyl stress through formation of protein carbonyls, but possibly also via oxidative stress through enhanced reactive oxygen species (ROS) formation.[2] Increased MG-derived glycation in diabetic patients seems to correlate with diabetic complications.[3] Both osteoporosis and osteoarthritis are classical age-related disorders. Osteoporosis is characterized by loss of bone mass, decreased bone density, increased bone fragility and resulting in fractures. Although it has been known that the estrogen deficiency is a most important factor of osteoporosis pathogenesis, emerging clinical and molecular evidences suggest that aging-associated immunosenescence and inflammation might have pivotal role in osteoporosis.[4] Highly reactive dicarbonyls such as MG attack the lysine, arginine and cysteine residues of long-lived proteins, such as collagens, to form irreversible advanced glycation end-products (AGEs).[5] Collagen cross-links resulted from accumulation of AGEs during aging is considered as another pathological mechanism of osteoporosis.[6] In addition, increased ROS from aging process also influence the generation and survival of osteoblasts.

MG-induced formation of protein adducts (AGEs) with cell surface or intracellular targets has been shown to initiate mitochondrial dysfunction[7] resulting in more production of ROS and further damage. Abnormal cellular accumulation of the MG occurs on exposure to high glucose concentrations, inflammation, cell aging and senescence. Increased MG modification of proteins is a likely consequence of oxidative stress and increased MG modification of mitochondrial proteins may also induce oxidative stress.[8] Glyoxalase I suppresses the formation of MG-derived AGEs. Glyoxalase I is part of the glyoxalase system that is present in the cytosol of all cells. The major function of glyoxalase I is the detoxification of α-ketoaldehydes, especially the potent and cytotoxic MG.[9] In glyoxalase I transgenic rats, overexpression of glyoxalase I against streptozotocin-induced diabetes is evidenced by decreased plasma AGEs and oxidative stress.[10] Recent studies have provided evidence that peroxisome proliferator-activated receptor γ coactivator 1α (PGC-1α) has a primary role in mitochondrial

biogenesis.[11] PGC-1α is a powerful coactivator of nuclear respiratory factor (NRF), and it has been postulated that increased transactivation of NRF-1 regulated genes could be the major mechanism by which PGC-1α induces an increase in mitochondrial biogenesis.[12] Previous studies also suggest that expression of mitochondrial biogenesis factors may be directly regulated by bioavailability of nitric oxide (NO), which is a short-lived radical that acts via a second messenger (cGMP) with many diverse actions in the nervous, vascular, and immune systems.[13,14] Several therapeutic methods that involve the scavenging of MG have been tested to prevent protein glycation and AGEs formation.[15] Aminoguanidine is an antiglycation agent that was shown to retard the deposition of AGEs. In addition, many antioxidant products, for example, trolox, triterpenesare, and soy isoflavones, were found to be effective in trapping MG and alleviating protein glycation.[16,17]

Flavonoids are part of a family of naturally occurring polyphenolic compounds and are one of the most prevalent classes of compounds in vegetables, nuts, fruits, and beverages such as coffee, tea, and red wine as well as medicinal herbs.[18] Chemically, flavonoids contain a common phenylchromanone structure (C_6–C_3–C_6) with at least one hydroxyl group substituent or a hydroxyl derivative such as a methoxy group. Liquiritigenin (7,4′-dihydroxyflavone), the primary active component of *Glycyrrhizae radix*, has a wide range of pharmacological activities. Liquritigenin is a metabolite of liquiritin and is known to be actually absorbed into the body.[19] The chemical structure of liquritigenin is shown in the Fig. 1. Liquiritigenin was metabolized to six metabolites (7,3′,4′-trihydroxyflavone, a hydroxyl quinine metabolite, two A-ring dihydroxymetabolites, 7,4′-dihydroxyflavone, and 7-hydroxychromone) in rat liver microsomes.[20] Recently, one oxidative metabolite of liquiritigenin (7,4′-dihydroxyflavone) was detected in human liver microsomes and identified as CYP3A4-mediated reaction.[21] Kim *et al*. demonstrated that liquiritigenin exerts cytoprotective effects against heavy metal-induced toxicity in cultured hepatocytes[22] and also protects against liver toxicity in rats.[23] Moreover, studies have shown that liquiritigenin is a selective agonist at the estrogen receptor-β[24] and that targeting this receptor may be associated with anti-inflammatory effects.[25] In our previous study, liquiritigenin inhibited the induction of bone resorbing cytokines and oxidative stress induced by antimycin A in MC3T3-E1 osteoblast cells.[26] In the present study, we investigated the protective mechanisms of liquiritigenin against MG-induced cytotoxicity was assessed in osteoblastic MC3T3-E1 cells.

2 Materials and methods

2.1 Materials

Liquiritigenin isolated from *G. radix* (> 98% purity by HPLC) was purchased from ChromaDex Inc. (Irvine, CA , USA). α-Modified minimal essential medium (α-MEM) and fetal bovine serum (FBS) were purchased from Gibco BRL (Grand Island, NY). Other reagents were of the highest commercial grade available and purchased from Sigma Chemical (St. Louis, MO, USA).

2.2 Cell culture

Osteoblastic MC3T3-E1 Subclone 4 line was obtained from the ATCC (USA). MC3T3-E1 cells were cultured at 37 °C in 5% CO_2 atmosphere in α-modified minimal essential medium (α-MEM; GIBCO). This cell line was isolated from the cloned but phenotypically heterogeneous MC3T3-E1 cell line and selected for high osteoblast differentiation and mineralization after growth in medium.[27] Unless otherwise specified, the medium contained 10% fetal bovine serum (FBS), 100 U/ml penicillin and 100 μg/ml streptomycin. Cells were seeded in well plates in α-MEM, which contains 5 mM β-glycerophosphate and 50 μg/ml ascorbic acid, supplemented with 10% FBS. After 48 h of seeding, cells were pre-incubated for 1 h with α-MEM containing 0.1% FBS and samples before treatment with MG for 48 h.

2.3 Cell viability

Surviving cells was counted by the MTT method. MTT 20 μl in phosphate buffered salt solution, pH 7.4 (5 mg/ml), was added to each well, and the plates were incubated for an additional 2 h. After the removal of solutions in the well, dimethyl sulfoxide was added to dissolve formazan products, and the plates were shaken for 5 min. The absorbance of each well was recorded on a microplate spectrophotometer at 570 nm.

2.4 Quantification of MG-modified proteins (adducts)

The quantification of MG-modified proteins (MG-protein adducts) was determined with an enzyme immunoassay kit purchased from Cell BioLabs Inc. (San Diego, CA). The MG protein adducts present in the sample or standard are probed with an anti-MG specific monoclonal antibody, followed by an Horseradish Peroxidase (HRP) conjugated secondary antibody. The quantity of MG adduct in protein samples was

determined by comparing its absorbance with that of a known MG-BSA standard curve.

2.5 Measurement of glyoxalase I activity

The cells were lysed and homogenized in PBS. Cell homogenates were centrifuged at 13,000 × *g* for 15 min at 4 °C and the supernatant was used for assay and protein content measurement. Glyoxalase I activity was measured using a modification from a previously published method.[28] To measure glyoxalase I activity, 50 µl of sample was loaded onto a UV microplate and 200 µl of reaction mix was added. Reaction mix consisted of 60 mM sodium phosphate buffer, pH 6.6, containing 4 mM GSH and 4 mM MG, and was preincubated for 10 min at 37°C. *S*-Lactoylglutathione synthesis was followed by measuring the absorbance at 240 nm for 5 min at 25°C.

2.6 Measurement of intracellular reactive oxygen species

Formation of intracellular ROS was measured using 2′,7′-dichlorodihydrofluorescin diacetate (H_2DCFDA).[29] Viable cells can deacetylate H_2DCFDA to the non-fluorescent derivative 2′,7′-dichlorofluorescin (DCF), which reacts with oxygen species, and can be measured to provide an index of intracellular oxidant production. In order to load the cells with the fluorescence dye, the cells were incubated with H_2DCFDA in Hank's solution at the final concentration of 10 µM for 45 min at 37 °C in the dark. Following washing with DPBS, ROS levels were determined by measuring the fluorescent intensity at excitation wavelength 485 nm and emission wavelength 530 nm.

2.7 Measurement of cardiolipin peroxidation

10-N-nonyl-Acridin Orange (NAO, Molecular Probes, Inc.), which binds to mitochondria cardiolipin, was used for the determination of cardiolipin. Decreases in the fluorescence of NAO in cells have been reported to reflect the peroxidation of intracellular cardiolipin because the fluorochrome loses its affinity for peroxidised cardiolipin. Cells were labeled with 5 µM NAO for 20 min. After washing, fluorescence was measured at excitation wavelength 485 nm and emission wavelength 530 nm using a Fluorescence microplate reader (Molecular Devices).

2.8 Determination of mitochondrial membrane potential

The JC-1 Mitochondrial Membrane Potential Assay Kit (Cayman Chemical Co., USA) was used to demonstrate

the changes in the mitochondrial membrane potential (MMP) in cells. JC-1 is a lipophilic and cationic dye that permeates plasma and mitochondrial membranes. The dye fluoresces red when it aggregates in healthy mitochondria with a high membrane potential, whereas it appears in a monomeric form and fluoresces green in mitochondria with a diminished membrane potential. Cells were incubated with the MMP-sensitive fluorescent dye JC-1 for 20 min at 37°C and washed twice in PBS, and then red (excitation, 550 nm; emission, 600 nm) and green (excitation, 485 nm; emission, 535 nm) fluorescence were measured using a fluorescence microplate reader (Molecular Devices). Mitochondrial depolarization (i.e., loss of MMP) manifests as a decrease in the red/green fluorescence ratio.

2.9 ATP measurement

The cells were lysed and homogenized in PBS. Cell homogenates were centrifuged at 13,000 × *g* for 15 min at 4 °C and the supernatant was used for assay and protein content measurement. The ATP concentra tion was determined by luciferase reaction using an EnzyLight™ ATP Assay Kit (BioAssay Systems, USA). This ATP assay kit provides a rapid method of measuring intracellular ATP. Protein concentrations were determined using the Bio–Rad protein assay reagent.

2.10 Measurement of nitric oxide (NO) generation

DAF-FM diacetate is a sensitive fluorescent indicator for the detection of NO. It is a cell-permeable derivative of DAF-FM. Upon entry into the cell, DAF-FM diacetate was transformed into the less cell-permeable DAF-FM by cellular esterases, thus preventing signal loss due to diffusion of the molecule from the cell. In the presence of oxygen, DAF-FM reacts with NO to yield the highly fluorescent triazolofluorescein. For NO detection,[30] cells were loaded with 5 μM DAF-FM diacetate (Invitrogen Corporation, Burlington, ON, Canada) for 2 h at 37°C. After removal of the excess probe, DAF-fluorescence intensity, which reflects the intracellular NO level, was measured with excitation at 495 nm and emission at 515 nm.

2.11 Measurement of PGC-1α levels

The cells were lysed and homogenized in PBS. Cell homogenates were centrifuged at 13,000 × *g* for 15 min at 4 °C and the supernatant was used for ELISA and protein content measurement. PGC-1α was measured using Mouse Peroxisome Proliferator Activated Receptor Gamma Coactivator 1 Alpha (PGC-1α) ELISA kit

(MyBioSource, USA). Protein concentrations were determined using the Bio–Rad protein assay reagent.

2.12 Measurement of TNF-α

TNF-α contents in the medium were measured with an enzyme immunoassay system (R&D system Inc., Minneapolis, MN, USA) according to the manufacturer's recommendation. Protein concentrations were determined using the BioRad protein assay reagent (Bio-Rad Laboratories, USA).

2.13 Statistical analysis

The results are expressed as mean ± SEM. Statistical significance was determined by analysis of variance and subsequently applying the Dunnett's t-test ($P<0.05$).

3 Results

3.1 Effect of liquiritigenin on cell viability of osteoblastic MC3T3-E1 cells

To determine whether liquiritigenin had a protective effect on the MG-induced cytotoxicity, cells were preincubated with liquiritigenin for 1 h and then cultured with 400 μM MG for 48 h. As shown in Fig. 2, MG (400 μM) treatment induced MC3T3-E1 cell death to nearly 50% compared with non-treated control cells, but liquiritigenin (0.01–1 μM) inhibited the MG-induced cytotoxicity. Aminoguanidine (300 μM), a carbonyl scavenger, also inhibited the effect of MG on cell viability.

3.2 Inhibitory effect of liquiritigenin on the formation of MG-induced protein adducts in MC3T3-E1 cells

Because MG is a glycating agent, we investigated whether incubation of MC3T3-E1 cells with liquiritigenin could reduce the formation of MG-induced protein adducts. As shown in Fig. 3, protein adducts accumulated in cells treated with 400 μM MG. However, pretreatment with liquiritigenin (1 μM) or aminoguanidine blocked protein adducts formation in MG-treated cells. The data indicated that liquiritigenin could block glucotoxicity in biological cells or tissues.

3.3 Effect of liquiritigenin on glyoxalase I activity in MG-treated MC3T3-E1 cells

MG is detoxified by the glyoxalase system, in which the enzyme glyoxalase I is the rate-limiting step. Thus, we examined the effect of liquiritigenin on the activity of glyoxalase I in osteoblastic MC3T3-E1 cells. As shown in Fig. 4, we found a significant decrease in the glyoxalase I activity of MC3T3-E1 cells treated with MG (400 μM). However, liquiritigenin (0.01–1 μM) or aminoguanidine increased the glyoxalase I activity inhibited by MG. These data demonstrate that liquiritigenin causes an increase in glyoxalase I activity.

3.4 Inhibitory effect of liquiritigenin on MG-induced oxidative stress in MC3T3-E1 cells

Evaluation of the increase in DCF fluorescence is a currently used method of measuring the production of intracellular ROS, particularly hydrogen peroxide.[31] In this study, we investigated whether ROS production was involved in the mechanism of MG-induced MC3T3-E1 cell death and estimated the inhibitory effect of liquiritigenin on ROS production. As illustrated in Fig. 5A, incubation of MC3T3-E1 cells with 400 μM MG resulted in an increase in ROS. However, a significant reduction in ROS was observed after pretreatment with 0.01–1 μM liquiritigenin or aminoguanidine. To obtain further evidence for oxidative stress within mitochondria, we assessed the oxidation of cardiolipin because this phospholipid exists in association with cytochrome *c* on the outer surface of the inner mitochondrial membrane. Because the fluorescent dye NAO binds to the nonoxidized form, but not to the oxidized form, of cardiolipin,[32] measurements of NAO fluorescence allow us to monitor the oxidation of cardiolipin in mitochondria . The results revealed that treatment with 400 μM MG decreased NAO fluorescence, indicating the induction of cardiolipin peroxidation (Fig. 5B). However, liquiritigenin (0.01–1 μM) or aminoguanidine reduced the cardiolipin peroxidation induced by MG. These data show that liquiritigenin reduces MG-induced ROS generation and oxidative stress within mitochondria.

3.5 Effect of liquiritigenin on mitochondrial functions in MG-treated MC3T3-E1 cells

Mitochondria play an essential role in the transduction of cell death signals, such as the permeability of transition pore opening and the collapse of the mitochondrial membrane potential (MMP). Therefore, the effect of liquiritigenin on MMP and ATP levels in MG-treated MC3T3-E1 cells was investigated. As shown in Figure 5, exposure of the cells to MG (400 μM) induced serious mitochondrial disruption in MC3T3-E1 cells. However, liquiritigenin (0.01–1 μM) or aminoguanidine treatment of MC3T3-E1 cells reduced the disruption of MMP by MG (Fig. 6A). In addition, liquiritigenin (0.1 and 1 μM) or aminoguanidine restored the ATP synthesis inhibited by MG (Fig. 6B). These results show that the cytoprotective effect of liquiritigenin results from mitochondrial

protection.

3.6 Effect of liquiritigenin on mitochondrial biogenesis factors in MG-treated MC3T3-E1 cells

It was reported that dysregulation of mitochondrial biogenesis is related with impaired nitric oxide (NO) bioavailability.[33] Thus, NO generation was evaluated by DAF-FM, a specific probe used to quantify low concentrations of intracellular NO. As shown in Fig. 7A, methylglyoxal (400 μM) decreased the production of NO in MC3T3-E1 cells, which was prevented by pretreatment with liquiritigenin (0.01–1 μM) or aminoguanidine. PGC-1α has been recognized to have a primary role in mitochondrial biogenesis/metabolism.[12] As shown in Fig. 7B, PGC-1α levels were increased by treatment with liquiritigenin (0.01–1 μM) or aminoguanidine. Our results suggest that liquiritigenin may reduce MG-induced dysfunction of mitochondria by enhancing mitochondrial biogenesis factors in osteoblastic MC3T3-E1 cells.

3.7 Effect of liquiritigenin on the production of inflammatory cytokine in MG-treated MC3T3-E1 cells

MG promotes the formation of proinflammatory cytokines in various cell types. Proinflammatory cytokine such as TNF-α has been demonstrated to increase osteoclastic activity. Thus, we also investigated whether liquiritigenin modulates the production of TNF-α in MG treated cells (Fig. 8). When 400 μM MG was added to cells, production of TNF-α increased significantly. However, MG-induced TNF-α production was significantly inhibited by treatment of liquiritigenin at the concentrations of 1 μM. Aminoguanidine (300 μM) also decreased MG-induced cytokine release.

4 Discussion

MG toxicity may be involved in diabetes-associated bone loss. Studies investigating the effects of diabetes on osteoporosis have shown that patients with type I diabetes have high rates of bone resorption and turnover and decreased bone mineral density.[34,35] Chan et al.[36] revealed that MG treatment triggers apoptotic biochemical changes in human osteoblasts. Furthermore, *in vivo* experiments showed that MG treatment could cause bone mineral density loss in an animal assay model, likely via MG-induced cytotoxicity.[36] Spontaneous and nonenzymatic glycation ubiquitously occurs between carbohydrate intermediates and proteins in biological systems, leading to the formation of AGEs. The amount of AGEs increases with age and is closely related to the

pathogenesis of various diseases.[37] Several pharmacological reagents, such as aminoguanidine, tenilsetam, carnosine, metformin, and pyridoxamine, have been investigated for inhibiting the formation of AGEs and the development of diabetic complications by trapping reactive dicarbonyl species.[38] However, all of these pharmaceutical agents have serious side effects. Thus, it is critical to develop effective and safe agents to protect diabetics from complications. Several dietary flavonoids have been shown to inhibit AGE formation through blocking the carbonyl or dicarbonyl groups and thus may prevent diabetes and its complications. For example, curcumin has been shown to inhibit protein glycation in erythrocytes and the formation of AGE as well as cross-linking of collagen in diabetic animals.[39] Lv et al.[38] have demonstrated that dietary flavonoids that have the same A ring structure as EGCG, phloretin, phloridzin, or genistein, such as quercetin, luteolin, and epicatechin, may also efficiently inhibit the formation of AGEs by trapping reactive dicarbonyl species. These results support the notion that AGE inhibitors from natural foodstuffs may be potential therapeutic agents for delaying and preventing diabetic complication. Our finding indicated that pretreatment with liquiritigenin in MG-treated MC3T3-E1 cells decreased the formation of protein adduct, which show that liquiritigenin could block MG-derived AGE formation in MC3T3-E1 cells and that might participate in the inhibitory mechanism of MC3T3-E1 cell damage. Therefore, liquiritigenin might contribute to the prevention of the development of diabetic bone diseases by blocking the MG-mediated intracellular glycation system.

Protein damage increases in periods of increased MG formation and decreased metabolism, such as metabolic stress associated with increased glucose metabolism,[40] inflammatory signalling.[41] and ageing and senescence.[42] Dicarbonyl stress is an imbalance between formation of dicarbonyl metabolites and enzymatic defences against them in favour of the former leading to increased cell damage. Protein damage by MG is suppressed by glyoxalase I which catalyses the GSH-dependent conversion of MG into *S*-D-lactoylglutathione. Further metabolism of *S*-D-lactoylglutathione to D-lactate is catalysed by glyoxalase II which restores GSH consumed in the glyoxalase I-catalysed reaction.[43] Our result indicates that the exogenous application of liquiritigenin increases the tolerance of cells to MG-induced damage by enhancing glyoxalase I activity. Glyoxalase I is ubiquitously distributed in all mammalian cells and plays an important role in the regulation of cell signals related to oxidative stress and AGE formation.[44] Overexpression of glyoxalase I inhibits intracellular AGE formation in bovine endothelial cells and prevents hyperglycemia-induced increases in macromolecular endocytosis.[45] In contrast, glyoxalase I deficiency is associated with increased levels of AGEs.[46] There is evidence of a progressive decline in glyoxalase I expression and activity in aging,[43] and glyoxalase I inducers

may counter this. Glyoxalase I inducers may prove useful in the treatment and prevention of early-stage vascular complications of diabetes. Therefore, dietary bioactive inducers of glyoxalase I such as liquiritigenin may provide resistance in the development of aging-related disorders or healthy aging.

MG-induced oxidative stress significantly contributes to the progression of diabetes and related complications.[47] MG induces the dysfunction of mitochondrial respiration[48] and increases ROS production.[49] Mitochondria are organelles often regarded as the cell's energy powerhouses, because they generate a majority of ATPs required for cell metabolism using specialized electron transport system.[50] Thus, mitochondrial dysfunction could lead to abnormalities in energy metabolism. There are reports showing that MG is toxic to cells by depleting ATP, modulation of mitochondrial membrane potential, induction of apoptosis and ROS-production.[51] Previously, Wu and Yen[52] found that the inhibitory capability of naturally occurring antioxidants against protein glycation was remarkably related to scavenging free radicals derived from glycoxidation. We have demonstrated that liquiritigenin inhibits MG-induced ROS generation and mitochondrial dysfunction. MG-induced oxidative stress, results from altered mitochondrial metabolism, which causes the overproduction of superoxide by the electron transport chain.[53] The increased generation of free radicals accelerates the oxidation of lipids, proteins, nucleic acids, and other molecules, altering the structure and function of mitochondrial cardiolipin. In liquiritigenin-pretreated cells, the level of peroxidized cardiolipin elevated by MG was significantly decreased, paralleled by an increase in markers of mitochondrial function. These results suggest that liquiritigenin may affect oxidative stress via effects on mitochondrial function.

Mitochondrial biogenesis is likely to be involved in the regulation of cell metabolism, redox regulation, and signal transduction. Impairment of mitochondrial biogenesis is frequently observed in diabetes and the metabolic syndrome[54] and is thus likely to contribute to cellular energetic imbalance, oxidative stress, and cell dysfunction in pathological conditions.[55] Mitochondria undergo constant biogenesis controlled primarily by the gene expression and post-translational modification of PGC-1α.[12] In the present study, we found that liquiritigenin increased PGC-1α level in MC3T3-E1 cells. Coactivation of PGC-1α induces nuclear respiratory factors, which promote the expression of most nuclear-encoding mitochondrial proteins, as well as mitochondrial transcription factor A that directly stimulates mitochondrial DNA replication and transcription.[56] Nitric oxide (NO) also plays a critical role in initiating and integrating signaling events underlying mitochondrial biogenesis in various tissues. Inhibition of NO synthesis significantly decreases mitochondrial content in the vasculature.[33] Treatment with NO donors increases mitochondrial mass in various cell types,

including brown adipocytes and 3T3-L1, U937, and HeLa cells.[57,58,13] We demonstrated that liquiritigenin prevents MG-induced inhibition of NO synthesis in MC3T3-E1 cells, suggesting that NO has a beneficial effect on MG-treated cells. Because NO appears to be a key regulator of mitochondrial content,[33] we attribute the liquiritigenin-induced mitochondrial biogenesis to the restoration of NO bioavailability. Pathways that regulate mitochondrial biogenesis have recently emerged as potential therapeutic targets for the amelioration of cell dysfunction and metabolic diseases.[59] Therefore, liquiritigenin may have the potential for new treatment approaches targeting mitochondria in metabolic diseases.

Accumulating evidence suggests that the pathophysiology of diabetes is analogous to chronic inflammatory states. Circulating levels of inflammatory cytokines such as tumor necrosis factor-α (TNF-α) are increased in diabetes.[60] Interestingly, MG administration induces inflammatory response, including increased expression of TNF-α and is associated with the onset of microvascular damage and other diabetes-like complications in rats.[61] Furthermore, MG stimulates the release of cytokines from neutrophils in non-diabetic subjects[62] and MG-formed AGEs increased the production of IL-6 and IL-8 in mesothelial cells.[63] Together with the above discussion that MG has the ability to increase oxidative stress, these indicated that the accumulation of MG was an important source not only for oxidative stress damage but also for inflammatory responses. In the current study, the levels of TNF-α were elevated in the MG-treated cells, while liquiritigenin treatment significantly prevented the elevation of TNF-α levels. Thus, the anti-inflammatory efficacy of liquiritigenin may at least be an important contribution to the improvement of bone impairment. Therefore, we speculate that liquiritigenin alleviates inflammatory reaction associated with diabetic complications.

In conclusion, we showed that liquiritigenin reduces MG-induced oxidative stress and glycation in osteoblastic MC3T3-E1 cells by enhancing glyoxalase I activity and mitochondrial biogenesis. Our study suggests that liquiritigenin might contribute to the prevention of the development of diabetic bone diseases by blocking the MG-mediated glycation.

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Fig. 1 Chemical structure of liquiritigenin

Fig. 2 Effect of liquiritigenin on the cell viability of osteoblasts. Osteoblasts were treated with liquiritigenin (Liq) or aminoguanidine (AG) in the absence (A) or presence (B) of 400 μM methylglyoxal (MG) for 48 h, and then cell viability was assessed by MTT assay. Data were expressed as a percentage of control. $^{\#}P<0.05$, compared with the untreated cells; $^{*}P<0.05$, compared with the cells treated with MG (400 μM) alone.

Fig. 3 MG-induced protein adduct in osteoblastic MC3T3-E1 cells. Osteoblasts were pre-incubated with liquiritigenin (Liq) or aminoguanidine (AG) before treatment with 400 μM methylglyoxal (MG) for 48 h. Data were expressed as a percentage of control. The control values for methylglyoxal-protein adduct was 0.076 ± 0.014 ng/mg. $^{\#}P<0.05$, compared with the untreated cells; $^{*}P<0.05$, compared with the cells treated with MG (400 μM) alone.

Fig. 4 Effect of liquiritigenin on glyoxalase I activity in MG-treated cells. Osteoblasts were pre-incubated with liquiritigenin (Liq) or aminoguanidine (AG) before treatment with 400 μM methylglyoxal (MG) for 48 h. Data were expressed as a percentage of control. The control value for glyoxalase I activity was 0.29 ± 0.001 ΔOD/min/mg. $^{\#}P<0.05$, compared with the untreated cells; $^{*}P<0.05$, compared with the cells treated with MG (400 μM) alone.

Fig. 5 Inhibitory effect of liquiritigenin on MG-induced oxidative stress in MC3T3-E1 cells. Osteoblasts were pre-incubated with liquiritigenin (Liq) or aminoguanidine (AG) before treatment with 400 μM methylglyoxal (MG) for 48 h. $^{\#}P<0.05$, compared with the untreated cells; $^{*}P<0.05$, compared with the cells treated with MG (400 μM) alone.

Fig. 6 Effect of liquiritigenin on the MG-induced mitochondrial dysfunction in osteoblastic MC3T3-E1 cells. Osteoblasts were pre-incubated with liquiritigenin (Liq) or aminoguanidine (AG) before treatment with 400 μM methylglyoxal (MG) for 48 h. Data were expressed as a percentage of control. The control values for (A) mitochondrial membrane potential (MMP) and (B) ATP level were 16.97 ± 2.64 red:green ratio and 1.496 ±

0.193 nmol/mg, respectively. $^{\#}P<0.05$, compared with the untreated cells; $^{*}P<0.05$, compared with the cells treated with MG (400 μM) alone.

Fig. 7 Effect of liquiritigenin on the level of nitric oxide and PGC-1α in MG-treated cells. Osteoblasts were pre-incubated with liquiritigenin (Liq) or aminoguanidine (AG) before treatment with 400 μM methylglyoxal (MG) for 48 h. The level of PGC-1α in the cell lysate was detected via ELISA. Data were expressed as a percentage of control. The control value for PGC-1α was 387.98 ± 5.39 ng/mg. $^{\#}P<0.05$, compared with the untreated cells; $^{*}P<0.05$, compared with the cells treated with MG (400 μM) alone.

Fig. 8 Effect of liquiritigenin on the level of TNF-α in MG-treated cells. Osteoblasts were pre-incubated with liquiritigenin (Liq) or aminoguanidine (AG) before treatment with 400 μM methylglyoxal (MG) for 48 h. The level of TNF-α in the cell medium was detected via ELISA. Data were expressed as a percentage of control. The control value for TNF-α was 3.5 ± 0.52 pg/mg. $^{\#}P<0.05$, compared with the untreated cells; $^{*}P<0.05$, compared with the cells treated with MG (400 μM) alone.

Fig. 1

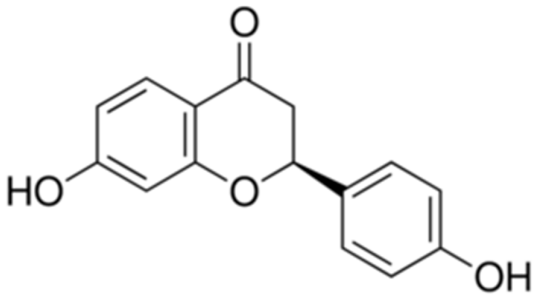

Fig. 2

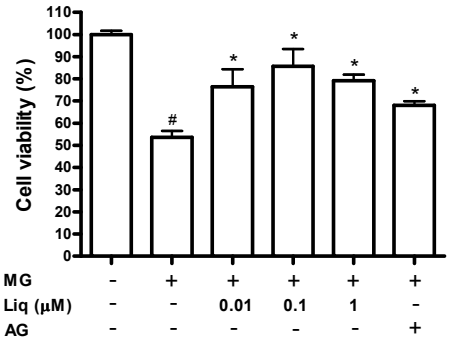

Fig. 3

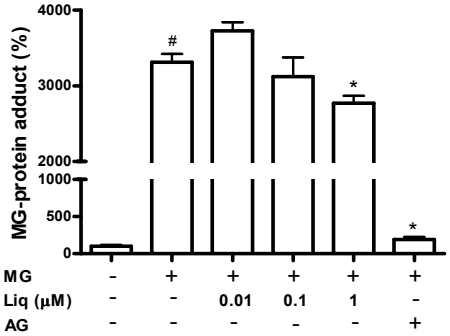

Fig. 4

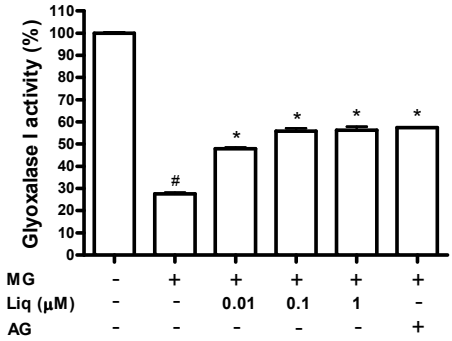

Fig. 5

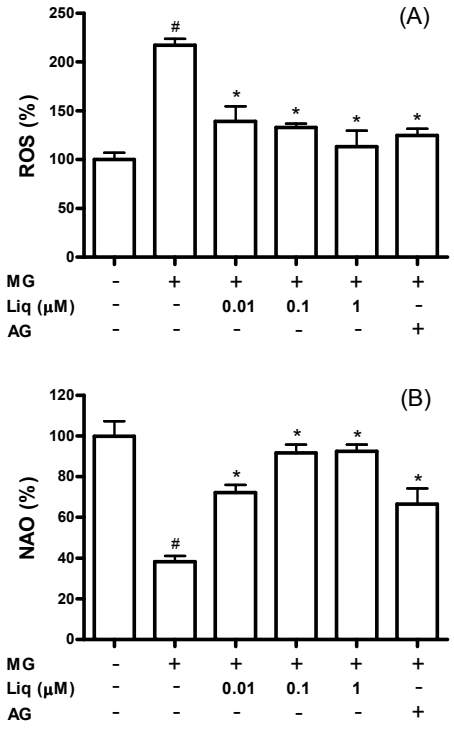

Fig. 6

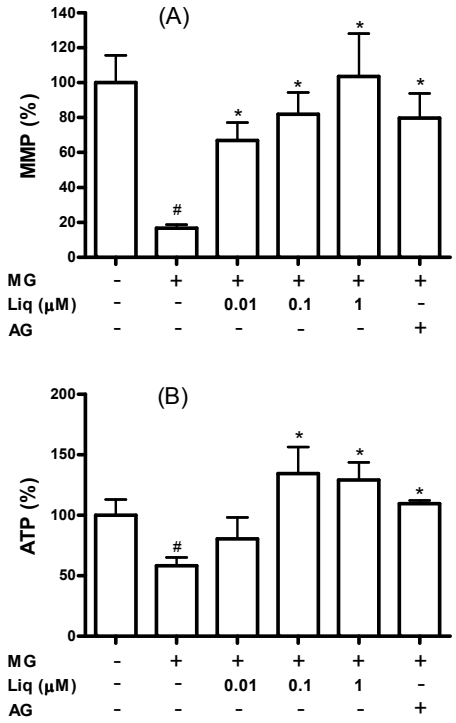

Fig. 7

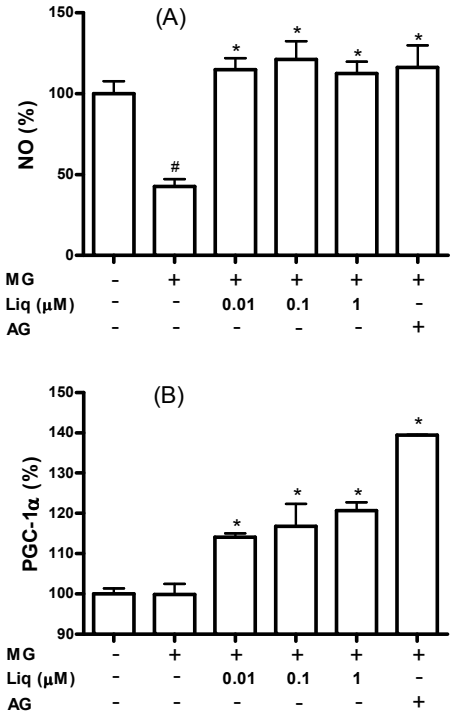

Fig. 8