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Effects of sappanchalcone on the cytoprotection and anti-inflammation *via* heme oxygenase-1 in human pulp and periodontal ligament cells

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ABSTRACT

Sappanchalcone has been demonstrated to possess several biological effects. However, the molecular mechanism underlying these effects is not fully understood. In this study, we examined the effects of sappanchalcone on hydrogen peroxide (H_2O_2)-induced cytotoxicity using human dental pulp (HDP) cells, and lipopolysaccharide (LPS)-induced inflammation using human periodontal ligament (HPDL) cells. Sappanchalone concentration proportionately increased heme oxygenase (HO)-1 protein expression and enzyme activity in both HDP and HPDL cells. It also protected HDP cells from H_2O_2-induced cytotoxicity and reactive oxygen species production. The cytoprotective effect of sappanchalcone was nullified by HO-1 inhibitor, Tin protoporphyrin (SnPP). Sappanchalcone is seen to inhibit LPS-stimulated nitric oxide (NO), prostaglandin E_2 (PGE_2), interlukine-1β (IL-1β), tumor necrosis factor-α (TNF-α), interlukine-6 (IL-6) and interlukine-12 (IL-12) release in addition to inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) expression in HPDL cells. SnPP, a specific inhibitor of HO-1, partly blocked sappanchalcone mediated suppression of inflammatory mediator production, in LPS-stimulated HPDL cells. HDP and HPDL cells treated with sappanchalcone exhibited the transient activation of c-Jun NH2-terminal kinase (JNK) and NF-E2-related factor-2 (Nrf2). The expression of HO-1 protein by sappanchalcone was significantly reduced by pretreatment with JNK inhibitor. In conclusion, induction of HO-1 is an important cytoprotective mechanism by which sappanchalcone protects HDP cells from H_2O_2 and in addition it also exhibits anti-inflammatory effects in LPS-stimulated HPDL cells. Thus, sappanchalcone could potentially be a therapeutic approach for periodontal, pulpal and periapical inflammatory lesion.

1. Introduction

Teeth with severe pulpal or periapical inflammation can result in the disruption of the structural integrity of the human dental pulp (HDP), human periodontal ligament (HPDL) and can cause progressive alveolar bone destruction. Intracanal medications have been known to reduce inflammatory response of periapical regions and also minimize wound exudation (Walton, 1984). Calcium hydroxide, paramonochlorophenol derivatives, chlorhexidine digluconate solutions and a combination of sodium lauryl sulfate with saturated calcium hydroxide solution have been used previously as root canal disinfectants (Walton, 1984; Barbosa et al., 1994; Siqueira and Lopes, 1999). However, high pH of calcium hydroxide makes it potentially cytotoxic and tends to dissolve soft tissues, which results in chronic inflammation and cell necrosis (Rams and Slots, 1996). Thus, newer developments in the line of cytoprotective and anti-inflammatory actions for intracanal medication have been recommended.

Previous studies have reported that agents like the peroxisome proliferator activated receptor gamma agonist (Yu et al., 2009), copper–zinc superoxide dismutase (Baumgardner and Sulfaro, 2001) and inducible nitric oxide synthase inhibitor (Kawashima et al., 2005) have a beneficial anti-inflammatory effect for the treatment of rat pulpitis. Natural products are now emerging to play a significant role in drug discovery and development, especially those directed against the inflammatory process. Plant products, such as *Prunella vulgaris* extract, rosmarinic acid (Zdarilová et al., 2009) and curcumin (Chen et al., 2008) inhibit lipopolysaccharide (LPS)-induced inflammatory mediators in human gingival fibroblasts and macrophages. Terrein, a bioactive fungal metabolite is known to reduce pulpal inflammation in HDP cells (Lee et al., 2008). In addition, propolis, a flavonoid-rich product of honey comb, exerts minimal toxicity on HPDL cells and HDP cells, a possible alternative as an intracanal antimicrobial agent (Al-Shaher et al., 2004).

Sappanchalcone is a bioactive flavonoid isolated from *Caesalpinia sappan* L., known to be used in traditional Chinese medicine. In Oriental traditional medicine, *C. sappan* is commonly used to treat emmeniopathy,

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sprains and convulsions for its analgesic and anti-inflammatory effects (Baek et al., 2000). Several evidences have demonstrated that sappanchalcone exerts many biological effects, including neuroprotective (Moon et al., 2009), anti-inflammatory (Washiyama et al., 2009), inhibition of antigen-induced beta hexosaminidase release (Yodsaoue et al., 2009) and anti-influenza viral activity (Liu et al., 2009). Furthermore, it is reported that sappanchalcone increased cellular resistance to oxidative injury caused by glutamate-induced cytotoxicity in mouse hippocampal cells *via* Nrf2-dependent HO-1 expression (Jeong et al., 2009). However, the molecular mechanism underlying sappanchalcone's anti-inflammatory and cytoprotective activity and its potential as pulp capping materials or root canal medicament for pulp and periapical disease still remain elusive.

This study was conducted to investigate the beneficial effects of sappanchalcone in pulp and periapical tissues, by demonstrating the effects of sappanchalcone on the hydrogen peroxide-induced cytotoxicity and LPS-induced inflammatory mediators by HDP and HPDL cell cultures *in vitro*. In addition, the potential involvement of HO-1 and related signal transduction mechanisms under the influence of sappanchalcone were also examined.

2. Materials and methods

2.1. Chemicals and reagents

Sappanchalcone was isolated from the heartwood of *C. sappan* as described by Jeong et al., 2009. Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), and other tissue culture reagents were purchased from Gibco BRL Co. (Grand Island, NY). Tin protoporphyrin IX (SnPP IX), an inhibitor of HO activity, was obtained from Porphyrin Products (Logan, UT). Primary antibodies, including HO-1, inducible nitric oxide synthase (iNOS), cyclooxygenase (COX-2), phosphorylated ERK1/2 (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). Enzyme-linked immunosorbent assay (ELISA) kits for prostaglandin E_2 (PGE_2), tumor necrosis factor-α (TNF-α), interlukine-1β (IL-1β), interlukine-6 (IL-6) and interlukine-12 (IL-12) were purchased from R&D system (Minneapolis, MN). LPS (from *P. gingivalis*), and all other chemicals were obtained from Sigma Chemical Co. (St. Louis, MO) unless indicated otherwise.

2.2. Culture of HDP cells and HPDL

The immortalized HDP and HPDL cell lines obtained by transfection with the telomerase catalytic subunit human telomerase reverse transcriptase gene were used for this study (Kitagawa et al., 2006, 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.

2.3. Cell viability assay

For the determination of cell viability, 50 mg/ml of 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) was added to 1 ml of cell suspension (1×10^4 cells/well in 96-well plates) for 4 h, and the formazan formed was dissolved in acidic 2-propanol; optical density was measured at 590 nm.

2.4. Reactive oxygen species measurement

For the measurement of reactive oxygen species, HDP cells (2.5×10^4 cells/well in 24-well plates) were treated with 1 mM hydrogen peroxide in the presence or absence of sappanchalcone or SnPP (HO inhibitor) and incubated for 8 h. After washing with PBS, the cells were stained with 10 μM 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).

2.5. Quantification of NO

Thawed 50 ml aliquots of culture supernatant were mixed with 50 ml Griess reagent: 5% phosphoric acid (Fisher Scientific, Fair Lawn, NJ, USA), 1% sulfanilamide and 0.1% *N*-naphthylethylenediamine (Sigma Aldrich, St. Louis MO, USA). Samples were incubated at room temperature for approximately 10 min and then read on an ELISA microplate plate reader (Bio-Rad, Hercules, CA, USA) at 570 nm.

2.6. Determination of PGE_2 levels

The culture medium of control and treated cells were collected, centrifuged, and stored at −70 °C until tested. The level of PGE_2 released into culture medium was quantified using a specific enzyme immunoassay (EIA) as per manufacturer's instructions (Amersham, Arlington Heights, IL, USA).

2.7. Cytokine production assay

After culture medium of control and treated cells were collected from wells, supernatant concentrations of TNF-α, IL-1β, IL-6 and IL-12 were measured by commercially available ELISA (BioSource International, Inc., Camarillo, CA).

2.8. Western blot analysis

Western blot analysis was performed by lysing cells in 20 mM Tris–HCl buffer (pH 7.4) containing protease inhibitor mixture (0.1 mM phenylmethanesulfonyl fluoride, 5 mg/ml aprotinin, 5 mg/ml pepstatin A, and 1 mg/ml chymostatin). Protein concentration was 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 then electrophoretically transferred onto a Hybond enhanced chemiluminescence (ECL) nitrocellulose membrane (Bio-Rad, Heracules, CA). The membrane was blocked with 5% skim milk and sequentially incubated with primary antibody (Santa Cruz Biotechnology) and horseradish peroxidase-conjugated secondary antibody followed by ECL detection (Amersham Pharmacia Biotech, Piscataway, NJ).

2.9. Preparation of nuclear and cytosolic fraction

Stimulated and control cells were homogenized (1:20, w:v) in PER-Mammalian Protein Extraction buffer (Pierce Biotechnology, Rockford, IL) containing freshly-added protease inhibitor cocktail I (EMD Biosciences, San Diego, CA) and 1 mM phenylmethylsulfonyl fluoride. Cell cytosols were prepared by centrifugation at $15{,}000\times g$ for 10 min at 4 °C. Nuclear and cytoplasmic extracts of HDP cells and HPDL cells were prepared, using NE-PER nuclear and cytoplasmic extraction reagents (Pierce Biotechnology). After treatment, cells (3×10^6 cells/ml in 60 mm dish) were collected and washed with phosphate-buffered saline (PBS). After centrifugation, cell lysis was performed at 4 °C by vigorous shaking for 15 min in RIPA buffer [150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris–HCl (pH 7.4), 50 mM glycerophosphate, 20 mM NaF, 20 mM EGTA, 1 mM DTT, 1 mM Na_3VO_4, and protease inhibitors]. After centrifugation at $14{,}800\times g$ for 15 min, the supernatant was separated and stored at −70 °C until use. Protein content was determined with BCA protein assay kit.

2.10. Assay for HO activity

HO enzyme activity was measured by the previously described method (Kutty and Maines, 1982). Briefly, microsomes from harvested