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Activation of PERK signaling through fluoride-mediated endoplasmic reticulum stress in OS732 cells

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ABSTRACT

Our proteomical analysis of osteoblasts exposed to fluoride revealed a distinctive upregulation of proteins in osteoblast. These upregulated proteins play key roles in the protein folding. The PRK-like ER kinase (PERK) signaling, one branch of unfolded protein response (UPR) to combat ER stress, is a transcription factor needed for osteoblast proliferation and differentiation. The mechanism of skeletal fluorosis by which fluoride regulates osteoblast is not fully defined. Here we studied the effect of fluoride on PERK signaling genes and x-box binding protein 1 (xbp-1) in OS7232 cells (human osteoblast-like cell line). Meantime, genes associated with bone turnover were examined in this study. We found that early and continuous fluoride exposure increased the binding immunoglobulin protein (BiP) expression and activated the PERK signaling pathway, resulting in activation of transcription factor 4 (ATF4) and nuclear factor erythroid 2-related factor 2 (Nrf2). The altered expression of cbfa1, osteoprotegerin (OPG)/nuclear factor kappa B ligand (RANKL) were viewed in this study. These results showed fluoride impelled a distinctive ER stress response in OS732 cells, primarily by activating PERK and PERK-dependent signaling. Little effects were viewed for activating xbp-1, a common target of the other two canonical sensors of ER stress, ATF6 and IRE1. In this study the altered expression of bone turnover genes were consistent with activation of ER stress and PERK signaling. This study proved that PERK signaling play major roles in action of fluoride on osteoblast, and suggested that bone response in skeletal fluorosis may be due in part to PERK signaling pathway.

1. Introduction

The skeletal fluorosis is endemic in some parts of the world because of lifelong ingestion of high amounts of fluoride in drinking water. Fluoride gets accumulated in the hard tissues of body and is characterized by dental changes and diffuse densification of bones. The underlying fundamental mechanisms of fluorosis broadly encompass all aspects of research that detected of cytokine, hormone, Ca^{2+} and oxidative stress (Li, 2009). The current data show a complicated picture of skeletal responses to fluoride exposure, for example, osteosclerosis, osteomalacia, osteoporosis and exostosis formation (Mithal et al., 1993). They are characterized by high bone turnover and enhanced proliferation and differentiation of osteoblasts (Boivin et al., 1989; Li et al., 2005).

In an attempt of further characterize the fundamental mechanism of fluoride-induced osteoblast toxicity, our laboratory previously performed the proteomical analysis of osteoblasts exposed to fluoride. Our previous study revealed distinctive upregulation of proteins, including thioredoxin, protein disulfide isomerase (PDI), and binding immunoglobulin protein (BiP, one member of heat shock 70-kDa protein family) (Xu et al., 2008a,b). These proteins play key roles in protein folding of endoplasmic reticulum (ER). Recent researches in fluorosis has an increased interest in ER stress (Ito et al., 2009; Kubota et al., 2005; Sharma et al., 2008). We sought to follow up this by mechanistically exploring potential ER stress in osteoblast-like cells induced by fluoride.

Stress to the endoplasmic reticulum is induced in response to cope with conditions such as the accumulation of misfolded and unfolded proteins, amino acid deprivation, and perturbations of the redox or energy status. The unfolded protein response (UPR) is regarded as a cellular tool to combat successfully ER stress. The UPR signaling is orchestrated by three different arms, each of which is initialized by a distinct sensor anchored in the ER as transmembrane protein and termed the PRK-like ER kinase (PERK),

Abbreviations: ATF4, activating transcription factor 4; ATF6, activating transcription factor 6; BiP, binding immunoglobulin protein; cbfa1, core binding factor alpha 1; DMEM, Dulbecco's modified Eagle's medium; ER, endoplasmic reticulum; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; IRE1, inositol-requiring enzyme 1; Nrf2, nuclear factor erythroid 2-related factor 2; OPG, osteoprotegerin; PDI, protein disulfide isomerase; PERK, PRK-like ER kinase; RANKL, nuclear factor kappa B ligand; ROS, reactive oxygen species; UPR, unfolded protein response; xbp-1, x-box binding protein 1.

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inositol-requiring enzyme 1 (IRE1), and activating transcription factor 6 (ATF6) (Shen et al., 2004). The most immediate response to ER stress, however, is provoked by the Ser/Thr kinase PERK (Harding et al., 2000). A major target of this striking bypass mechanism is represented by activating transcription factor 4 (ATF4). Besides ATF4 expression, activated PERK has also been reported to activate directly the nuclear factor erythroid 2-related factor 2 (Nrf2), which is a strong inducer of antioxidant and phase II-related genes (Cullinan and Diehl, 2004). Further, Wei et al. (2008) have reported PERK as a novel regulator of skeletal development and osteoblast biology.

Here, we reported that fluoride exposure activated the PERK signaling pathway resulting in activation of ATF4 and Nrf2, and upregulated expression of bone turnover associated genes in OS732 cells (human osteoblast-like cell line). Our presented results suggested that PERK signaling pathway probably played a major role in the pathogenesis of skeletal fluorosis.

2. Materials and methods

2.1. Cell culture

The human osteoblast-like cell line (OS732 cells), derived from a human osteosarcoma, was maintained in Dulbecco's modified Eagle's medium (DMEM) (DMEM, Gibco, Grand Island, NY, USA), supplemented with 10% fetal bovine serum and penicillin/streptomycin mixture (100 U/mL penicillin G, 100 μg/mL streptomycin sulfate) and cultured in a humidified atmosphere of 5% CO_2 and 95% air. The experiment was designed to include 3 fluoride-treated groups ($n = 5$) and one control group ($n = 5$). Cells were seeded (2×10^6 cells per well) into 6-well plates and treated with DMEM (2% calf serum) containing 1.0, 4.0 and 10.0 mg/L of fluoride for 1, 4, and 10 days, respectively. Cells in the control group were cultured with DMEM (2% calf serum, fluoride-free) for the same period. At the end of each treated period, cells were washed twice with ice-cold 0.9% NaCl, then lysed into debris after being treated with TRIzol reagent (Invitrogen, Groningen, NL).

2.2. Reverse transcription and real-time PCR

Total RNA was extracted using TRIzol reagent (Invitrogen Inc., USA) and quantified by scanning spectrophotomer. First-strand cDNA was synthesized from 1 μg of total RNA with the use of Oligo $(dT)_{18}$ primer (Takara, Japan) and Rever Tra Ace (Toyobo Japan). Quantitative PCR (qPCR) was performed in a total reaction volume of 20 μL with an SYBR PCR master mix (Toyobo, Japan) per well. An ABI 7500 thermocycler (Applied Biosystems, Foster City, CA) was used for PCR. The reaction conditions were as follows: 95 °C preheat for 1 min, followed by 40 cycles of 95 °C for 15 s (denaturation), 57 °C for 20 s (annealing) and 72 °C for 32 s (elongation). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as reference.

The qPCR primer pairs are as follows (Sun et al., 2009; Wu et al., 2009; Xuan et al., 2009; Clements et al., 2006): BiP (NM_005347.3, 134 bp): forward, TCCTATGTCGCCTTCACT, reverse, ACAGACGGGTCATTCCAC; ATF4 (NM_001675.2, 166 bp): forward, GTCTCCGTGAGCGTCCAT, reverse, CAGAAGCCAACTCCCATTAG; Nrf2 (NM_006164.3, 105 bp): forward, AGTGGATCTGCCAACTACTC, reverse, CATCTACAAACGGGAATGTCTG; PERK (NM_004836.4, 240 bp): forward, GGCTTGAAAGCAGTTAG, reverse, GGACAGTTGCCTTACAGA; core binding factor alpha 1 (cbfa1) (NM_004348.3, 178 bp): forward, CCACCACTCACTACCACACCTACC, reverse, CATGGCGGAAGCATTCTGGAAGG; osteoprotegerin (OPG) (NM_002546.3, 219 bp): forward, GGGAAAGAAAGTGGGAGCAGAAGAC, reverse, GTGAAGCTGTGAAGGAACCTGATGG; nuclear factor kappa B ligand (RANKL) (NM_003701.3, 273 bp): forward, CAGCGTCGCCCTGTTCTTCTATTTC, reverse, TCTAACCATGAGCCATCCACCATCG; GAPDH (NM_002046.3, 221 bp): forward, TGGTCTCCTCTGACTTCAAC, reverse, GTGAGGGTCTCTCTCTTCCT. The comparative expression level (fold change) was gained by transforming the logarithmic values to absolute values using $2^{-\Delta\Delta C_T}$ method (Livak and Schmittgen, 2001).

2.3. Semi-quantity PCR

The method of reverse transcription was same as above. A 289-base-long region encompassing the splice site of xbp-1 was amplified by PCR using the following primers (Samali et al., 2010): forward, TTACGAGAGAAAACTCATGGCC; reverse, GGGTCCAAGTTGTCCAGAATGC. GAPDH was used as internal control. PCR products were separated on a 2% agarose gel, stained with ethidium bromide, scanned and quantified by using the Tanon Gis-1000 UV transilluminator and digital image gel analytical system.

2.4. Statistical analysis

The data were presented as mean ± standard deviation (SD). Data were subjected to analysis of variance (ANOVA) followed by the Newman–Keuls multiple comparisons test by using SPSS ver. 18 (SPSS Inc., Chicago, IL, USA). Significance was set at $p < 0.05$.

3. Results

3.1. Assessment of ER stress

BiP is a central regulator of ER homeostasis because of its multiple roles in protein folding and activating transmembrane ER stress sensors. BiP is thought as one of ER stress markers. OS732 cells were treated with various concentrations of fluoride (1.0, 4.0 and 10.0 mg F^-/L) for different periods (1, 4 and 10 days). The expression of BiP in OS732 cells exposed to fluoride is shown in Fig. 1. It was significantly upregulated of BiP in 4 mg F^-/L group for 1-day exposure than that in the control, but there was a marked downregulation in 4 mg F^-/L and 10 mg F^-/L group for 4-day exposure. However, the expression of BiP significantly increased in 4 mg F^-/L and 10 mg F^-/L group for 10 days exposure. These data indicated that early and continuous exposure to fluoride induced varying degrees of ER stress in cells (Fig. 1).

3.2. Assessment of gene experiment of PERK signal pathway

The signaling pathway mediated by PERK/eIF-2α/ATF4 or Nrf2 has been implicated in response to ER stress. To further study the possible mechanism mediated by UPR-dependent PERK activation, we assessed the effect of fluoride exposure on expression of PERK, ATF4 and Nrf2 in OS732 cells. The expression of PERK significantly increased in 1, 4 mg F^-/L groups for 1-day exposure. Similarly, the upregulated expression of PERK was viewed in 4, 10 mg F^-/L groups for 10-day exposure. On the other hand, 1, 4 mg/L of fluoride decreased its mRNA expression in 4-day exposure (Fig. 2).

ATF4 and Nrf2 acted as the downstream signaling pathways of PERK. It showed the significant upregulation of ATF4 in 10 mg F^-/L groups for 10-day exposure. The expression of Nrf2 markedly increased in OS732 treated by 4 mg/L of fluoride for 1 day and 4, 10 mg/L of fluoride for 10-day exposure. However, there was markedly decrease in expression of ATF4 and Nrf2 in OS732 cells treated with different concentrations of fluoride for 4 days. Their trends of gene expression were similar to the altered expression of PERK. It suggested that PERK signal pathway was sensitive to the fluoride-induced ER stress (Fig. 3).

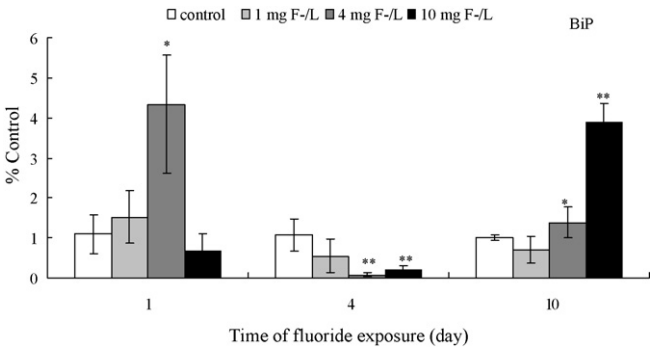

Fig. 1. Expression of BiP in OS732 cells exposed to fluoride. OS732 cells (a human osteoblast-like cell line) were exposed to fluoride at different concentrations (0–10 mg F^-/L) and lysed at various time points, as indicated. Using reverse transcriptase real-time quantitative PCR, the fold induction of BiP was determined relative to that of untreated control cells using GAPDH for normalization. Mean values with standard deviation bars for fold induction from at least three independent experiments are depicted. $^*p \leq 0.05$; $^{**}p \leq 0.01$.