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Sodium fluoride induces apoptosis in odontoblasts via a JNK-dependent mechanism

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ABSTRACT

Sodium fluoride (NaF) is widely used for the treatment of dental caries and dentin hypersensitivity. However, its pro-apoptotic effect on odontoblasts may lead to harmful side-effects. The purpose of this study was to evaluate the pro-apoptotic effects of NaF in odontoblasts and elucidate the possible underlying molecular mechanisms. NaF generated cytotoxic effects in odontoblast-lineage cell (OLC) in a dose- and time-dependent manner. Exposure of cells to 4 mM NaF for 24 h induced caspase-3 activation, ultrastructural alterations, and resulted in the translocation of Bax to the mitochondria and the release of cytochrome c from the mitochondrial inter-membrane space into the cytosol, indicating that fluoride-mediated apoptosis is mitochondria-dependent. Fluoride treatment also increased phosphorylation of JNK and ERK, but not p38, and apoptosis induced by fluoride was notably or partly suppressed by treatment with JNK or ERK inhibitors, respectively.

Taken together, these findings suggest that NaF induces apoptosis in OLC odontoblasts through a JNK-dependent mitochondrial pathway.

1. Introduction

Odontoblasts perform a crucial function in dentin formation by producing organic matrix and contributing to mineralization. Odontoblasts deposit new layers of reactionary/reparative dentine and play a role in preventing dentine sensitivity. Apoptosis is a key mechanism by which the life span of odontoblasts is regulated. External stimuli, such as abrasion, dental caries, and cavity preparation induce apoptosis in odontoblasts and promote pulp stem cell migration and differentiation into newly formed odontoblasts (Kitamura et al., 2001; Mitsiadis and De Bari, 2008; Smith, 2002; Toit et al., 2008), resulting in the secretion of reparative dentin. Temperature stimulation and changes in external and internal pressure can also initiate apoptotic events, inducing secondary dentin production (Bronckers et al., 2000; Harada et al., 2008; Kitamura et al., 2003). Decreased numbers of odontoblasts resulting from apoptosis could lead to the reorganization of odontoblasts into a single layer and subsequent secondary dentin deposition (Franquin et al., 1998). Multiple studies have shown that certain toxic substances, such as 2-Hydroxyethyl methacrylate(HEMA), polymethylmethacrylate (PMMA), fluoride and resin monomer in dental materials may also lead to uncontrolled apoptosis in odontoblasts (El-kholany et al., 2011; Hoppe et al., 2011; Krifka et al., 2012; Yamada et al., 2009).

Fluoride is a key ingredient of numerous dental products that are used daily, such as dentifrices used for preventing dentin hypersensitivity and remineralization of dental caries (Burwell et al., 2009; Lee et al., 2010). Currently, many commercial dental products contain high concentrations of fluoride. For example, an in-office product sold under the brand name Gluma® (Heraeus Kulzer, Inc., South Bend, IN, USA) contains fluoride at a concentration of over 1450–4500 ppm (Cummins, 2009). When this type of product is applied to dentin exposed in open tubule orifices or patent tubules, the local concentration of fluoride may reach as high as 5000 ppm (Duane, 2012; Nordstrom and Birkhed, 2010). Concentrations of fluoride at this level may produce significant toxic effects on odontoblasts that cannot be ignored. The cytotoxic effects induced inflammation on odontoblasts (Goldberg et al., 2008) are the link between initial inflammation/apoptosis and cell commitment in the pulp reparative process (Horst et al., 2011).

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Abundant evidence has demonstrated the pro-apoptotic effect of NaF in various cell types, including ameloblasts, osteoblasts, neural progenitor cells and odontoblasts (Bronckers et al., 2009; Karube et al., 2009; Kubota et al., 2005; Ma et al., 2012; Qu et al., 2008; Yan et al., 2009). However, it has also been reported that the levels of fluoride used in preventing dentin hypersensitivity might initiate apoptosis (Jacinto-Alemán et al., 2010). A even lower concentration of fluoride can selectively affect odontoblast gene expression ex vivo (Wurtz et al., 2008) and down-regulate dentine sialophosphoprotein (DSPP) mRNA in vivo. Fluoride-induced abnormalities in the dentine of deer teeth is characterized by the presence of interglobular dentine and regular bands of hypo- and hypermineralized dentine and giant tubules (Richter et al., 2010). Thus, the pro-apoptotic effect of fluoride might be key contributor to dentine abnormalities induced by high concentrations of NaF (Lyaruu et al., 2008). The purpose of this study was to evaluate the cytotoxic and apoptotic effects of NaF on odontoblast cells and to clarify the underlying mechanism of these effects.

2. Materials and methods

2.1. Chemicals

Fetal bovine serum (FBS), Dulbecco's modified Eagle's medium (DMEM) and trypsin were obtained from Gibco (Grand Island, NY, USA). Methyl thiazolyl tetrazolium (MTT), sodium fluoride (NaF), dimethyl sulfoxide (DMSO) and Hoechst 33258 were obtained from Sigma–Aldrich Co. (St. Louis, MO, USA).

2.2. Cell culture

Odontoblast-lineage cells (OLC), a novel odontoblast-lineage cell line established from mouse embryo tooth germs in E18.5 mouse mandibles, kindly provided by Dr. Arany, (Arany et al., 2006). OLCs were detected to express dentin matrix proteins and were also positive for novel odontoblast phenotype markers such as Lhx6 and Lhx7.

OLCs exhibited long cellular processes, suggesting cell polarization, which is similar to the columnar shaped, polarized mature odontoblast cell shape.

They were grown in Alpha Modification of Minimum Essential Medium Eagle (α-MEM) with 10% heat-inactivated FBS, 1% penicillin/streptomycin, and 2 mM L-glutamine (Gibco, Invitrogen Corp., Carlsbad, CA, USA) in a humidified atmosphere of 5% CO_2 and 95% air at 37 °C.

Cells were incubated with serum-free medium containing the indicated concentrations of NaF for 6–48 h at 37 °C. SP600125 (the specific JNK inhibitor) (Bennett et al., 2001) and PD98059(the specific ERK inhibitor) (Zelivianski et al., 2003) were dissolved in dimethyl sulfoxide (DMSO). Unless indicated, cells were pre-incubated with serum-free medium containing DMSO (0.2%) or each compound for 1 h, and then treated with 4 mM NaF for 24 h.

2.3. Cell viability assay

Drug cytotoxicity was determined using the colorimetric MTT assay. Briefly, cells (1×10^5 cells/well) were plated into 96-well plates (Costar, Cambridge, MA, USA) containing 100 μl of the completed growth medium in the absence or presence of increasing concentrations of drugs (1 to 6 mM) at 37 °C in 5% CO_2 for 6–48 h. MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide) (50 μl/well, 5 mg/ml in PBS) was then added and incubated for 4 h at 37 °C. The cells were further treated with 0.1 N acidic isopropanol to dissolve formazan crystals and the absorbance was measured at 570 nm in an ELISA reader. All experiments were repeated at least three times, with triplicates in each experiment.

2.4. TUNEL assay

A single cell suspension of odontoblast OLC cells was used for detecting apoptotic cells by in situ the terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL) assay, which labels the 3′-OH ends of DNA cut by endonucleases activated during apoptosis. TUNEL staining was performed with the In Situ Cell Death Detection Kit (Roche Applied Science) according to the manufacturer's instructions. Briefly, OLC odontoblasts were cultured on coverslips in 12 well plates to ~60% confluence and then placed in serum-free medium for 2 h. After treatment with 4 mM of NaF for 12–48 h, the cells were fixed in 4% paraformaldehyde (PFA), permeabilized in 0.2% Triton X-100, blocked in 2% bovine serum albumin (BSA) and 3% normal goat serum, and incubated with 50 μl TUNEL reaction mixture containing terminal deoxynucleotidyl transferase in a humidified atmosphere for 1 h at 37 °C in the dark. Rinsed slides were then stained with Hoechst 33258 in phosphate-buffered saline for nuclear counterstaining and washed again with phosphate-buffered saline. DNase-treated cell used as positive control according Uygun discribed. The slides were mounted with Vectashield Mounting Medium (H-1000) (Vector Laboratories, USA). Stained sections were analyzed using standard fluorescence microscopy (Olympus, Japan).

2.5. Ultramicrostructural observation

To evaluate the morphologic alterations in odontoblast cells following NaF exposure, the ultramicrostructure of OLC cells was observed by Transmission Electron Microscopy (TEM). Treated cell lysates were fixed with 2.5% glutaraldehyde overnight at 4 °C followed by fixation with 2% osmium tetroxide at 4 °C for 2 h (Carlson, 2000). After two washes in distilled water, the cells were stained with 1% uranyl acetate for 30 min, dehydrated in 95% and 100% ethanol, and then embedded in Epon 812. Ultrathin sections were stained with uranyl acetate and lead citrate. The sections were then observed under a transmission electron microscope (JEM-1200EX, JEOL, Japan) at 80 kV.

2.6. Mitochondrial membrane potential

The mitochondrial membrane potential ($\Delta\psi m$) was assayed with the cationic dye JC-1 (5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolylcarbocyanine iodide) (Beyotime, Jiangsu, China) according to the manufacturer's directions. Briefly, cells were incubated with the JC-1 reagent in the dark for 25 min at 37 °C. Carbonyl cyanide p-(trifluoromethoxy) phenylhydrazone (FCCP) (Sigma–Aldrich Co. St. Louis, MO) at 2.5 μM served as a positive control for collapse of mitochondrial membrane potential. After washing twice by centrifugation with ice-cold staining buffer, the cells were resuspended in 500 μl culture medium and images were obtained using a laser scanning confocal microscope (Carl Zeiss, Germany; excitation at 488 nm, emission at 529 and 590 nm). Loss of mitochondrial membrane potential was indicated by the loss of red fluorescence and increase in green fluorescence. The ratio of red-to-green fluorescence in 20 random cells was calculated by measuring the average intensities of the emitted fluorescence using FV1000 Viewer software and Adobe Photoshop CS4.

2.7. Western blot analysis

OLC cells were harvested and lysed with lysis buffer on ice for 20 min. Equal amounts of protein were separated by 12% SDS-PAGE and transferred onto nitrocellulose membranes. After incubation in blocking buffer (5% nonfat dry milk in TBS-T) for 1 h at room temperature, the membrane was probed with primary antibodies overnight at 4 °C. The blots were the incubated with the appropriate secondary antibody for 1 h at room temperature and visualized using the West Pico Chemiluminescent kit (Pierce, Rockford, IL, USA). The primary antibodies and second antibodies were obtained and used at the indicated dilutions same as our group reported previously (Cai et al., 2007; Luo et al., 2008; Zhang et al., 2010; Zheng et al., 2008).

2.8. Detection of cytochrome c release and translocation of Bax

OLC cells were cultured in 100-mm dishes until 80% confluence was reached and then exposed to NaF for 24 h. The cytosolic and mitochondrial fractions were isolated using a mitochondria isolation kit (Pierce) according to the manufacturer's instructions and protocols previously reported (Zhao and Herdegen, 2009). Protein concentration was determined by a Bio-Rad protein assay kit and equal amounts from each fraction were separated by SDS-PAGE. Western blotting was carried out with antibodies specific to Bax, Bcl-2, cytochrome c, cox-4 and β-actin.

2.9. Statistics

All data are expressed as the mean ± S.D. of each independent experiment. Differences between the control and the treatment groups were analyzed using one-way ANOVA with a post hoc Dunnett's test. The significance level was set as 0.05.

3. Results

3.1. NaF decreases cell viability and induces apoptosis in odontoblast cells

The cytotoxicity of NaF was determined by the MTT assay. As shown in Fig. 1a, NaF is cytotoxic to OLC cells in a dose-and time-dependent manner when administered at concentrations ranging from 1 to 6 mM for 6 to 48 h (Fig. 1a). Reduction of cell viability reached approximately 50% after 24 h treatment with 4 mM NaF.

Apoptosis of OLC cells induced by NaF was assessed by in situ TUNEL assay. A large number of TUNEL-positive cells were present in cells exposed to 4 mM NaF (Fig. 1b), and quantitative analysis showed that fluoride induced a significant increase in apoptotic