Fluoride induces endoplasmic reticulum stress and inhibits protein synthesis and secretion

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Fluoride is anticariogenic and is recommended by the U.S. Public Health Service for addition to drinking water at a concentration of 0.7–1.2 ppm, such that an average of 1 mg F– is consumed per day [Centers for Disease Control and Prevention (CDC) 1995]. However, higher levels of F– exposure can result in dental fluorosis, which is manifested as mottled, discolored, porous enamel that is susceptible to decay (DenBesten 1999). High F– doses can cause skeletal fluorosis that may result in bone fracture (Boivin et al. 1989). High F– doses may also cause renal toxicity (Zager and Iwata 1997), epithelial lung cell toxicity (Thranen et al. 2001), and reproductive defects (Ghosh et al. 2002). Among these, attention has been focused on the role of F– in dental fluorosis, because the most apparent effects of excess F– ingestion in an individual are white spots (mild fluorosis) or dark stains (moderate to severe fluorosis) on the teeth.

Ameloblasts are epithelial cells that are responsible for enamel formation. The three major stages of the ameloblast life cycle, namely, secretory, transition, and maturation, correspond to distinct steps in enamel development. During the secretory stage, the ameloblasts are tall and columnar, and they secrete large amounts of proteins that form a matrix within which thin enamel ribbons of hydroxypatite crystallize. Once the enamel ribbons attain their full length, ameloblasts enter the transition stage, when they decrease in height and experience a reduction in Golgi complex and rough endoplasmic reticulum (ER). During the maturation stage, the ameloblasts secrete KLK4 (Simmer et al. 1998) to help degrade the enamel proteins, which are then resorbed from the maturing enamel. It is during the maturation stage that the enamel ribbons grow in width and thickness to form mature hardened enamel. Normal enamel is composed of about 96% mineral and 4% organic content (Robinson et al. 1988). Excess F– ingestion during tooth formation causes an increase in the protein content and a decrease in overall mineral content of the enamel (Robinson et al. 2004; Robinson and Kirkham 1990; Wright et al. 1996). F– ions have been suggested to adversely affect the precipitation of hydroxypatite that forms the mineralized enamel (Aoba and Fejerskov 2002). However, two observations suggest that F–-mediated toxicity also involves genetic responses. First, different inbred strains of mice with similar overall levels of F– in their enamel differ in their susceptibilities to fluorosis (Everett et al. 2002). Second, no correlation was found between the concentration of F– in enamel and the severity of dental fluorosis (Vieira et al. 2004). These results clearly suggest a genetic basis for susceptibility to fluorosis (Yan et al. 2007). We have previously shown that F– induces ER stress in ameloblasts, thereby compromising their function during enamel formation (Kubota et al. 2005).

Proteins to be secreted are translocated into the ER for posttranslational modification, folding, and assembly. The ER is a quality control organelle in which individual proteins must adopt a stable conformation; unfolded or misfolded proteins are prevented from traversing the secretory pathway (Hammond and Helenius 1995). Factors that compromise ER homeostasis initiate an ER-to-nucleus signaling pathway, termed the unfolded protein response (UPR). Activation of the UPR serves three major functions: a) it results in transcriptional up-regulation of molecular chaperones such as BiP/GRP78 that help augment the folding capacity of the ER; b) it transiently attenuates protein translation via phosphorylation of the translation initiation factor, eucaryotic initiation factor-2, subunit alpha (eIF2α) phosphorylation, thereby allowing cells to cope with the existing protein load; and c) it activates components of the ER-associated degradative pathway (ERAD) to degrade the accumulated misfolded proteins. If these pathways succeed in alleviating cell stress, the cell survives; if not, the cell undergoes apoptosis via caspase activation. Indeed, ER stress is associated with several diverse diseases, including diabetes, neurodegenerative disorders (Gow and Sharma 2003), arsenite exposure (Lu et al. 2001), and heavy metal–induced toxicity (Hiramatsu et al. 2007).

Recently, the reporter construct secreted alkaline phosphatase (SEAP) (Berger et al. 1988) was used to detect and quantify ER stress in real time (Hiramatsu et al. 2006b). SEAP traverses the secretory pathway (Larlemus et al. 2006), and its activity can be detected at very low levels (0.2 pg/mL).
Because SEAP is a secreted protein, medium supernatant can be assayed for SEAP activity in a real-time fashion. Most importantly, transfection of cells with SEAP does not, by itself, cause ER stress (Hiramatsu et al. 2006b). SEAP secretion is decreased only by ER stress-inducing agents such as tunicamycin that blocks N-linked glycosylation or thapsigargin that functions as an inhibitor of Ca\(^{2+}\) ATPase (Hiramatsu et al. 2007). Cytokines that do not cause ER stress, such as tumor necrosis factor-\(\alpha\), transforming growth factor-\(\beta\), or interleukin-1\(\beta\), do not decrease SEAP activity (Hiramatsu et al. 2006b).

Thus, SEAP is specifically sensitive to ER stress-inducing agents. SEAP has been used to detect ER stress induced by heavy metals such as nickel, cadmium, and cobalt in cell lines as well as in mice (Hiramatsu et al. 2007).

In this study, we demonstrate that F\(^{-}\) concentrations as low as 2.4 ppm can induce ER stress in LS8 cells, which results in the inhibition of protein secretion; we also identify PKR-like ER kinase (PERK)-mediated phosphorylation of eIF2\(\alpha\) as a signaling pathway responsible for F\(^{-}\)-mediated inhibition of protein synthesis.

Materials and Methods

Production of stable transfecants, pSEAP2-Control (Great EscAPe SEAP Reporter System 3) and pTK-Hygromycin (both from Clontech, Mountain View, CA, USA) were cotransfected into LS-8 cells using Lipofectamine-LTX reagent and Lipofectamine Plus Reagent (both from Invitrogen, Carlsbad, CA, USA). After 24 hr, cells were washed and grown in alpha-MEM medium (Invitrogen) containing 750 \(\mu\)g/mL Hygromycin B (Invitrogen). Positive clones, isolated using cloning rings, were assayed for SEAP activity using the Great EscAPe SEAP Chemiluminescence Kit 2.0 (Clontech). Clone 10 showed the highest activity and was used for all experiments. For negative control, LS-8 cells were stably transfected with pTK-Hygromycin and pSEAP2-Basic (Great EscAPe SEAP Reporter System 3; Clontech), that lacks the SV40 early promoter and enhancer sequences present in pSEAP2-Control. Cells were maintained in alpha-MEM containing 10% fetal bovine serum and 750 \(\mu\)g/mL Hygromycin B during all experiments.

SEAP activity assay. We measured SEAP activity using the Great EscAPe SEAP Chemiluminescence Kit 2.0 according to the manufacturer’s instructions. Briefly, 25 \(\mu\)L cell supernatant was mixed with 75 \(\mu\)L of 1x dilution buffer in 96-well plates and incubated at 65\(^{\circ}\)C for 30 min. Plates were chilled for 3 min, and 100 \(\mu\)L SEAP substrate was added. After incubating at room temperature for 30 min, samples were measured for chemiluminescence on a Victor 1420 multilabel counter (PerkinElmer, Waltham, MA, USA); data are expressed in relative light units (RLU). All experiments were performed in triplicate and were repeated three times.

Immunoblotting. Cells were grown in 100-mm plates and treated with varying doses of sodium F\(^{-}\) for either 6 or 24 hr. Supernatant was collected for assessing the quantity and activity of SEAP released into the medium. To detect UPR proteins and intra- cellular SEAP, cells were washed twice with phosphate-buffered saline (PBS, pH 7.4) and lysed with Complete Lysis-M reagent (Roche Diagnostics, Mannheim, Germany) containing protease and phosphatase inhibitors (Roche Diagnostics). We determined the protein concentration using the BCA assay kit (Pierce, Rockford, IL, USA). A total of 10–30 \(\mu\)g protein was loaded per lane onto 4–20% polyacrylamide gels (Bio-Rad Laboratories, Hercules, CA, USA). Proteins were transferred onto nitrocellulose membranes (Schleicher and Schuell, Whatman, Germany), blocked with 5% nonfat dried milk, and probed with primary antibodies in blocking solution overnight at room temperature. Blots were washed with PBS containing 0.1% Tween-20 (PBST) and incubated with secondary antibodies conjugated to horseradish peroxidase for 1 hr at room temperature. After washing with PBST, bands were developed with ECL Advance Western Blotting kit (GE Healthcare, Piscataway, NJ, USA). We used the following primary antibodies: goat anti-PLAP (placental alkaline phosphatase; 1:1000) and goat anti-BiP (immunoglobulin heavy chain binding protein; 1:1000) from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA); rabbit anti-eIF2\(\alpha\) (1:1000, [pS52]; BioSource); mouse anti-actin (1:500; Sigma, St. Louis, MO, USA). Secondary antibodies were purchased from Southern Biotech (Birmingham, AL, USA).

Immunocytochemistry and immunohistochemistry. LS8-SEAP cells were grown on four-chamber tissue culture-treated glass slides (BD Biosciences, Bedford, MA, USA) and treated with 0.125 mM (2.4 ppm) sodium fluoride (NaF) for 24 hr. Cells were fixed with 2% paraformaldehyde for 30 min and permeabilized with 0.2% Triton X-100 for 30 min. After washing with PBS (pH 7.4), cells were blocked with 10% goat serum in PBS, secondary antibody (Alexa 488-conjugated antibody; 1:1000; Invitrogen) in blocking solution was added for 2 hr. After washing with PBS containing 0.1% Tween-20 (PBST) and incubated with secondary antibodies conjugated to horseradish peroxidase for 1 hr at room temperature. After washing with PBST, bands were developed with ECL Advance Western Blotting kit (GE Healthcare, Piscataway, NJ, USA). We used the following primary antibodies: goat anti-PLAP (placental alkaline phosphatase; 1:1000) and goat anti-BiP (immunoglobulin heavy chain binding protein; 1:1000) from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA); rabbit anti-eIF2\(\alpha\) (1:1000, [pS52]; BioSource); mouse anti-actin (1:500; Sigma, St. Louis, MO, USA). Secondary antibodies were purchased from Southern Biotech (Birmingham, AL, USA).

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Cell proliferation assay. LS8-SEAP cells were plated at a density of 2,500 cells in 96-well plates. After 18 hr, medium was changed and cells were incubated for either 6 or 24 hr in 100 \(\mu\)L medium containing varying doses of F\(^{-}\). For measuring cell proliferation, 10 \(\mu\L\) WST-1 (Roche Diagnostics) was added, and the resulting absorbance was measured after 3 hr at 440 nm on an EL800 Universal Microplate Reader (Biotek Instruments, Inc, Winookski, VT, USA). All experiments were performed in triplicate and repeated three times.

Statistics. We performed one-way analysis of variance with Bonferroni's posttest using GraphPad Prism, version 5.00 for Windows (GraphPad Software, San Diego CA, USA).
for lactate dehydrogenase, a cytoplasmic enzyme that is released into the medium on cell death, showed no significant F−-induced cell cytotoxicity at 6 hr (data not shown).

Thus, the observed decrease in SEAP activity did not correlate to cell proliferation or cell death. Treatment of cells with sodium chloride did not have any significant effect on SEAP activity, suggesting that the F− ion was responsible for the observed effects (Figure 1B). Tunicamycin also decreased SEAP activity, confirming previous reports that SEAP can be used to detect ER stress (Hiramatsum et al. 2006b). Therefore, F− treatment attenuated the secretion of SEAP from LS8-SEAP cells.

**Lack of direct inhibition of SEAP activity.**

Flouride, at a high concentration of 50 mM (950 ppm), is commonly used as a serine/threonine phosphatase inhibitor during cell lysis. Therefore, it is possible that F− directly inhibits the alkaline phosphatase activity of SEAP without causing an ER stress-mediated decrease in SEAP secretion. To address this issue, we collected cell-free medium containing SEAP from LS8-SEAP cells grown in the absence of F−. Recombinant SEAP thus obtained was then incubated with different doses of F− for 6 and 24 hr. As shown in Figure 2, no significant decrease in SEAP activity was observed (p > 0.05) until the F− concentration reached 50–100 mM. Thus, the decrease in the SEAP activity observed in our experiments can be attributed to reduced SEAP secretion and not to direct inhibition of phosphatase activity by F−.

**Intracellular accumulation of SEAP and activation of the UPR.** Any factor that disturbs ER homeostasis could lead to the intracellular accumulation of misfolded or unfolded proteins, causing ER stress. As shown in Figure 3A, treatment of LS8-SEAP cells with F− results in a dose-dependent increase in the intracellular accumulation of SEAP protein. Conversely, secretion of SEAP into the medium decreases (Figure 3B). These immunoblot results indicate that F− interferes with the secretion of SEAP and, presumably, other endogenous secretory proteins. We also demonstrate a concurrent induction of the ER stress-induced UPR signaling pathway along with the observed decrease in SEAP secretion (Figure 4). The UPR proximal sensor, PERK, is a transmembrane serine/threonine kinase activated by autotransphosphorylation (Harding et al. 2000). Activated PERK phosphorylates eIF2α (reviewed by Kimball 1999). This leads to transient translation attenuation, allowing the cells to cope with the proteins that have already accumulated in the ER. As shown in Figure 4A, PERK is activated (phosphorylated) in LS8-SEAP cells treated with 0.125 mM (2.4 ppm) F−. Immunoblots show translational induction of the molecular chaperone BiP, as well as phosphorylation of the PERK target eIF2α (Figure 4B) within 6 hr of F− treatment. BiP remains induced after 24 hr of treatment, especially in cells treated with higher doses of F− (≥19 ppm).

We next asked if maturation stage ameloblasts from mice drinking F−-treated water (0, 25, 50, or 100 ppm in drinking water for approximately 3–4 weeks) initiated phosphorylation of eIF2α in vivo. As shown in Figure 5, eIF2α was phosphorylated at the lowest dose tested (25 ppm). The amount of eIF2α phosphorylation in vivo correlated positively with F− dose, suggesting an increase in the magnitude of ER stress with increasing doses of F−.

**Discussion**

Fluorosed enamel is characterized by hypomineralization, increased protein content, and greater surface and subsurface porosity. The most significant characteristic of fluorosed enamel is its increased protein content (Holland 1979a). Several studies have pointed toward F−-mediated inhibition of protein secretion and/or synthesis (Conconi et al. 1966; Godchaux and Arwood 1976; Helgeland 1976; Holland 1979a, 1979b; Lin et al. 1966; Vesco and Colombo 1970; Zhou et al. 1996). For example, F− in the drinking water of rats inhibited protein removal from early maturation-stage incisor enamel (DenBesten 1986; DenBesten and Thariani 1992; Zhou et al. 1996). F− has also been shown to inhibit insulin secretion in rats (Menoyo et al. 2005; Rigalli et al. 1990). However, a direct mechanism for F−-induced inhibition of protein secretion remains to be elucidated.

In this article, we show that NaF decreases secreted SEAP activity in a dose-dependent manner (Figure 1A). The effect is mediated only by NaF and not by NaCl (Figure 1B), suggesting that F− is responsible for the observed decrease in protein secretion. We also demonstrate that the low concentrations of F− used in our experiments do not directly inhibit recombinant SEAP activity.
Fluoride inhibits protein secretion

(Figure 2). Thus, F– does not interfere with our assay system.

Accumulation of excess protein within the ER is a hallmark of ER stress. We found that with an increase in F– dose, increasing quantities of SEAP accumulate intracellularly (Figure 3). Thus, the observed decrease in SEAP secretion is at least partially due to ER stress-mediated protein retention within the cells. Our results with F– are similar to reports using the well-characterized ER-stress inducer thapsigargin. SEAP also accumulated in the ER after thapsigargin treatment (Hiramatsu et al. 2006a).

F– induces ER stress and initiates the UPR, as demonstrated by the induction of the molecular chaperone BiP and by phosphorylation of PERK and eIF2α (Figure 4). We also demonstrated phosphorylation of eIF2α in vivo in ameloblasts of mice treated ad libitum with F– at doses of ≥ 25 ppm (Figure 5). It must be noted that a higher F– dose is required to cause fluorosis in a mouse (25 ppm) compared with a human (~ 2 ppm). This may be because the continuously erupting mouse incisor ameloblast progresses from the secretory stage to the final maturation stage in a matter of weeks, whereas this progression occurs over several years for human teeth. Thus, human ameloblasts have a much longer exposure to F– present in drinking water than do mouse ameloblasts. Second, rodents do appear to more efficiently clear F– from their bodies compared with humans (Angmar-Mansson and Whitford 1984). The immunostaining for phosphorylated eIF2α observed in maturation stage ameloblasts exposed to 25 ppm F– is highly significant, because 25 ppm F– is the threshold concentration where F–-susceptible mice will have fluorosis (Everett et al. 2002).

F– induced ER stress and subsequent inhibition of protein secretion is consistent with prior in vivo studies demonstrating F–-mediated disruption in the export of proteins from the ER (Kruger 1968; Matsuo et al. 1996, 2000). Furthermore, 100 ppm F– in rat drinking water delays by as much as 30% the modulatory cycle of the apical ends of ameloblasts between a ruffle-ended and smooth-ended morphology during the maturation stage of enamel development (Smith et al. 1993). This modulation is thought to assist the ameloblasts in removing H+ ions from the enamel matrix, and its inhibition by F– is consistent with a decrease in the translation of proteins required for the modulation to occur. Taken together, these observations support our results suggesting that F– causes ER stress in ameloblasts and induces the UPR, which initiates eIF2α phosphorylation with subsequent attenuation of protein synthesis and secretion.

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