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Butein protects human dental pulp cells from hydrogen peroxide-induced oxidative toxicity via Nrf2 pathway-dependent heme oxygenase-1 expressions

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ABSTRACT

Rhus verniciflua Stokes is a plant that is native to East Asian countries, such as Korea, China, and Japan. Butein, a plant polyphenol, is one of the major active components of *R. verniciflua*. Reactive oxygen species (ROS), produced via dental adhesive bleaching agents and pulpal disease, can cause oxidative stress. Here, we found that butein possesses cytoprotective effects on hydrogen peroxide (H_2O_2)-induced dental cell death. H_2O_2 is a representative ROS and causes cell death through necrosis in human dental pulp (HDP) cells. H_2O_2-induced cytotoxicity and production of ROS were blocked in the presence of butein, and these effects were dose dependent. Butein also increased heme oxygenase-1 (HO-1) protein expression and HO activity. In addition, butein-dependent HO-1 expression was required for the inhibition of H_2O_2-induced cell death and ROS generation. Furthermore, butein treatment caused nuclear accumulation of nuclear factor-E2-related factor 2 (Nrf2) and increased the promoter activity of antioxidant response elements (AREs). Treatment of HDP cells with a c-Jun NH2-terminal kinase (JNK) inhibitor also reduced butein-induced HO-1 expression, and butein treatment led to increased JNK phosphorylation. These results indicate that butein may be used to prevent functional dental cell death and thus may be useful as a pulpal disease agent.

1. Introduction

Human dental pulp (HDP) is the living connective tissue in the center of a tooth made up of cells called odontoblasts. The primary function of the dental pulp is the formation of dentin by odontoblasts. The dentine–pulp complex is formed by differentiated and undifferentiated cell populations, including odontoblasts, odontoblast-like cells, and pulp stem cells (Sloan and Smith, 2007). Cells are damaged by imbalances in the systems generating and scavenging reactive oxygen species (ROS). In addition, ROS, produced by dental adhesive bleaching agents and pulpal disease, can cause oxidative stress in dental cells (Min et al., 2008). The inducible protein heme oxygenase-1 (HO-1) is expressed in response to oxidative stress and exhibits various cytoprotective effects, which are mostly associated with the different by-products of heme catabolism, with the exception of its ability to remove pro-oxidant heme molecules. HO-1 degrades heme to generate carbon monoxide (CO), biliverdin, and free iron (Montellano, 2000). HO-1 and its enzymatic by-products play key roles in regulating biological responses, including oxidative stress and inflammation (Ryter et al., 2002). Previously, we reported that the HO-1 pathway is involved in the adaptation of dental cells, including HDP cells and periodontal ligament (PDL) cells, to stressful conditions and mediates their recovery from injurious events (Jeong et al., 2010a,b; Min et al., 2006). The mitogen-activated protein kinase (MAPK) pathway is one of the most common signaling pathways; it functions to coordinate the cellular response to a variety of extracellular stimuli. MAPKs play important roles in cellular processes, such as proliferation, stress responses, apoptosis, and immune defense (Liu et al., 2007). MAPK activation also modulates the expression of several genes and proteins, including HO-1 (Iles et al., 2005). Among the various anti-oxidative enzymes, nuclear factor-E2-related factor 2 (Nrf2) plays a key role in protection of cells against oxidative stress (Baird and Dinkova-Kostova, 2011). Nrf-2 is a basic leucine zipper transcription factor that is localized to the cytoplasm, bound to its inhibitor protein Kelch-like ECH-associated protein 1 (Keap 1), under normal conditions (Tkachev et al., 2011). The complex is disrupted by exposure to several stimuli, and free Nrf2 translocates to the nucleus to form heterodimers with small oncogene family proteins. This leads to the selective recognition of the antioxidant response element (ARE) on target genes, resulting in the regulation

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of gene expression of phase II detoxifying enzymes, including HO-1. It is well known that nuclear translocation of activated Nrf2 is an important upstream contributor to induction of HO-1 expression (Numazawa et al., 2003).

Rhus verniciflua Stokes (Anacadiaceae) is a plant that is native to East Asian countries, such as Korea, China, and Japan, and is used as a natural dye, food additive, and herbal medicine; in this context, it has been reported to improve blood circulation and prevent blood stasis (Kitts and Lim, 2001; Samoszuk et al., 2005). Butein (3,4,2′,4′-tetrahydroxychalcone), a plant polyphenol, is one of the major active components of *R. verniciflua*. Butein is known to have many pharmacological effects in various *in vitro* and *in vivo* models (Kang et al., 2004; Sogawa et al., 1994; Lee et al., 2002; Yu et al., 1995; Iwashita et al., 2000), including antioxidant and anti-inflammatory properties (Sogawa et al., 1994; Lee et al., 2002). However, to date, no studies have described the protective effects of butein in dental cells. In the present study, we found that butein possesses cytoprotective effects on H_2O_2-induced dental cell death. Our data showed that butein inhibited H_2O_2-induced cell death and ROS production through regulation of HO-1 expression. These results indicate that butein, a natural compound isolated from *R. verniciflua*, may be used to prevent functional dental cell death and thus may be useful as a pulpal disease agent.

2. Materials and methods

2.1. Preparation of butein

Butein (>95% pure) was isolated from the bark of *R. verniciflua* as described by Kang et al. (2004). Dried *R. verniciflua* bark was purchased from the University Oriental herbal drugstore, Iksan, Korea in March 2004, and a sample specimen (No. WP 04-12) was deposited at the Herbarium of the College of Pharmacy, Wonkwang University, Korea. Butein (NNMBP1) was deposited at the New Natural Material Bank of the College of Pharmacy, Wonkwang University, Korea. Butein was dissolved in dimethyl sulfoxide (DMSO) to make a 30 mM solution (stock solution) and then diluted with medium. The final DMSO concentration in each experimental and control well was kept constant at 0.1%. This final DMSO concentration had no relevant effects on cellular growth or survival in our assay.

2.2. Chemicals and reagents

Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), and other tissue culture reagents were purchased from Gibco BRL Co. (Gaithersburg, MD, USA). Tin protoporphyrin IX (SnPP IX), an inhibitor of HO activity, was obtained from Porphyrin Products (Logan, UT). Primary antibodies, including HO-1, phosphorylated extracellular signal-regulated kinase 1/2 (p-ERK), phosphorylated JNK (p-JNK), or phosphorylated p38 (p–p38) and secondary antibodies used for western blot analysis were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Tissue culture plates (96-well) and other tissue culture dishes were obtained from Falcon (Biosciences, Oxford, UK). All other chemicals, including 3′-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and DMSO, were obtained from Sigma Chemical Co. (St. Louis, MO, USA), unless otherwise noted.

2.3. Cell culture

Immortalized HDP cell lines obtained by transfection with the telomerase catalytic subunit human telomerase reverse transcriptase gene were used for this study (Kitagawa et al., 2007). Cells were cultured in DMEM supplemented with 10% FBS, 100 U/mL penicillin, and 100 μg/mL streptomycin in a humidified atmosphere of 5% CO_2 at 37 °C.

2.4. MTT assay for cell viability

The effects of various experimental treatments on cell viability were evaluated by determining mitochondrial reductase functionality on the basis of the reduction of a tetrazolium salt, MTT, into formazan crystals (Mosmann, 1983). The formation of formazan is proportional to the number of functional mitochondria in living cells. After the treatment with hydrogen peroxide (H_2O_2) (1 mM) for 12 h, cells were washed twice with PBS, and the MTT solution was added at a final concentration of 50 μg/mL to each well. After 4 h incubation at 37 °C, the medium was discarded and the formazan blue that formed in the cells was dissolved in 150 μL DMSO. The optical density was determined with a microplate reader at 570 nm, and the optical density of control (untreated) cells was taken as 100% viability.

2.5. Measurement of ROS

For the measurement of ROS, HDP cells (2.5×10^4 cells/well in 24-well plates) were treated with 1 mM H_2O_2 in the presence or absence of butein, cobalt protoporphyrin (CoPP, a HO-1 inducer) or SnPP (an HO inhibitor) and incubated for 8 h. After washing with PBS, the cells were stained with 10 μM dichlorofluorescein diacetate (DCFDA) in Hanks' balanced salt solution for 30 min in the dark. Cells were then washed twice with PBS and extracted with 1% Triton X-100 in PBS for 10 min at 37 °C. Fluorescence was recorded with an excitation wavelength of 490 nm and an emission wavelength of 525 nm (Spectramax Gemini XS; Molecular Devices, Sunnyvale, CA).

2.6. Western blot analysis

Western blot analysis was performed by lysing cells in 20 mM Tris–HCl buffer (pH 7.4) containing a protease inhibitor mixture (0.1 mM phenylmethanesulfonyl fluoride, 5 mg/mL aprotinin, 5 mg/mL pepstatin A, and 1 mg/mL chymostatin). Protein concentrations were determined using the Lowry protein assay kit (P5626; Sigma). An equal amount of protein for each sample was resolved using 7% or 12% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE), and proteins were then electrophoretically transferred onto a Hybond enhanced chemiluminescence (ECL) nitrocellulose membrane (Bio-Rad, Hercules, CA). The membrane was blocked with 5% skim milk and sequentially incubated with anti-HO-1, anti-Nrf2, anti-phospho JNK, anti-JNK, anti-phospho ERK, anti-ERK, anti-phospho p38, anti-p38, anti-actin or anti-lamin B at 4 °C overnight (all antibodies were used at a 1:1000 dilution and were purchased from Santa Cruz Biotechnology, CA, USA). Immunoreactive bands were visualized by horseradish peroxidase-conjugated secondary antibodies (1:1000 dilution, Santa Cruz Biotechnology) followed by ECL detection (Amersham Pharmacia Biotech, Piscataway, NJ) and were quantified using an imaging program (Image Gauge v3.12 software, Fujifilm, Tokyo, Japan).

2.7. Plasmids, transfections, and luciferase assays

To construct the antioxidant response element (ARE)-luciferase vector, tandem repeats of double-stranded oligonucleotides spanning the Nrf2 binding site (5′-TGACTCAGCA-3′) were introduced into the restriction sites of the pGL2 promoter plasmid (Madison, WI). All transfection experiments were performed using lipofectamine reagent (Invitrogen, Carlsbad, CA) according