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Kaempferol protects MC3T3-E1 cells through antioxidant effect and regulation of mitochondrial function

Eun Mi Choi *

Department of Food and Nutrition, Education Graduate School, Kyung Hee University, 1, Hoegi-dong, Dongdaemun-gu, Seoul 130-701, South Korea

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ABSTRACT

Kaempferol, a natural flavonoid present in fruits, vegetables, and teas, provides beneficial effects for human health. In this study, we investigated the protective effects of kaempferol on antimycin A (AMA)-induced toxicity in osteoblast-like MC3T3-E1 cells. Exposure of MC3T3-E1 cells to AMA caused significant cell viability loss, as well as mitochondrial membrane potential dissipation, complex IV inactivation, intracellular calcium ($[Ca^{2+}]_i$) elevation, and reactive oxygen species (ROS) production. Pretreatment with kaempferol prior to AMA exposure significantly reduced AMA-induced cell damage by preventing mitochondrial membrane potential dissipation, complex IV inactivation, $[Ca^{2+}]_i$ elevation, and ROS production. Kaempferol also induced the activation of PI3K (phosphoinositide 3-kinase), Akt (protein kinase B), and CREB (cAMP-response element-binding protein) inhibited by AMA, which result demonstrates that kaempferol utilizes the PI3K/Akt/CREB pathway to augment metabolic activity inhibited by AMA. All these data indicate that kaempferol may reduce or prevent osteoblasts degeneration in osteoporosis or other degenerative disorders.

1. Introduction

Aging is a multi-faceted process associated with several functional and structural deficits, and also often associated with major defects on the skeleton itself. Aging-related bone diseases are comprised of three major classes: the erosion of the joints or osteoarthritis; an increase in bone resorption leading to osteoporosis, a low bone mass disease with high risk of fracture; and metastatic bone disease that relates to breast cancer in women or prostate cancer in men. Osteoarthritis and osteoporosis are not only the most frequent degenerative diseases of the skeleton, they are also the most frequent degenerative diseases in developed countries (Cooper and Melton, 1996). In aging, there are alterations in Ca^{2+} handling that may affect the bioenergetic and mitochondrial functions and may contribute to cell death process. The production of reactive oxygen species (ROS) may cause a leak of Ca^{2+} from stores in the intracellular compartments. Transient increase in Ca^{2+} is essential element in the control of many physiological processes. However, sustained increases in Ca^{2+} may contribute to oxidative stress and cell death. It is well known that intracellular Ca^{2+} overload causes mitochondrial Ca^{2+} accumulation, change in the mitochondrial pH, increase of the ROS production, decrease or complete loss of mitochondrial membrane potential and opening of the mitochondrial permeability transition pore.

Several reports showed that inhibitors of the mitochondrial electron transport chain induce cell injuries *in vitro* and *in vivo* (Brouillet et al., 1993; Smith and Bennett, 1997). Mitochondria energy metabolism is extremely sensitive to impairment by free radicals and that mitochondrial oxidative stress limits metabolic recovery (Fiskum et al., 2004). Damage of mitochondria leads to cell death, because mitochondria are involved in energy metabolism and calcium homeostasis. Chemicals generating ROS, such as rotenone and antimycin A (AMA), decrease cell viability and reduce mitochondrial membrane potential. AMA is an inhibitor of mitochondrial electron transport via its binding to complex III. Though it is generally accepted that mitochondrial complexes have the potential to generate superoxide anions leading to oxidative stress, complex III is a major site for ROS generation (Dawson et al., 1993) since inhibition of complex III by antimycin A in isolated liver mitochondria results in the accumulation of an ubisemiquinone intermediate with subsequent superoxide anion production (Garcia-Ruiz et al., 1995).

Kaempferol belongs to the flavonol group and is present at high levels in broccoli, chives, and kale. Recently, food flavonoids have attracted great interest owing to their apparent beneficial effects on human health. The health-promoting effects of flavonoids have been attributed to their antioxidant activities. Wattel et al. (2003) demonstrated that flavonols such as kaempferol decrease osteoclastic bone resorption *in vitro* by targeting directly the mature osteoclast by a mechanism involving, at least in part, the estrogen receptor (ER). Miyake et al. (2003) reported the promoting effect of kaempferol on the differentiation and mineralization of murine

* Tel.: +82 010 4246 8023; fax: +82 2 968 0260.
E-mail address: cheunmi@hanmail.net

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pre-osteoblastic cell line. Prouillet et al. (2004) showed that the kaempferol increases the alkaline phosphatase activity, and that this effect is dependent upon both the ERK pathway and the ER activation. A model commonly used to study osteogenic development is the MC3T3-E1 osteoblast-like cell line (Quarles et al., 1992). The MC3T3-E1 cell culture system represents a very useful model for studying the process of osteoblast function. Therefore, we investigated the protective effects of kaempferol against AMA-induced cytotoxicity using osteoblast-like MC3T3-E1 cells.

2. Materials and methods

2.1. Materials

Kaempferol and antimycin A (AMA) 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). Other reagents were of the highest commercial grade available and purchased from Sigma Chemical (St. Louis, MO, USA).

2.2. Cell culture

Murine osteoblast-like MC3T3-E1 cells were cultured at 37 °C in 5% CO_2 atmosphere in α-MEM. Unless otherwise specified, the medium contained 10% heat-inactivated FBS, 100 U/ml penicillin and 100 μg/ml streptomycin. The cultures have fully undergone differentiation in the presence of ascorbic acid and β-glycerophosphate for at least 7 days (Fatokun et al., 2006). The cells were treated, at confluence, with culture medium containing 10 mM β-glycerophosphate and 50 μg/ml ascorbic acid to initiate differentiation. After 3 days as a point of early differentiation, the medium was removed and the cell monolayer was gently washed twice with DPBS. Then the medium was replaced with media supplemented with kaempferol. After 1 h, AMA was added in the medium and further incubated
for 48 h.

2.3. Metabolic activity

The overall metabolic activity in the cell populations was determined via reduction of 3-(4,5-dimethyl-thiazol-2yl)-2,5-diphenyl tetrazolium bromide (MTT) by NAD-dependent dehydrogenase activity to form a colored reaction product. MTT (0.5 mg/ml in PBS) 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. Measurement of complex IV activity

Microplate assay for complex IV activity (MitoSciences, USA) was used to determine the activity of the cytochrome *c* oxidase enzyme. Complex IV is immunocaptured within the wells and activity is determined colorimetrical by following the oxidation of reduced cytochrome *c* as an absorbance decrease at 550 nm. Protein concentrations were determined using the BioRad protein assay reagent.

2.5. Determination of mitochondrial membrane potential

JC-1 mitochondrial membrane potential assay kit (Cayman Chemical Co., USA) was used to demonstrate the changes in the mitochondrial membrane potential in cells. JC-1 is a lipophilic and cationic dye, which permeates plasma and mitochondrial membranes. The dye fluoresces red when aggregates in healthy mitochondria with high membrane potential, whereas it appears in monomeric form and fluoresces green in mitochondria with diminished membrane potential. Cells were incubated with the mitochondrial membrane potential-sensitive fluorescent dye JC-1 for 20 min at 37 °C, washed twice in PBS, and then the "red" (excitation 550 nm, emission 600 nm) and "green" (excitation 485 nm, emission 535 nm) fluorescence were measured. Mitochondrial depolarization (i.e., loss of mitochondrial membrane potential) manifests itself by a decrease in the red/green fluorescence ratio.

2.6. Intracellular Ca^{2+} measurement

Cells were treated with 5 μM Fura-2/AM for 1 h at 37 °C. Following washing with DPBS, fluorescence emission (510 nm) was monitored with the excitation wavelength of 340 and 380 nm.

2.7. Measurement of intracellular reactive oxygen species

Cells were loaded with 5 μM 2′,7′-dichlorofluorescin diacetate (DCFH-DA) for 1 h. Following washing with DPBS, ROS levels were determined by measuring the fluorescent intensity at excitation wavelength 485 nm and emission wavelength 530 nm.

2.8. Measurement of mitochondrial superoxide

Mitochondrial superoxide levels were detected using MitoSOX™ Red mitochondrial superoxide indicator (Invitrogen Molecular Probes, Carlsbad, CA). MitoSOX™ Red (Ex/Em = 510 nm/580 nm) is a fluorogenic dye for highly selective detection of superoxide in the mitochondria of cells. Cells were incubated with 5 μM MitoSOX™ Red at 37 °C for 20 min according to the instructions of the manufacturer. After the washing of cells, the MitoSOX™ Red fluorescences were detected.

2.9. Measurement of nitrotyrosine content, and thioredoxin reductase activity

Cells were lysed by homogenization in lysis buffer [20 mM MOPS, 50 mM sodium fluoride, 1 mM sodium vanadate, 5 mM EGTA, 2 mM EDTA, 1% NP-40, 1 mM DTT, 1 mM benzamidine, 1 mM PMSF, and 1% protease inhibitor cocktail (pH 7.2)]. After centrifugation at 10,000g for 15 min at 4 °C, supernatant was used as cell lysates for assay. As a marker of peroxynitrite effect on cell proteins, nitrotyrosine content was measured by using an ELISA kit (Northwest Life Science, USA). The activity of thioredoxin reductase was measured using enzyme activity assay kit (Cayman Chemical, USA).

2.10. Measurement of PI3K and Akt activities and phosphorylated CREB

PI3K activity in cell lysates was evaluated using PI3 kinase ELISA kit (Echelon Biosciences, USA). The Akt (PKB) activity in cell lysates was assayed according to manufacturer's instructions (Assay Designs, USA). Phosphorylated CREB was evaluated using cell-based phospho-CREB (S133) immunoassay (R&D Systems. USA).

2.11. Statistical analysis

The results are expressed as mean ± S.E.M. ($n = 6$). 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 kaempferol on the metabolic activity of MC3T3-E1 cells in the presence of AMA

The overall metabolic activity in the cell populations was determined via reduction of MTT by NAD-dependent dehydrogenase activity to form a colored reaction product. The MTT assay provided information about the overall metabolic activity, because it was shown that most cellular reduction of MTT is dependent on the reduced pyridine nucleotides. In our system, the concentration at which 50% growth inhibition (IC50) for AMA in osteoblastic MC3T3-E1 cells was 70 μM (data not shown), and 70 μM was chosen as the optimal dose of AMA for the experiments of -AMA-induced cell damages. Also, kaempferol did not result in cytotoxicity at the concentrations of less than 0.3 μM, and so these concentrations of kaempferol were pretreated in the cells. AMA (70 μM) significantly decreased the metabolic activity of osteoblasts, whereas kaempferol pretreatment (0.03–0.3 μM) increased metabolic activity compared with AMA-treated cells (Fig. 1). Our result demonstrates that kaempferol can decrease osteoblast death induced by AMA.

3.2. Effect of kaempferol on the AMA-induced mitochondrial dysfunction in MC3T3-E1 cells

To investigate the mitochondrial function of osteoblasts, mitochondrial membrane potential, complex IV activity, and intracellular calcium concentration ($[Ca^{2+}]_i$) were assayed after osteoblasts were treated with AMA in the presence or absence of kaempferol. The mitochondrial membrane potential is a marker of mitochon-