Cytotechnology (2016) 68:2539–2552
DOI 10.1007/s10616-016-9977-y

ORIGINAL ARTICLE

Luteolin alleviates methylglyoxal-induced cytotoxicity in osteoblastic MC3T3-E1 cells

Kwang Sik Suh · Suk Chon · Eun Mi Choi

Received: 2 February 2016 / Accepted: 22 April 2016 / Published online: 24 May 2016

Abstract Methylglyoxal (MG), a reactive sugar-derived metabolite, exerts harmful effects by inducing oxidative stress, which aggravates a series of diabetic complications, including osteoporosis. The present study was performed to examine the effects of luteolin, a dietary polyphenolic flavonoid, on MG-induced cytotoxicity in MC3T3-E1 osteoblastic cells. Pretreatment of MC3T3-E1 osteoblastic cells with luteolin prevented MG-induced cell death and production of tumor necrosis factor-alpha, intracellular reactive oxygen species, mitochondrial superoxide, and cardiolipin peroxidation. In addition, luteolin increased the levels of glutathione and nuclear factor erythroid 2-related factor 2 (Nrf2) and decreased the inhibition of heme oxygenase-1 activity by MG. Pretreatment with luteolin prior to MG exposure reduced MG-induced mitochondrial dysfunction and increased the peroxisome proliferator-activated receptor γ co-activator 1α (PGC-1α) and nitric oxide levels, suggesting that luteolin may induce mitochondrial biogenesis. Taken together, these observations indicated that luteolin has potential as a preventive agent against the development of diabetic osteopathy related to MG-induced oxidative stress in diabetes.

Keywords Reactive oxygen species · Glutathione · Osteoblasts · Nitric oxide · Mitochondrial function

Abbreviations

AG	Aminoguanidine
AGEs	Advanced glycation end products
DAF-FM DA	4-Amino-5-methylamino-2′,7′-difluorofluorescein diacetate
H_2DCFDA	2′,7′-Dichlorodihydrofluorescin diacetate
HO-1	Heme oxygenase-1
MG	Methylglyoxal
MMP	Mitochondrial membrane potential
NAO	10-N-nonyl-Acridine Orange
NO	Nitric oxide
Nrf2	Nuclear factor erythroid 2-related factor 2
PGC-1α	Peroxisome proliferator-activated receptor γ co-activator 1α
RAGE	Receptor for AGE
ROS	Reactive oxygen species
sRAGE	Soluble form of receptor for advanced glycation end products
TNF-α	Tumor necrosis factor-alpha

Kwang Sik Suh and Suk Chon have contributed equally to this article.

K. S. Suh
Research Institute of Endocrinology, Kyung Hee University Hospital, 1, Hoegi-dong, Dongdaemun-gu, Seoul 130-702, Republic of Korea

S. Chon · E. M. Choi (✉)
Department of Endocrinology and Metabolism, School of Medicine, Kyung Hee University, 1, Hoegi-dong, Dongdaemun-gu, Seoul 130-701, Republic of Korea
e-mail: cheunmi@hanmail.net

Introduction

Methylglyoxal (MG) is a highly reactive dicarbonyl metabolite produced during glucose metabolism (Wu et al. 2011) and is a major precursor of advanced glycation end products (AGEs) involved in the pathogenesis of diabetes and inflammation. Proinflammatory cytokines can be generated through receptor for AGE (RAGE) activation, and are related to the modulation of inflammatory molecules through oxidative stress (Wu et al. 2011). The level of the soluble form of RAGE (sRAGE) has been suggested to be a useful chemical biomarker of cardiovascular disease in type 2 diabetes (Colhoun et al. 2011). The toxic effects of MG are supposed to be mediated through MG-derived AGE. The accumulation of AGEs initiates a cascade of signal transduction events and activates damage responses, such as apoptosis, inflammation, and oxidative stress (Zhao et al. 2010; Dalal et al. 2011). Many diseases such as diabetes, cataract formation, hypertension, and uremia are proposed to be intimately linked with MG-derived AGE (Talukdar et al. 2009). Oxidative stress is increased during diabetes; reactive oxygen species (ROS) have been reported to be generated as a result of hyperglycemia, which causes the secondary complications of diabetes (Shanmugam et al. 2003). MG cytotoxicity may be involved in diabetes-associated bone defects. It has been shown that patients with type I diabetes have high rates of bone resorption and turnover and decreased bone mineral density (Kayath et al. 1998; Nicodemus and Folsom 2001). Recently, we reported that MG has harmful effects on osteoblastic cells through a mechanism involving oxidative stress and mitochondrial dysfunction (Suh et al. 2014). Previous studies suggested that peroxisome proliferator-activated receptor γ coactivator 1α (PGC-1α) increases mitochondrial biogenesis (Ojuka et al. 2003), and the expression of mitochondrial biogenesis factors may be directly regulated by the bioavailability of nitric oxide (NO; Nisoli et al. 2005). Excessive production of mitochondrial ROS due to impaired mitochondrial biogenesis seems to play a pivotal role in the development of aging-induced diseases (Ungvari et al. 2008). Moreover, nuclear factor erythroid 2-related factor 2 (Nrf2) has been shown to attenuate oxidative damage by expression of heme oxygenase-1 (HO-1; Chartoumpekis et al. 2011). The main function of Nrf2 is to activate the antioxidant response and induce transcription of genes that are able to counteract the harmful effects of oxidative damage, thus restoring intracellular homeostasis (Villeneuve et al. 2010).

MG can readily bind to amino groups, thereby modifying biological molecules to form covalently cross-linked aggregates (Finotti et al. 2001). Therefore, the scavenging of reactive dicarbonyl species has been used as a therapeutic strategy to prevent protein modification and AGE formation (Wang and Ho 2012; Peng et al. 2011). Several pharmacological agents, such as aminoguanidine (Thomas et al. 2005), tenilsetam (Webster et al. 2005), carnosine (Blatnik et al. 2008), metformin (Beisswenger and Ruggiero-Lopez 2003), and pyridoxamine (Nagaraj et al. 2002), have been developed to inhibit the formation of AGEs and prevent the development of diabetic complications. However, all of these pharmaceutical agents have side effects. Therefore, it is critical to develop effective and safe agents to protect against diabetic complications.

Flavonoids have attracted considerable interest because of their potential beneficial effects on human health, and they have been reported to reduce the risk of developing chronic diseases. Luteolin (3′,4′,5,7-tetrahydroxyflavone) is a flavone that exists in various types of plants. It has been reported that luteolin exerts various beneficial effects, including cardiovascular protection, anti-inflammatory activity, and anticancer activity (Shi et al. 2007; Funakoshi-Tago et al. 2011; Lang et al. 2012). In addition, recent studies showed that luteolin can trap MG and has a significant inhibitory effect on MG-mediated protein modification, indicating that this agent can serve as a scavenger of reactive dicarbonyl species (Shao et al. 2014; Wu and Yen 2005). Our previous study showed that luteolin protects osteoblastic MC3T3-E1 cells against antimycin A-induced cytotoxicity through improved mitochondrial function and activation of the PI3K/Akt/CREB pathway (Choi 2011). Therefore, the present study was performed to investigate the protective effects of luteolin against MG-induced cytotoxicity in MC3T3-E1 osteoblastic cells.

Materials and methods

Materials

Luteolin, aminoguanidine, and methylglyoxal were purchased from Sigma Chemical (St. Louis, MO,

USA). α-Modified minimal essential medium (α-MEM) and fetal bovine serum (FBS) were purchased from Gibco BRL (Grand Island, NY, USA). Other reagents were of the highest grade commercially available and purchased from Sigma Chemical (St. Louis, MO, USA).

Cell culture

The MC3T3-E1 Osteoblastic Subclone 4 cell line was obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). The cells were cultured at 37 °C under a 5 % CO_2 atmosphere in α-modified minimal essential medium (α-MEM; Gibco BRL). Unless otherwise specified, the medium contained 10 % fetal bovine serum (FBS), 100 U/ml penicillin, and 100 μg/ml streptomycin. Cells were seeded in 24-well plates in α-MEM containing 5 mM β-glycerophosphate and 50 μg/ml ascorbic acid, supplemented with 10 % FBS. 48 h after seeding, cells were preincubated for 1 h with α-MEM containing 0.1 % FBS and samples before treatment with MG for 48 h.

Cell viability

The MC3T3-E1 cells were seeded in a 24-well plate at a density of 2×10^4 cells/well. After 48 h, the cells were preincubated for 1 h with different concentrations of luteolin before treatment with MG for 48 h. Then cell viability was assessed by MTT assay. MTT assay is particularly useful for this type of analysis. Tetrazolium salts are cleaved to formazan by the succinate-tetrazolium reductase system which belongs to the respiratory chain of the mitochondria, and is only active in metabolically intact cells. Briefly, 20 μl of MTT in Dulbecco's phosphate-buffered saline (DPBS), were added to each well, and incubated for 2 h. After removal of the solution in the well, dimethyl sulfoxide (DMSO) 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 (Zenyth 3100 multimode detector, Anthos Labtec Instruments, Wals-Siezenheim, Austria) at 570 nm.

Measurement of TNF-α

Cells were seeded in a 24-well plate at a density of 2×10^4 cells/well. After 48 h, the cells were preincubated for 1 h with different concentrations of luteolin before treatment with MG for 48 h. TNF-α contents in the medium were measured with an enzyme immunoassay system according to the manufacturer's protocol (R&D Systems Inc., Minneapolis, MN, USA). Protein concentrations were determined using the BioRad protein assay reagent (Bio-Rad Laboratories, Hercules, CA, USA).

Measurement of RAGE and sRAGE levels

Cells were seeded in a 24-well plate at a density of 2×10^4 cells/well. After 48 h, the cells were preincubated for 1 h with different concentrations of luteolin before treatment with MG for 48 h. The cells were lysed and homogenized in PBS. Cell homogenates were centrifuged at $13{,}000 \times g$ for 15 min at 4 °C and the supernatant was used for ELISA and protein content measurement. RAGE and sRAGE were assayed using a Mouse RAGE ELISA kit (RayBio, Norcross, GA, USA), sRAGE ELISA kit (MyBioSource, San Diego, CA, USA), and ELISA kit (USCN Life Science, Wuhan, China). Protein concentrations were determined using Bio-Rad protein assay reagent.

Measurement of intracellular reactive oxygen species

Cells were seeded in a 96-black well plate at a density of 10^4 cells/well in culture medium. After 24 h, the cells were preincubated for 1 h with different concentrations of luteolin before treatment with MG for 48 h. Formation of intracellular ROS was measured using 2′,7′-dichlorodihydrofluorescin diacetate (H2DCFDA; Jakubowski and Bartosz 2000). Viable cells can deacetylate H2DCFDA 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. To load the cells with the fluorescence dye, they were incubated with H2DCFDA in Hank's solution at a final concentration of 10 μM for 45 min at 37 °C in the dark. Following washing with Dulbecco's phosphate-buffered saline (DPBS), ROS levels were determined by measuring the fluorescence intensity at an excitation wavelength of 485 nm and emission wavelength of 530 nm.

Measurement of mitochondrial superoxide

Cells were seeded in a 96-black well plate at a density of 10^4 cells/well in culture medium. After 24 h, the

cells were preincubated for 1 h with different concentrations of luteolin before treatment with MG for 48 h. Mitochondrial superoxide levels were determined using MitoSOX^{TM} Red mitochondrial superoxide indicator (Invitrogen Molecular Probes, Carlsbad, CA, USA). MitoSOX^{TM} Red is a fluorogenic dye for highly selective detection of superoxide in the mitochondria of cells (Schroeder et al. 2007). Cells were incubated with 2 μM MitoSOX^{TM} Red at 37 °C for 20 min. Fluorescence (Ex/Em = 510/580 nm) was measured after washing the cells on a microplate spectrophotometer (Zenyth 3100 multimode detector, Anthos Labtec Instruments, Austria).

Measurement of cardiolipin peroxidation

Cells were seeded in a 96-black well plate at a density of 10^4 cells/well in culture medium. After 24 h, the cells were preincubated for 1 h with different concentrations of luteolin before treatment with MG for 48 h. 10-N-nonyl-Acridine Orange (NAO; Molecular Probes, Carlsbad, CA, USA), which binds to mitochondrial cardiolipin, was used for 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 peroxidized cardiolipin. Cells were labeled with 5 μM NAO for 20 min. After washing, fluorescence was measured at an excitation wavelength of 485 nm and emission wavelength of 530 nm using a fluorescence microplate reader (Molecular Devices, Sunnyvale, CA, USA).

Measurement of reduced glutathione

Cells were seeded in a 24-well plate at a density of 2×10^4 cells/well. After 48 h, the cells were preincubated for 1 h with different concentrations of luteolin before treatment with MG for 48 h. Cells were lysed by homogenization in cold buffer containing 50 mM MES (2-(*N*-morpholino) ethanesulfonic acid) or phosphate (pH 6.7) and 1 mM EDTA. After centrifugation at 10,000×*g* for 15 min at 4 °C, the supernatant was used for the assay. Glutathione was measured using a glutathione assay kit (BioAssay Systems, Hayward, CA, USA) according to the manufacturer's instructions. Determination of glutathione is based on the reaction of 5,'5-dithiobis-2-nitrobenzoic acid (DTNB) with glutathione, which yields a yellow-colored chromophore, 5-thionitrobenzoic acid (TNB), with a maximum absorbance at 412 nm. Concentrations of glutathione were determined with reference to a freshly prepared standard curve.

Measurement of heme oxygenase-1

Cells were seeded in a 6-well plate at a density of 10^5 cells/well in culture medium. After 48 h, the cells were preincubated for 1 h with different concentrations of luteolin before treatment with MG for 48 h. Heme oxygenase-1 in the cell extract was determined using a Mouse Heme Oxygenase-1 Enzyme Immunoassay (EIA) Kit (Takara Bio Inc., Otsu, Japan), which is based on a sandwich method that utilizes anti-heme oxygenase-1 monoclonal antibodies to detect mouse heme oxygenase-1. The amount of mouse heme oxygenase-1 can be quantified by measuring the absorbance using an EIA plate reader. Accurate sample concentration of mouse heme oxygenase-1 can be determined by comparing their specific absorbance with those obtained for standards plotted on a standard curve.

Measurement of nuclear Nrf2

Cells were seeded in a 6-well plate at a density of 10^5 cells/well in culture medium. After 48 h, the cells were preincubated for 1 h with different concentrations of luteolin before treatment with MG for 48 h. After harvesting, cells were resuspended in ice-cold hypotonic buffer and allowed the cells on ice for 15 min. Cells were treated with 0.5 % Nonidet P-40 and centrifuged for 30 s at 4 °C in a microcentrifuge. Supernatant was removed and the nuclear pellet was resuspended in lysis buffer. After centrifugation (14,000×*g*, 10 min, 4 °C), the supernatant (nuclear extract) was stored at −80 °C. NF-E2-related factor (Nrf2) in the nuclear extract was measured using a TransAM Nrf2 Kit (Active Motif, Carlsbad, CA, USA), which included a 96-well plate on which had been immobilized an oligonucleotide containing the antioxidant response elements (AREs) consensus binding site. The active form of Nrf2 present in the nuclear extract binds specifically to this oligonucleotide. The primary antibody used to detect Nrf2 recognizes an epitope on Nrf2 protein upon DNA binding. Addition of an HRP-conjugated secondary antibody provides a sensitive colorimetric readout that can be easily quantified by spectrophotometry.

Determination of mitochondrial membrane potential

Cells were seeded in a 96-black well plate at a density of 10^4 cells/well in culture medium. After 24 h, the cells were preincubated for 1 h with different concentrations of luteolin before treatment with MG for 48 h. A JC-1 Mitochondrial Membrane Potential Assay Kit (Cayman Chemical Co., Ann Arbor, MI, 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 across the plasma and mitochondrial membranes. The dye shows red fluorescence when it aggregates in healthy mitochondria with a high membrane potential, whereas it appears in monomeric form and shows green fluorescence 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, followed by measurement of red (excitation, 550 nm; emission, 600 nm) and green (excitation, 485 nm; emission, 535 nm) fluorescence using a microplate reader (Molecular Devices). Mitochondrial depolarization (i.e., loss of MMP) manifests as a decrease in the red/green fluorescence ratio.

ATP measurement

Cells were seeded in a 24-well plate at a density of 2×10^4 cells/well. After 48 h, the cells were preincubated for 1 h with different concentrations of luteolin before treatment with MG for 48 h. The cells were lysed and homogenized in PBS. Cell homogenates were centrifuged at 13,000×*g* for 15 min at 4 °C and the supernatants were used for assay and protein content measurement. The ATP concentration was determined by luciferase reaction using an EnzyLight™ ATP Assay Kit (BioAssay Systems, Hayward, CA, USA). This ATP assay kit provides a rapid method of measuring intracellular ATP. Protein concentrations were determined using the Bio-Rad protein assay reagent.

Measurement of PGC-1α levels

Cells were seeded in a 6-well plate at a density of 10^5 cells/well in culture medium. After 48 h, the cells were preincubated for 1 h with different concentrations of luteolin before treatment with MG for 48 h. The cells were lysed and homogenized in PBS. Cell homogenates were centrifuged at 13,000×*g* for 15 min at 4 °C and the supernatants were used for ELISA and protein content measurement. PGC-1α was measured using a Mouse Peroxisome Proliferator Activated Receptor Gamma Coactivator 1 Alpha (PGC-1α) ELISA kit (MyBioSource). Protein concentrations were determined using the Bio-Rad protein assay reagent.

Measurement of nitric oxide (NO) generation

Cells were seeded in a 96-black well plate at a density of 10^4 cells/well in culture medium. After 24 h, the cells were preincubated for 1 h with different concentrations of luteolin before treatment with MG for 48 h. DAF-FM diacetate, a cell-permeable derivative of DAF-FM, is a sensitive fluorescent indicator for detection of NO. Upon entry into the cell, DAF-FM diacetate is 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 (Wang et al. 2006), cells were loaded with 5 μM DAF-FM diacetate (Invitrogen Corp., 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.

Statistical analysis

The results are expressed as means ± SEM. Statistical significance was determined by analysis of variance and subsequent application of Dunnett's test. In all analyses, $P < 0.05$ was taken to indicate statistical significance. The analysis was performed using SAS statistical software.

Results

Luteolin reduces cytotoxicity induced by MG

Osteoblastic MC3T3-E1 cells treated with 400 μM MG for 48 h exhibited significant cytotoxicity, as determined by MTT assay (Fig. 1). The purpose of

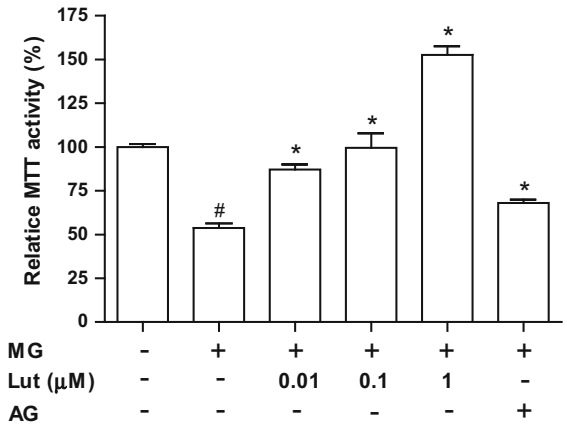

Fig. 1 Effects of luteolin on the viability of osteoblasts. Osteoblasts were treated with luteolin (Lut) or aminoguanidine (AG) 1 h before exposure to 400 μM MG for 48 h. $^{\#}P < 0.05$, compared to untreated cells; $^{*}P < 0.05$, compared to cells treated with MG alone

our study was focused on the mitochondrial function. The principal of MTT assay is based on succinyl dehydrogenase (Complex II) present in the mitochondria of viable cells. The measured absorbance directly correlates to the number of viable cells. Cell proliferation and viability assays are of particular importance for routine applications in cell biology. The viability of MC3T3-E1 cells was significantly increased by pretreatment with luteolin (0.01–1 μM) 1 h before exposure to MG for 48 h ($P < 0.05$). Aminoguanidine (AG, 300 μM), a carbonyl scavenger, also inhibited the effect of MG on cell viability. The protective effect of luteolin against MG-induced cytotoxicity was greater than that of AG. To evaluate the effect of luteolin itself on cell survival, cells were incubated in medium containing 0.5 % FBS with increasing concentrations of luteolin (0.01–10 μM) and then cell viabilities were determined using MTT assay. Lutelin at concentrations of 10 μM or lower did not influence cell viability, while higher doses (>10 μM) were cytotoxic in a dose-dependent manner (data not shown). Therefore, we chose the non-toxic concentration of luteolin (0.01–1 μM) for all subsequent cell culture experiments. In our previous study, the IC_{50} of MG was measured as 400 μM for 48 h and MG has detrimental effects on MCT3E-1 osteoblastic cells through a mechanism involving oxidative stress and mitochondrial dysfunction (Suh et al. 2014).

Luteolin reduces MG-induced TNF-α production

MG promotes the formation of proinflammatory cytokines in various cell types. Therefore, we investigated whether luteolin modulates the production of TNF-α in MG-treated cells (Fig. 2). 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 with luteolin at concentrations of 0.01–1 μM. AG also decreased MG-induced TNF-α release.

Effects of luteolin on cellular RAGE and sRAGE levels in MG-treated MC3T3-E1 cells

Ligand-induced upregulation of RAGE is involved in various pathophysiological processes and high sRAGE could result from increased production of the cleaved-form of RAGE (Goldin et al. 2006). The level of sRAGE was significantly higher in MG-treated cells than in control cells, while RAGE level was very low and was not significantly different from that in controls (Fig. 3). sRAGE level induced by MG was reduced by pretreatment with luteolin (1 μM) or AG.

Luteolin inhibits MG-induced oxidative stress in MC3T3-E1 cells

We examined whether luteolin could attenuate MG-induced cell death by decreasing intracellular ROS

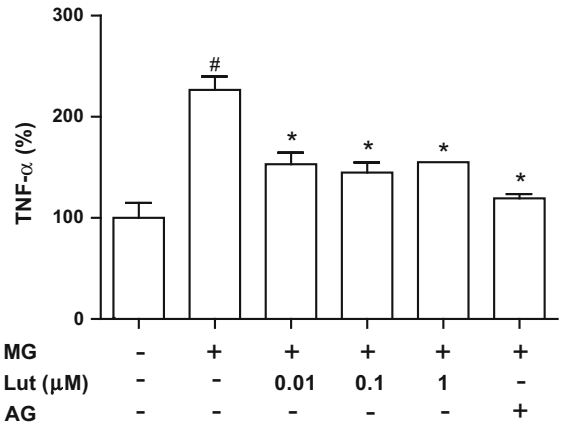

Fig. 2 Effects of luteolin on TNF-α level in MG-treated MC3T3-E1 cells. Osteoblasts were treated with luteolin (Lut) or aminoguanidine (AG) 1 h before exposure to 400 μM MG for 48 h. $^{\#}P < 0.05$, compared to untreated cells; $^{*}P < 0.05$, compared to cells treated with MG alone

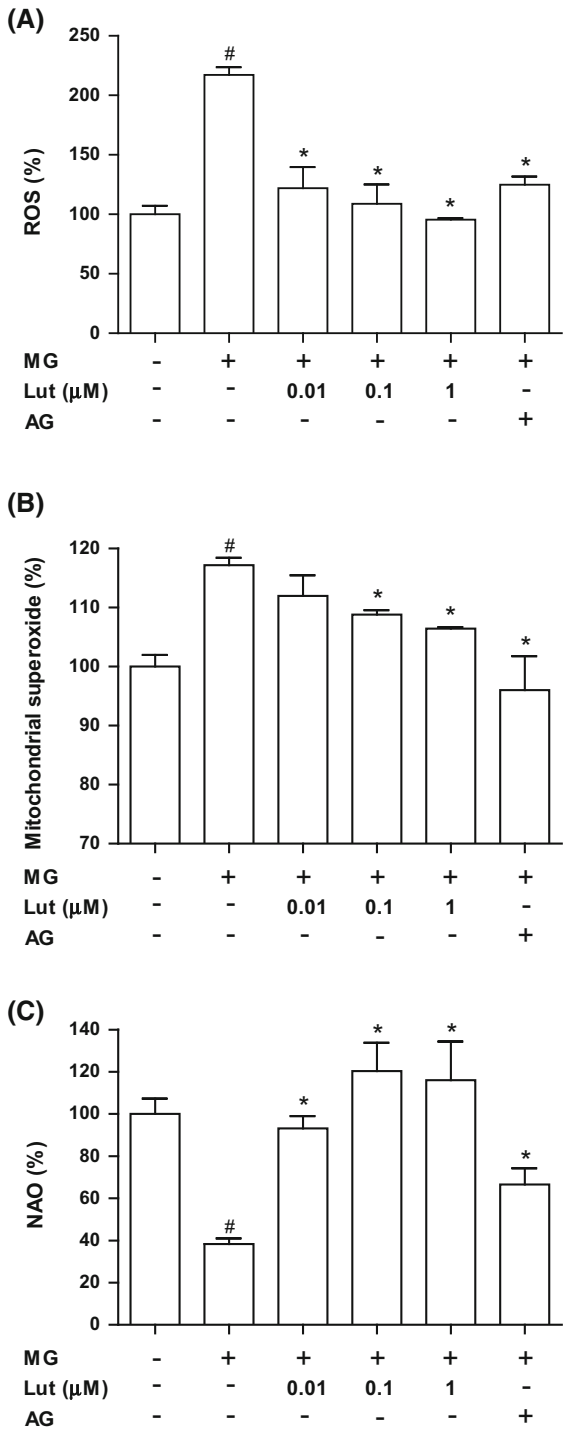

Fig. 4 Inhibitory effect of luteolin on MG-induced oxidative stress in MC3T3-E1 cells. Osteoblasts were treated with luteolin (Lut) or aminoguanidine (AG) 1 h before exposure to 400 μM MG for 48 h. **a** The data show changes in levels of ROS, which was measured by the DCF fluorescence method. **b** Mitochondrial superoxide levels were detected using MitoSOX™ Red mitochondrial superoxide indicator. **c** Cardiolipin oxidation was measured using 5 μM NAO. $^{\#}P < 0.05$, compared to untreated cells; $^{*}P < 0.05$, compared to cells treated with MG alone

production. As shown in Fig. 4a, exposure of MC3T3-E1 cells to 400 μM MG for 48 h increased DCF fluorescence. Preincubation of MC3T3-E1 cells with 0.01–1 μM luteolin or AG 1 h prior to MG insult prevented intracellular oxidation of the fluorescent probe. MitoSOX localizes to the mitochondria and served as a fluoroprobe for selective detection of superoxide in these organelles (Mukhopadhyay et al. 2007). MG (400 μM) significantly increased mitochondrial superoxide production compared to untreated cells. However, pretreatment with luteolin (0.1 and 1 μM) or AG decreased mitochondrial superoxide production induced by MG treatment (Fig. 4b). To obtain further evidence of oxidative stress within mitochondria, we examined the oxidation of cardiolipin because this phospholipid is present in association with cytochrome c on the outer surface of the inner mitochondrial membrane. As the fluorescent dye NAO binds to the nonoxidized form of cardiolipin,

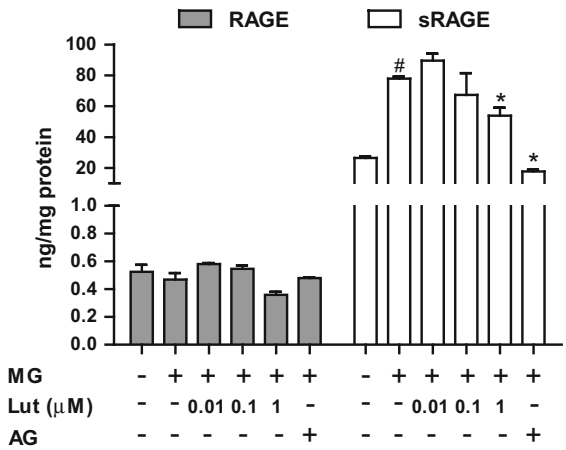

Fig. 3 Effects of luteolin on RAGE and sRAGE levels in MG-treated cells. Osteoblasts were treated with luteolin (Lut) or aminoguanidine (AG) 1 h before exposure to 400 μM MG for 48 h. $^{\#}P < 0.05$, compared to untreated cells; $^{*}P < 0.05$, compared to cells treated with MG alone

but not to the oxidized form (Petit et al. 1992), measurements of NAO fluorescence allow us to monitor the oxidation of cardiolipin in mitochondria. Treatment with 400 μM MG decreased NAO

fluorescence, indicating the induction of cardiolipin peroxidation (Fig. 4c). However, luteolin (0.01–1 μM) or AG decreased the cardiolipin peroxidation induced by MG. These data indicated that luteolin decreases MG-induced ROS generation and oxidative stress within mitochondria.

Luteolin-mediated protective action involves the GSH and Nrf2/HO-1 pathway

Glutathione content reflects the amount of substrate available to act as an antioxidant. MG treatment seems to have little effect on the GSH content of cells. However, the level of glutathione after luteolin exposure for 48 h was significantly increased at concentrations of 0.01–1 μM compared with controls, indicating that luteolin-induced cytoprotective effects are associated with increased glutathione levels (Fig. 5a). As HO-1 is an important component of cellular defense against oxidative stress, we examined whether luteolin could alter the level of the antioxidant enzyme HO-1. As shown in Fig. 5b, the levels of HO-1 were decreased in cells treated with MG alone as compared with vehicle controls ($P < 0.05$). However, luteolin (0.01–1 μM) markedly increased the levels of HO-1 in MC3T3-E1 cells. Thus, luteolin appears to prevent oxidative damage by inducing HO-1 expression. Nrf2 is a master regulator against oxidative stress and controls the transcription of several antioxidant genes (Nguyen et al. 2009). To elucidate the potential mechanisms underlying the luteolin-mediated protective effect against oxidative stress, we examined the nuclear level of the Nrf2 transcription factor. As shown in Fig. 5c, MG treatment slightly decreased the nuclear Nrf2 level, although the difference was not statistically significant. Luteolin (0.1 and 1 μM) pretreatment significantly increased Nrf2 accumulation in the nucleus compared with the MG-treated group. Therefore, luteolin is thought to show antioxidant and cytoprotective effects via activation of the Nrf2/HO-1 signaling pathway.

Luteolin increases mitochondrial functions in MG-treated MC3T3-E1 cells

Mitochondria are important regulators of cellular processes and survival that may play key roles in aging-

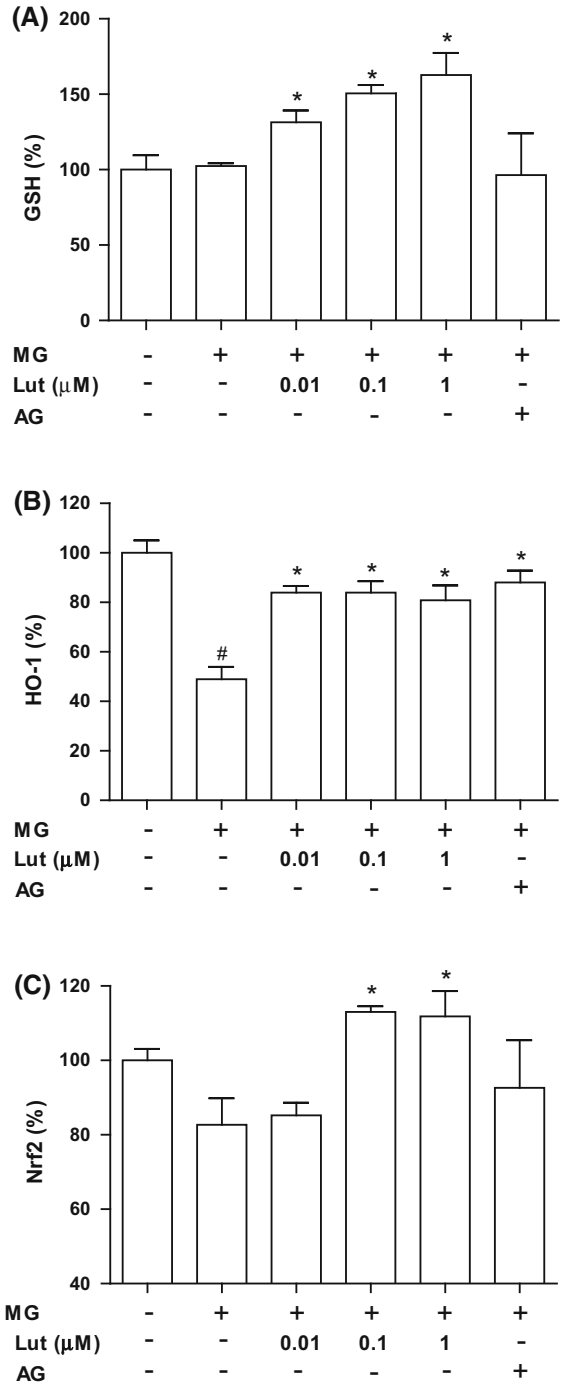

Fig. 5 Effects of luteolin on reduced glutathione (GSH), HO-1, and Nrf2 levels in osteoblastic MC3T3-E1 cells. Osteoblasts were treated with luteolin (Lut) or aminoguanidine (AG) 1 h before exposure to 400 μM MG for 48 h. *$P < 0.05$, compared to cells treated with MG alone

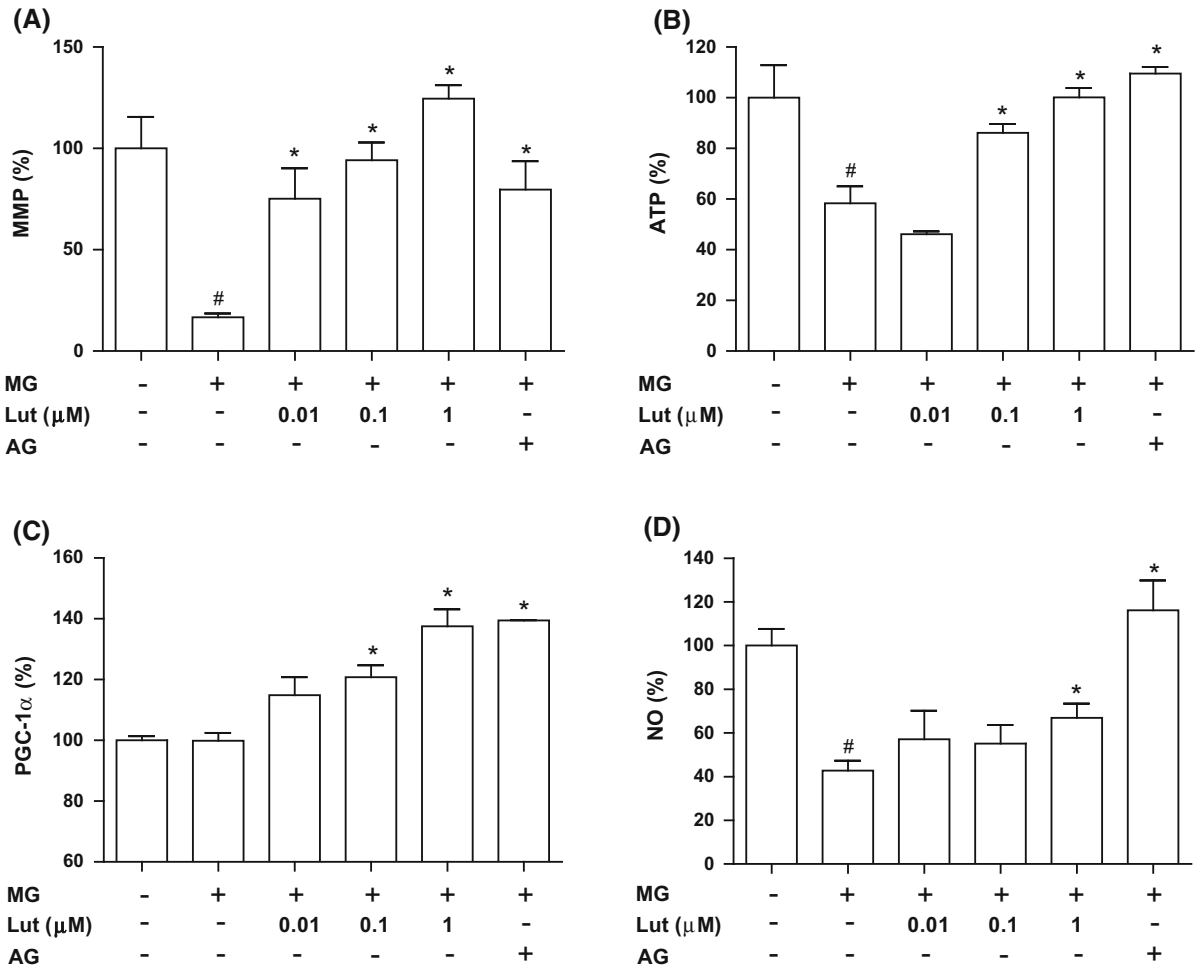

Fig. 6 Effects of luteolin on the MG-induced mitochondrial dysfunction in osteoblastic MC3T3-E1 cells. Osteoblasts were treated with luteolin (Lut) or aminoguanidine (AG) 1 h before exposure to 400 μM MG for 48 h. (**A**) Changes in mitochondrial membrane potential (MMP) were monitored by loading with the fluorescent probe JC-1. (**B**) The ATP concentrations were determined by luciferase reaction. (**C**) Intracellular PGC-1α levels were measured using ELISA. (**D**) Cells were loaded with DAF-FM-DA as an indicator of nitric oxide. $^{\#}P < 0.05$, compared to untreated cells; $^{*}P < 0.05$, compared to cells treated with MG alone

related diseases. As shown in Fig. 6a, exposure of MC3T3-E1 cells to MG (400 μM) induced marked mitochondrial disruption. However, luteolin (0.01–1 μM) or AG treatment of MC3T3-E1 cells reduced the disruption of MMP by MG. In addition, luteolin (0.1 and 1 μM) or AG restored the ATP synthesis inhibited by MG (Fig. 6b). These results showed that the cytoprotective effect of luteolin is due to protection of mitochondria. PGC-1α has been shown to play a primary role in mitochondrial biogenesis/metabolism (Wu et al. 1999). As shown in Fig. 6c, PGC-1α levels were increased by treatment with luteolin (0.1 and 1 μM) or AG. It was also reported that dysregulation of mitochondrial biogenesis is related to impaired nitric oxide (NO) bioavailability (Addabbo et al. 2009). Therefore, NO generation was evaluated by DAF-FM, a specific probe used to quantify low concentrations of intracellular NO. As shown in Fig. 6d, MG (400 μM) decreased the production of NO in MC3T3-E1 cells, which was prevented by pretreatment with luteolin (1 μM) or AG. Our results suggest that luteolin may reduce MG-induced dysfunction of mitochondria by enhancing mitochondrial biogenesis factors in osteoblastic MC3T3-E1 cells.

Discussion

Many natural agents capable of scavenging free radicals have been identified, and have been proposed as therapeutic agents to counteract oxidative stress-induced diseases (Vitaglione et al. 2004). Certain flavonoids show more effective inhibition of AGE formation than aminoguanidine, a prototype therapeutic agent for prevention of formation of AGE (Wu and Yen 2005). For example, luteolin, rutin, (—)-epigallocatechin-3-gallate (EGCG), and quercetin showed significant inhibitory effects against MG-mediated AGE formation (Wu and Yen 2005). In addition, phloretin or phloridzin exhibited inhibitory effects against arginine residue modification by MG (Odani et al. 1998), which indicated that direct capture of the reactive α-dicarbonyl species, such as MG, would effectively inhibit AGE formation and eventually prevent diabetic complications. Our previous study reported that luteolin (0.03–0.3 μM) protects MC3T3-E1 cells from antimycin A (an inhibitor of the mitochondrial electron transport chain)-induced cell damage through the improved mitochondrial function and activation of PI3 K/Akt/CREB pathway (Choi 2011). In the present study, we demonstrated that luteolin suppressed MG-induced MC3T3-E1 cell death by decreasing TNF-α release, oxidative stress, and mitochondrial dysfunction. Mitochondrial dysfunction and oxidative stress can provoke and potentiate inflammatory responses. Proinflammatory cytokines, including TNF-α, can induce oxidative stress and contribute to the pathophysiology of various diseases (Pignatelli et al. 2008). Our previous study reported that luteolin (1 μM) caused a significant elevation of collagen content, alkaline phosphatase (ALP) activity, and osteocalcin secretion in MC3T3-E1 cells by modulating the inflammatory mediators including TNF-α and interleukin-6 (Choi 2007). TNF-α stimulates ROS in mitochondria by altering membrane permeability and by inhibiting the electron transport chain, thereby causing mitochondrial damage (Mariappan et al. 2007). TNF-α decreases MnSOD protein levels, thereby causing a functional decline in the respiratory chain and increased ROS generation, coupled with an inability to efficiently scavenge free radicals, which may perpetuate mitochondrial dysfunction. Further, TNF-α present in the cytosol may penetrate the damaged mitochondrial membrane through an as yet unknown mechanism, causing increased production of mitochondrial superoxide. Mitochondrial superoxide could then induce further production of cytosolic superoxide by NAD(P)H oxidase, thereby initiating a ROS-induced ROS mechanism. TNF-α alters the cellular redox state, decreases the expression of four complex I subunits by increasing mitochondrial superoxide production and depleting ATP synthesis, and decreases oxygen consumption, thereby resulting in mitochondrial damage and thus leading to cellular dysfunction (Mariappan et al. 2009). RAGE activates pathways responsible for acute and chronic inflammation. The soluble type of this receptor, sRAGE, which acts as a decoy receptor, is a novel biomarker that has been linked to severity of sepsis (Yamagishi and Matsui 2010). Other studies have linked sRAGE to chronic (Miniati et al. 2011) or acute lung injury (Liu et al. 2012), coronary artery disease (Raposeiras-Roubin et al. 2010), and other diseases (Yamagishi and Matsui 2010). We speculate that decreased levels of sRAGE by luteolin may reduce bone injury caused by chronic glucotoxicity, possibly in the bone tissue of diabetic patients.

Nrf2 induces expression of antioxidant enzymes, such as HO-1, glutamate-cysteine ligase, catalase, glutathione dismutase, and superoxide dismutase (Zenkov et al. 2013). Upon oxidative stress, Nrf2 escapes Kelch-like ECH-associated protein 1 (Keap1)-mediated proteosomal degradation and translocates into the nucleus (Li and Kong 2009). In the nucleus, Nrf2 forms a heterodimer with small Maf proteins (Itoh et al. 1997) that bind to antioxidant response elements (AREs) and induce a number of Nrf2-dependent drug processing genes. HO-1 is the rate-limiting enzyme in the degradation pathway of pro-oxidant heme into biliverdin/bilirubin (both known antioxidants; Dore 2005). The HO pathway has been reported to be active and to operate as a fundamental defense mechanism for cells exposed to oxidant challenge (Scapagnini et al. 2004). Nrf2 also mediates cytoprotection and modulates expression of several detoxification genes that encode antioxidant proteins, such as GSH system and thioredoxin, regulators of the intracellular redox environment (Kim et al. 2010; Arredondo et al. 2010). Luteolin increased the level of GSH, the major antioxidant, produced by MC3T3-E1 cells under MG-induced oxidative stress. GSH serves as a substrate for GSH synthesis (Pope et al. 2008). Metal chelators, SOD,

and antioxidants (e.g., resveratrol) inhibit the oxidation of GSH (Pope et al. 2008). Moreover, the increase in GSH level confers cytoprotection in degenerative diseases (Lee et al. 2010). Under oxidative conditions, luteolin induced increases in GSH and HO-1 levels. In addition, luteolin decreased DCFH oxidation and prevented mitochondrial superoxide release and cardiolipin peroxidation. These observations suggest that luteolin has strong antioxidant properties. Our data regarding ROS production indicated the involvement of the HO-1 signaling pathway in the protective mechanism of luteolin against MG-induced oxidative stress. Although its mechanism of action is not yet fully understood, luteolin may exert its effects by antioxidant/scavenger activity, by modulation of HO-1 expression or by anti-inflammatory effects.

Mitochondria are the main organelles that produce ROS. Defective mitochondria may be involved in human diseases with underlying inflammatory pathologies, such as diabetes mellitus and cardiac dysfunction (Nisoli et al. 2007; Patti and Corvera 2010). Zhou et al. (2011) and Nakahira et al. (2011) reported that after an inflammatory stimulus, the accumulation of damaged mitochondria precipitates an increase in mitochondrial ROS production, which in turn enhances inflammasome activation. Mitochondrial dysfunction may affect several pathways that have been implicated in bone degradation, including oxidative stress, increased cytokine-induced osteoblast inflammation and matrix catabolism, and increased apoptosis. A decrease in MMP following intense ROS production induces mitochondrial disruption, inhibition of mitochondrial respiratory chain, reduction of ATP synthesis, and cell death (Choi et al. 2000). In the present study, MG treatment resulted in significant damage to the mitochondrial membrane and was accompanied by a decrease in ATP production. In contrast, treatment with luteolin reduced mitochondrial damage and restored ATP production. Luteolin was shown to impact mitochondrial function in osteoblasts (Choi 2011), but its role in mitochondrial biogenesis remains poorly defined. PGC-1α has been shown to have a primary role in mitochondrial biogenesis/metabolism (Puigserver et al. 2001). PGC-1α represents an upstream inducer of genes of mitochondrial metabolism by positively affecting the activities of some hormone nuclear receptors and nuclear transcription factors (Finkel 2006). In the present study, luteolin increased PGC-1α level, suggesting the potential for new treatment approaches targeting mitochondria in metabolic diseases. In addition, nitric oxide plays a critical role in initiating and integrating signaling events underlying mitochondrial biogenesis. Importantly, inhibition of nitric oxide synthesis significantly decreases mitochondrial content in the vasculature (Addabbo et al. 2009). Previous studies also suggested that expression of mitochondrial biogenesis factors might be directly regulated by the bioavailability of nitric oxide (Nisoli et al. 2005). We demonstrated that luteolin prevents MG-induced inhibition of nitric oxide synthesis in MC3T3-E1 cells, suggesting that nitric oxide has a beneficial effect on MG-treated cells. Luteolin-induced mitochondrial biogenesis would correct cellular impairment and may reduce mitochondrial ROS production in cells.

In diabetics, MG plays a noxious role by inducing oxidative stress, which causes and exacerbates a series of complications including low-turnover osteoporosis. Excess accumulation of AGEs is known to lead to stiffness, brittleness and overall alterations in tissue biomechanics of collagen rich tissues (Karim et al. 2013; Tang et al. 2008). Furthermore, T2DM (type 2 diabetes mellitus) is associated with changes in bone quality (Hamann et al. 2012) leading to increased bone fragility and brittleness and therefore is considered to be a risk factor for vertebral or other bone fractures (Kilpadi et al. 2013; Yamaguchi and Sugimoto 2011). In vivo studies on partially insulin-deficient rats, demonstrated that diabetes induced deleterious changes on long-bone micro-architecture (Tolosa et al. 2013). Further, T2DM negatively affected femur and vertebrae in Zucker Diabetic Fatty rats (Hamann et al. 2014). Recent studies by our group showing that transgenic mice that overexpress thioredoxin-1 are resistant to streptozotocininduced diabetic osteopenia (Hamada et al. 2009) clearly demonstrate that oxidative stress plays a crucial role in the development of diabetic osteopenia. Since diabetes, osteoporosis, and accumulation of AGEs are frequent consequences of aging, early treatments to reduce AGE-induced ROS and Inflammation may have broad public-health implications. We conclude that the oral ingestion of AGE inhibitory and anti-inflammatory luteolin provide a safe and efficacious treatment that may slow the progression of diabetes-induced changes within the bone.

In summary, we demonstrated the cytoprotective effects of luteolin against MG in osteoblastic MC3T3-E1

cells and the putative involvement of Nrf2/HO-1 activation in this cytoprotection by luteolin. Luteolin markedly prevented the increases in ROS production and TNF-α levels induced by MG in MC3T3-E1 cells. In addition, we demonstrated that markers of mitochondrial biogenesis were significantly upregulated by luteolin treatment. These results suggest that enhancement of mitochondrial biogenesis may contribute to the luteolin-induced cytoprotection in osteoblastic MC3T3-E1 cells and that luteolin may be useful in preventing the development of diabetic osteopathy.

Acknowledgments This research was supported by the Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Education (NRF-2013R1A1A2A10004361) and by a grant of the Korea Health Technology R&D Project through the Korea Health Industry Development Institute (KHIDI), funded by the Ministry of Health & Welfare, Republic of Korea. (Grant Number: HI14C-2700).

Compliance with ethical standards

Conflict of interest The authors declare that there are no conflicts of interest.

References

Addabbo F, Ratliff B, Park HC, Kuo M, Ungvari Z, Csiszar A, Krasnikov B, Sodhi K, Zhang F, Nasjletti A, Goligorsky MS (2009) The Krebs cycle and mitochondrial mass are early victims of endothelial dysfunction: proteomic approach. Am J Pathol 174:34–43

Arredondo F, Echeverry C, Abin-Carriquiry JA, Blasina F, Antunez K, Jones DP, Go YM, Liang YL, Dajas F (2010) After cellular internalization, quercetin causes Nrf2 nuclear translocation, increases glutathione levels, and prevents neuronal death against an oxidative insult. Free Radic Biol Med 49:738–747

Beisswenger P, Ruggiero-Lopez D (2003) Metformin inhibition of glycation processes. Diabetes Metab 29:6S95–6S103

Blatnik M, Frizzell N, Thorpe SR, Baynes JW (2008) Inactivation of glyceraldehyde-3-phosphate dehydrogenase by fumarate in diabetes: formation of S-(2-succinyl)cysteine, a novel chemical modification of protein and possible biomarker of mitochondrial stress. Diabetes 57:41–49

Chartoumpekis DV, Ziros PG, Psyrogiannis AI, Papavassiliou AG, Kyriazopoulou VE, Sykiotis GP, Habeos IG (2011) Nrf2 represses FGF21 during long-term highfat diet-induced obesity in mice. Diabetes 60:2465–2473

Choi EM (2007) Modulatory effects of luteolin on osteoblastic function and inflammatory mediators in osteoblastic MC3T3-E1 cells. Cell Biol Int 31:870–877

Choi EM (2011) Luteolin protects osteoblastic MC3T3-E1 cells from antimycin A-induced cytotoxicity through the improved mitochondrial function and activation of PI3K/Akt/CREB. Toxicol In Vitro 25:1671–1679

Choi IY, Lee SJ, Ju C, Nam W, Kim HC, Ko KH, Kim WK (2000) Protection by a manganese porphyrin of endogenous peroxynitrite-induced death of glial cells via inhibition of mitochondrial transmembrane potential decrease. Glia 31:155–164

Colhoun HM, Betteridge DJ, Durrington P, Hitman G, Neil A, Livingstone S, Charlton-Menys V, Bao W, Demicco DA, Preston GM, Deshmukh H, Tan K, Fuller JH (2011) Total soluble and endogenous secretory receptor for advanced glycation end products as predictive biomarkers of coronary heart disease risk in patients with type 2 diabetes: an analysis from the CARDS trial. Diabetes 60:2379–2385

Dalal M, Semba RD, Sun K, Crasto C, Varadhan R, Bandinelli S, Fink JC, Guralnik JM, Ferrucci L (2011) Endogenous secretory receptor for advanced glycation end products and chronic kidney disease in the elderly population. Am J Nephrol 33:313–318

Dore S (2005) Unique properties of polyphenol stilbenes in the brain: more than direct antioxidant actions; gene/protein regulatory activity. Neurosignals 14:61–70

Finkel T (2006) Cell biology: a clean energy programme. Nature 444:151–152

Finotti P, Pagetta A, Ashton T (2001) The oxidative mechanism of heparin interferes with radical production by glucose and reduces the degree of glycooxidative modifications on human serum albumin. Eur J Biochem 268:2193–2200

Funakoshi-Tago M, Nakamura K, Tago K, Mashino T, Kasahara T (2011) Anti-inflammatory activity of structurally related flavonoids, Apigenin, Luteolin and Fisetin. Int Immunopharmacol 11:1150–1159

Goldin A, Beckman JA, Schmidt AM, Creager MA (2006) Advanced glycation end products: sparking the development of diabetic vascular injury. Circulation 114:597–605

Hamada Y, Fujii H, Kitazawa R, Yodoi J, Kitazawa S, Fukagawa M (2009) Thioredoxin-1 overexpression in transgenic mice attenuates streptozotocininduced diabetic osteopenia: a novel role of oxidative stress and therapeutic implications. Bone 44:936–941

Hamann C, Kirschner S, Gunther KP, Hofbauer LC (2012) Bone, sweet bone—osteoporotic fractures in diabetes mellitus. Nat Rev Endocrinol 8:297–305

Hamann C, Picke AK, Campbell GM, Balyura M, Rauner M, Bernhardt R, Huber G, Morlock MM, Günther KP, Bornstein SR, Glüer CC, Ludwig B, Hofbauer LC (2014) Effects of parathyroid hormone on bone mass, bone strength, and bone regeneration in male rats with type 2 diabetes mellitus. Endocrinology 155:1197–1206

Itoh K, Chiba T, Takahashi S, Ishii T, Igarashi K, Katoh Y, Oyake T, Hayashi N, Satoh K, Hatayama I, Yamamoto M, Nabeshima Y (1997) An Nrf2/small Maf heterodimer mediates the induction of phase II detoxifying enzyme genes through antioxidant response elements. Biochem Biophys Res Commun 236:313–322

Jakubowski W, Bartosz G (2000) 2,7-Dichlorofluorescin oxidation and reactive oxygen species: what does it measure? Cell Biol Int 24:757–760

Karim L, Tang SY, Sroga GE, Vashishth D (2013) Differences in non-enzymatic glycation and collagen cross-links

between human cortical and cancellous bone. Osteoporos Int 24:2441–2447

Kayath MJ, Tavares EF, Dib SA, Vieira JG (1998) Prospective bone mineral density evaluation in patients with insulin-dependent diabetes mellitus. J Diabet Complications 12:133–139

Kilpadi KL, Eldabaje R, Schmitz JE, Ehler B, Thames TA, Joshi AP, Simmons JW 3rd, Michalek JE, Fajardo RJ (2013) Type 2 diabetes is associated with vertebral fractures in a sample of clinic- and hospital-based latinos. J Immigr Minor Health 16:440–449

Kim JW, Lim SC, Lee MY, Lee JW, Oh WK, Kim SK, Kang KW (2010) Inhibition of neointimal formation by trans-resveratrol: role of phosphatidyl inositol 3-kinasedependent Nrf2 activation in heme oxygenase-1 induction. Mol Nutr Food Res 54:1497–1505

Lang Y, Chen D, Li D, Zhu M, Xu T, Zhang T, Qian W, Luo Y (2012) Luteolin inhibited hydrogen peroxide-induced vascular smooth muscle cells proliferation and migration by suppressing the Src and Akt signalling pathways. J Pharm Pharmacol 64:597–603

Lee M, Cho T, Jantaratnotai N, Wang YT, McGeer E et al (2010) Depletion of GSH in glial cells induces neurotoxicity: relevance to aging and degenerative neurological diseases. FASEB J 24:2533–2545

Li W, Kong AN (2009) Molecular mechanisms of Nrf2-mediated antioxidant response. Mol Carcinog 48:91–104

Liu YW, Zhu X, Li W, Wang JY, Wei YQ, Yin XX (2012) Ginsenoside Re attenuates diabetesassociated cognitive deficits in rats. Pharmacol Biochem Behav 101:93–98

Mariappan N, Soorappan RN, Haque M, Sriramula S, Francis J (2007) TNF-α-induced mitochondrial oxidative stress and cardiac dysfunction: restoration by superoxide dismutase mimetic Tempol. Am J Physiol Heart Circ Physiol 293:H2726–H2737

Mariappan N, Elks CM, Fink B, Francis J (2009) TNF-induced mitochondrial damage: a link between mitochondrial complex I activity and left ventricular dysfunction. Free Radic Biol Med 46:462–470

Miniati M, Monti S, Basta G, Cocci F, Fornai E, Bottai M (2011) Soluble receptor for advanced glycation end products in COPD: relationship with emphysema and chronic corpulmonale: a case-control study. Respir Res 12:37

Mukhopadhyay P, Rajesh M, Kashiwaya Y, Haskó G, Pacher P (2007) Simple quantitative detection of mitochondrial superoxide production in live cells. Biochem Biophys Res Commun 358:203–208

Nagaraj RH, Sarkar P, Mally A, Biemel KM, Lederer MO, Padayatti PS (2002) Effect of pyridoxamine on chemical modification of proteins by carbonyls in diabetic rats: characterization of a major product from the reaction of pyridoxamine and methylglyoxal. Arch Biochem Biophys 402:110–119

Nakahira K, Haspel JA, Rathinam VAK, Lee SJ, Dolinay T, Lam HC, Englert JA, Rabinovitch M, Cernadas M, Kim HP, Fitzgerald KA, Ryter SW, Choi AM (2011) Autophagy proteins regulate innate immune responses by inhibiting the release ofmitochondrial DNA mediated by the NALP3 inflammasome. Nat Immunol 12:222–230

Nguyen T, Nioi P, Pickett CB (2009) The Nrf2-antioxidant response element signaling pathway and its activation by oxidative stress. J Biol Chem 284:13291–13295

Nicodemus KK, Folsom AR (2001) Type 1 and type 2 diabetes and incident hip fractures in postmenopausal women. Diabetes Care 24:1192–1197

Nisoli E, Tonello C, Cardile A, Cozzi V, Bracale R, Tedesco L, Falcone S, Valerio A, Cantoni O, Clementi E, Moncada S, Carruba MO (2005) Calorie restriction promotes mitochondrial biogenesis by inducing the expression of eNOS. Science 310:314–317

Nisoli E, Clementi E, Carruba MO, Moncada S (2007) Defective mitochondrial biogenesis: a hallmark of the high cardiovascular risk in the metabolic syndrome? Circ Res 100:795–806

Odani H, Shinzato T, Usami J, Matsumoto Y, Brinkmann Frye E, Baynes JW, Maeda K (1998) Imidazolium crosslinks derived from reaction of lysine with glyoxal and methylglyoxal are increased in serum proteins of uremic patients: evidence for increased oxidative stress in uremia. FEBS Lett 427:381–385

Ojuka EO, Jones TE, Han DH, Chen M, Holloszy JO (2003) Raising Ca^{2+} in L6 myotubes mimics effects of exercise on mitochondrial biogenesis in muscle. FASEB J 17:675–681

Patti ME, Corvera S (2010) The role of mitochondria in the pathogenesis of type 2 diabetes. Endocr Rev 31:364–395

Petit JM, Maftah A, Ratinaud MH, Julien R (1992) 10N-nonyl acridine orange interacts with cardiolipin and allows the quantification of this phospholipid in isolated mitochondria. Eur J Biochem 209:267–273

Peng X, Ma J, Chen F, Wang M (2011) Naturally occurring inhibitors against the formation of advanced glycation end-products. Food Funct 2:289–301

Pignatelli P, Cangemi R, Celestini A, Carnevale R, Polimeni L, Martini A, Ferro D, Loffredo L, Violi F (2008) Tumour necrosis factor α upregulates platelet CD40 L in patients with heart failure. Cardiovasc Res 78:515–522

Pope SA, Milton R, Heales SJ (2008) Astrocytes protect against copper-catalysed loss of extracellular glutathione. Neurochem Res 33:1410–1418

Puigserver P, Rhee J, Lin J, Wu Z, Yoon JC, Zhang CY, Krauss S, Mootha VK, Lowell BB, Spiegelman BM (2001) Cytokine stimulation of energy expenditure through p38 MAP kinase activation of PPARgamma coactivator-1. Mol Cell 8:971–982

Raposeiras-Roubin S, Rodino-Janeiro BK, Grigorian-Shamagian L, Moure-Gonzalez M, Seoane-Blanco A, Varela-Roman A, Alvarez E, Gonzalez-Juanatey JR (2010) Soluble receptor of advanced glycation end products levels are related to ischaemic aetiology and extent of coronary disease in chronic heart failure patients, independent of advanced glycation end products levels: new roles for soluble RAGE. Eur J Heart Fail 12:1092–1100

Scapagnini G, Butterfield DA, Colombrita C, Sultana R, Pascale A, Calabrese V (2004) Ethyl ferulate, a lipophilic polyphenol, induces HO-1 and protects rat neurons against oxidative stress. Antioxid Redox Signal 6:811–818

Schroeder P, Pohl C, Calles C, Marks C, Wild S, Krutmann J (2007) Cellular response to infrared radiation involves retrograde mitochondrial signaling. Free Radic Biol Med 43:128–135

Shanmugam N, Reddy MA, Guha M, Natarajan R (2003) High glucose-induced expression of proinflammatory cytokine and chemokine genes in monocytic cells. Diabetes 52:1256–1264

Shao X, Chen H, Zhu Y, Sedighi R, Ho CT, Sang S (2014) Essential structural requirements and additive effects for flavonoids to scavenge methylglyoxal. J Agric Food Chem 62:3202–3210

Shi R, Huang Q, Zhu X, Ong YB, Zhao B, Lu J, Ong CN, Shen HM (2007) Luteolin sensitizes the anticancer effect of cisplatin via c-Jun NH2-terminal kinase-mediated p53 phosphorylation and stabilization. Mol Cancer Ther 6:1338–1347

Suh KS, Choi EM, Rhee SY, Kim YS (2014) Methylglyoxal induces oxidative stress and mitochondrial dysfunction in osteoblastic MC3T3-E1 cells. Free Radical Res 48:206–217

Talukdar D, Chaudhuri BS, Ray M, Ray S (2009) Critical evaluation of toxic versus beneficial effects of methylglyoxal. Biochemistry (Mosc) 74:1059–1069

Tang SY, Sharan AD, Vashishth D (2008) Effects of collagen crosslinking on tissue fragility. Clin Biomech (Bristol, Avon) 23:122–123

Thomas MC, Baynes JW, Thorpe SR, Cooper ME (2005) The role of AGEs and AGE inhibitors in diabetic cardiovascular disease. Curr Drug Targets 6:453–474

Tolosa MJ, Chuguransky SR, Sedlinsky C, Schurman L, McCarthy AD, Molinuevo MS, Cortizo AM (2013) Insulin-deficient diabetes-induced bone microarchitecture alterations are associated with a decrease in the osteogenic potential of bone marrow progenitor cells: preventive effects of metformin. Diabetes Res Clin Pract 101:177–186

Ungvari ZI, Labinskyy N, Gupte SA, Chander PN, Edwards JG, Csiszar A (2008) Dysregulation of mitochondrial biogenesis in vascular endothelial and smooth muscle cells of aged rats. Am J Physiol Heart Circ Physiol 294:H2121–H2128

Villeneuve NF, Lau A, Zhang DD (2010) Regulation of the Nrf2-Keap1 antioxidant response by the ubiquitin proteasome system: an insight into cullin-ring ubiquitin ligases. Antioxid Redox Signal 13:1699–1712

Vitaglione P, Morisco F, Caporaso N, Fogliano V (2004) Dietary antioxidant compounds and liver health. Crit Rev Food Sci Nutr 44:575–586

Wang Y, Ho CT (2012) Flavour chemistry of methylglyoxal and glyoxal. Chem Soc Rev 41:4140–4149

Wang H, Meng QH, Chang T, Wu L (2006) Fructose-induced peroxynitrite production is mediated by methylglyoxal in vascular smooth muscle cells. Life Sci 79:2448–2454

Webster J, Urban C, Berbaum K, Loske C, Alpar A, Gartner U, de Arriba SG, Arendt T, Munch G (2005) The carbonyl scavengers aminoguanidine and tenilsetam protect against the neurotoxic effects of methylglyoxal. Neurotoxic Res 7:95–101

Wu CH, Yen GC (2005) Inhibitory effect of naturally occurring flavonoids on the formation of advanced glycation endproducts. J Agric Food Chem 53:3167–3173

Wu Z, Puigserver P, Andersson U, Zhang C, Adelmant G, Mootha V, Troy A, Cinti S, Lowell B, Scarpulla RC (1999) Mechanisms controlling mitochondrial biogenesis and respiration through the thermogenic coactivator PGC-1. Cell 98:115–124

Wu CH, Huang SM, Lin JA, Yen GC (2011) Inhibition of advanced glycation endproduct formation by foodstuffs. Food Funct 2:224–234

Yamagishi S, Matsui T (2010) Soluble form of a receptor for advanced glycation endproducts (sRAGE) as a biomarker. Front Biosci 2:1184–1195

Yamaguchi T, Sugimoto T (2011) Bone metabolism and fracture risk in type 2 diabetes mellitus. Endocr J 58:613–624

Zenkov NK, Menshchikova EB, Tkachev VO (2013) Keap1/Nrf2/ARE redoxsensitive signaling system as a pharmacological target. Biochemistry (Mosc) 78:19–36

Zhao Z, Liu J, Shi B, He S, Yao X, Willcox MD (2010) Advanced glycation end product (AGE) modified proteins in tears of diabetic patients. Mol Vis 16:1576–1584

Zhou R, Yazdi AS, Menu P, Tschopp J (2011) A role for mitochondria in NLRP3 inflammasome activation. Nature 469:221–225