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Protective effects of honokiol against methylglyoxal-induced osteoblast damage

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ABSTRACT

Honokiol is an active compound isolated from *Magnolia officinalis* that has been used without notable side effects in traditional medicine. We investigated the effects of honokiol against methylglyoxal (MG)-induced cytotoxicity in MC3T3-E1 osteoblast cells and the possible molecular mechanism(s) involved. The results showed that honokiol alleviated MG-induced cell death and the production of intracellular ROS, mitochondrial superoxide, cardiolipin peroxidation, and inflammatory cytokines. MG induction of the soluble receptor for advanced glycation end product (AGE) was reduced by pretreatment with honokiol. Furthermore, honokiol increased the levels of Nrf2 and increased the levels of glutathione and the activity of glyoxalase I. Pretreatment with honokiol prior to MG exposure reduced MG-induced mitochondrial dysfunction and alleviated MG-induced reduction of nitric oxide and PGC1α levels, suggesting that honokiol may induce mitochondrial biogenesis. It was concluded that honokiol could be useful in the attenuation of MG-induced cell damage.

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1. Introduction

Methylglyoxal (MG), a three-carbon α-ketoaldehyde, is formed by the enzymatic and non-enzymatic fragmentation of glyceraldehyde-3-phosphate and dihydroxyacetone phosphate. It is also formed during ketone body metabolism from acetone and in the catabolism of threonine [1]. Methylglyoxal can react with proteins under physiological conditions, initially by reversible reactions and subsequently by irreversible reactions, to form cross-links between amino acids [2]. Ultimately, there is modification of DNA and long-lived proteins, such as collagen and elastin, all of which accelerate the aging process and trigger the onset of various diseases. Because MG is highly cytotoxic, the body has developed several detoxification mechanisms. One of these is the glyoxalase enzyme system, which functions primarily to remove MG [3]. Using glutathione (GSH) as a cofactor, glyoxalase I catalyzes the formation of S-2-hydroxyacylglutathione from MG. Glyoxalase II converts S-2-hydroxyacylglutathione into D-lactate and GSH. NF-E2-related factor (Nrf2) is critical in the oxidative stress signaling pathway. Nrf2 is a basic leucine zipper transcription factor that binds to the antioxidant-responsive element (ARE) and may serve as a master regulator of the entire antioxidant system in cellular defense pathways [4].

Formation of advanced glycation end products (AGEs) results from an endogenous process that leads to posttranslational modifications of proteins, with evidence suggesting a role for AGEs in the development of degenerative conditions, including diabetic complications. Among the many reactive carbonyl compounds and AGE precursors, MG contributes significantly to intracellular AGE formation [5]. Furthermore, advanced glycation and oxidative stress are closely linked [6]. All steps of glycoxidation generate reactive oxygen species (ROS), some of which intersect with lipid peroxidation pathways [7]. AGEs may upregulate the membrane-anchored receptor for AGEs (RAGE). Engagement of RAGE by AGEs can activate transcription factor NF-κB, which further increases oxidative stress by increasing ROS formation [8]. RAGE is expressed in both osteoclasts and osteoblasts and contributes to bone remodeling [9].

Furthermore, the soluble form of RAGE (sRAGE) could reflect tissue RAGE expression and has been proposed as a potential biomarker for cardiovascular disease in diabetic patients [10,11]. Additionally, MG-induced ROS and AGEs can impair mitochondrial function, resulting in further ROS production and damage. Increased mitochondrial production of ROS appears to be a key

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event in the development of aging-induced pathologies [12]. Mitochondria undergo constant biogenesis, controlled primarily by the gene expression and post-translational modification of peroxisome proliferator-activated receptor γ co-activator 1α (PGC-1α) [13]. It has been suggested that expression of mitochondrial biogenesis factors may be directly regulated by the bioavailability of nitric oxide (·NO) [14].

Magnolia officinalis is an important medicinal herb used widely in traditional medicine to treat fever, headache, anxiety, and nervous disturbances [15]. Honokiol is an active compound isolated from *M. officinalis* [16] that has been used without notable side effects in traditional medicine [17]. Honokiol has been reported to have several pharmacological properties, including anti-inflammatory [18], antioxidant [19], and anti-bacterial [20] effects. It has been reported that honokiol isolated from *M. officinalis* stimulates osteoblast function and protects osteoblasts against antimycin A-induced mitochondrial dysfunction [21,22]. However, no report has described the effects of honokiol on reactive carbonyl compound-induced glucotoxicity in osteoblastic cells. MG toxicity may be involved in diabetes-associated bone loss. Studies investigating the effects of diabetes on osteoporosis have shown that patients with diabetes have high rates of bone resorption and turnover and decreased bone mineral density [23]. Additionally, Chan et al. [24] revealed that MG treatment triggers apoptotic biochemical changes in human osteoblasts and showed that MG treatment could cause bone mineral density loss in an animal model. In the present study, we investigated the protective mechanisms of honokiol against MG-induced cytotoxicity in osteoblastic MC3T3-E1 cells.

2. Materials and methods

2.1. Materials

Honokiol, isolated from *M. officinalis*, 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, USA). Other reagents were of the highest commercial grade available and purchased from Sigma Chemical (St. Louis, MO, USA).

2.2. Cell culture

Murine osteoblastic MC3T3-E1 cell line was obtained from the American Type Culture Collection (USA). MC3T3-E1 cells were cultured at 37 °C in a 5% CO2 atmosphere in α-MEM (GIBCO). Unless otherwise specified, the medium contained 10% 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. At 48 h after 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 were evaluated quantitatively using MTT assay. This assay is based on the ability of viable cells to convert soluble 3-(4,5-dimethyl-thiazol-2yl)-2,5-diphenyl tetrazolium bromide (MTT) into an insoluble dark blue formazan reaction product. MTT (20 μL) in phosphate-buffered saline, pH 7.4 (5 mg/mL), was added to each well, and the plates were incubated for an additional 2 h. After removal of the solution from the well, dimethyl sulfoxide was added to dissolve the 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. LDH cytotoxicity assay

Cytotoxicity was assessed by measuring the release of the cytosolic enzyme lactic dehydrogenase (LDH) from damaged cells. Release of LDH was used as a marker for an evaluation of cell membrane integrity. LDH activity was measured in the supernatants using an *in vitro* cytotoxicity assay kit (Promega, USA) in accordance with the manufacturer's protocol. The absorbance was determined at 490 nm using a plate reader. Cells in the positive control group were treated with Triton X-100 solution (Sigma Aldrich, Germany), and those in the negative control group were incubated in culture medium alone. Percentage of cytotoxicity was calculated as: (experimental value − negative control)/(positive control − negative control) × 100.

2.5. Measurement of glyoxalase I activity

The cells were lysed and homogenized in PBS. Cell homogenates were centrifuged (13,000*g*, 15 min, 4 °C), and the supernatant was used for assays and protein content measurements. Glyoxalase I activity was measured using a modification of a previously published method [25]. To measure glyoxalase I activity, 50 μL sample were loaded onto a UV microplate and 200 μL reaction mixture added. The reaction mixture consisted of 60 mM sodium phosphate buffer, pH 6.6, containing 4 mM GSH and 4 mM MG and was pre-incubated for 10 min at 37 °C. *S*-lactoylglutathione synthesis was evaluated by measuring the absorbance at 240 nm for 5 min at 25 °C.

2.6. Measurement of reduced GSH

Cells were lysed by homogenization in cold buffer containing 50 mM MES or phosphate (pH 6.7) and 1 mM EDTA. After centrifugation (10,000*g*, 15 min, 4 °C), the supernatant was used for the assay. GSH was measured using BioAssay Systems' QuantiChrom™ Glutathione Assay Kit (BioAssay Systems, Hayward, CA, USA), which is designed to accurately measure reduced glutathione in biological samples. DTNB (5′,5-dithiobis-2-nitrobenzoic acid) reacts with reduced glutathione to form a yellow product. The optical density, measured at 412 nm, is directly proportional to glutathione concentration in the sample.

2.7. Measurement of TNF-α and IL-6

TNF-α and IL-6 contents in the medium were measured using an enzyme immunoassay system (R&D Systems Inc., Minneapolis, MN, USA), according to the manufacturer's protocol. Protein concentrations were determined using the Bio-Rad protein assay reagent (Bio-Rad Laboratories, USA).

2.8. Measurement of RAGE and sRAGE levels

The cells were lysed and homogenized in PBS. Cell homogenates were centrifuged (13,000*g*, 15 min, 4 °C), and the supernatant was used for ELISA and protein content measurements. RAGE and sRAGE were measured using the Mouse RAGE ELISA kit (RayBio, USA) and sRAGE ELISA kit (MyBioSource, USA) ELISA kit (USCN Life Science, USA). Protein concentrations were determined using the Bio-Rad protein assay reagent.

2.9. Measurement of intracellular ROS

Steady-state ROS level was measured using 2′,7′-dichlorodihydrofluorescein diacetate (H2DCFDA) [26]. Viable cells can deacetylate H2DCFDA into the non-fluorescent derivative 2′,7′-

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