RESEARCH ARTICLE

Fluoride-induced c-Fos expression in MC3T3-E1 osteoblastic cells

Mamiko Iwatsuki and Masato Matsuoka

Department of Hygiene and Public Health I, Tokyo Women’s Medical University, Tokyo, Japan

Abstract
Excessive systemic exposure to fluoride leads to disturbances of bone homeostasis. c-Fos is known to be essential in bone development by affecting osteoblast and osteoclast differentiation. In this study, we examined the effects of fluoride exposure on c-Fos expression and its regulatory signaling pathways in MC3T3-E1 mouse osteoblast cell line. c-fos mRNA level, c-Fos protein level and c-Fos DNA-binding activity were markedly increased, with a peak at 2 or 4 h, in MC3T3-E1 cells exposed to sodium fluoride (NaF). Fra-1 protein, another member of Fos family, was also elevated, whereas FosB and Fra-2 proteins remained unchanged. NaF further induced phosphorylation of mitogen-activated protein kinases (MAPKs), including extracellular signal-regulated protein kinase 1/2 (ERK1/2), ERK5, c-Jun NH2-terminal kinase and p38. NaF-induced expression of c-Fos protein was markedly suppressed with U0126, the inhibitor of both activated and non-activated forms of MAPK/ERK kinase 1/2 (MEK1/2) and BIX02189, the MEK5 inhibitor, but partially with SP600125, the JNK inhibitor and SB203580, the p38 inhibitor. Therefore, ERK1/2 and ERK5 signal transduction pathways are important for accumulating c-Fos. siRNA targeting against the mouse c-fos gene further enhanced NaF-induced up-regulation of osteoprotegerin (OPG), an inhibitor of osteoclastogenesis, suggesting that c-Fos might negatively regulate OPG expression induced by fluoride in osteoblastic cells.

Keywords
C-Fos, fluoride, MC3T3-E1 osteoblast cell line, mitogen-activated protein kinases, osteoprotegerin

Introduction
Fluorides are naturally occurring and present ubiquitously in the environment. They are found in dust, industrial waste and burning coal (Perumal et al., 2013; Prystupa, 2011). Although fluoride is essential for maintaining bone structure, excessive systemic exposure to fluoride leads to disturbances in bone homeostasis called skeletal fluorosis, which is accompanied by osteosclerosis, osteoporosis, osteomalacia or osteopenia (Everett, 2011; Perumal et al., 2013). Fluoride acts on osteoblasts and osteoclasts that are responsible for bone formation and resorption, respectively (Barbier et al., 2010; Everett, 2011; Prystupa, 2011). However, the molecular mechanisms of skeletal fluorosis have not been fully clarified. Thus, it is important to determine the key genes or proteins that regulate osteogenesis in response to fluoride exposure.

The immediate early gene c-fos is the cellular homolog of the retroviral gene v-fos that was isolated originally from murine osteosarcomas (Curran et al., 1983; Dutschewald et al., 2009; Van Beveren et al., 1983). Members of the Fos family, including c-Fos, FosB, Fra-1 and Fra-2, dimerize with the Jun family members (c-Jun, JunB and JunD) to form the activator protein-1 (AP-1) transcription factor complex that participates in cell proliferation, death, survival, differentiation and stress responses (Milde-Langosch, 2005; Shaulian & Karin, 2002). Ectopic c-Fos expression in transgenic mice triggers the transformation of osteoblasts preceding osteosarcoma formation (Grigoriadis et al., 1993; Wagner & Eferl, 2005). Conversely, mice without c-Fos lack mature osteoclasts and suffer from severe osteopetrosis (Dutschewald et al., 2009; Johnson et al., 1992; Wang et al., 1992). These findings indicate that c-Fos is essential in bone development by affecting osteoblast and osteoclast differentiation.

In this study, we determined the level of c-fos mRNA, c-Fos protein and c-Fos DNA-binding activity in MC3T3-E1 mouse osteoblastic cells exposed to sodium fluoride (NaF). Mitogen-activated protein kinases (MAPKs), including extracellular signal-regulated protein kinase 1/2 (ERK1/2), c-Jun NH2-terminal kinase (JNK) and p38, increased c-fos transcription in response to a diverse array of extracellular stimuli (Tulchinsky, 2000; Whitmarsh & Davis, 1996). Furthermore, post-translational phosphorylation of c-Fos protein by MAPKs stabilized c-Fos (Coronella-Wood et al., 2004; Ferrara et al., 2003; Iwatsuki et al., 2011; Okazaki & Sagata, 1995; Terasawa et al., 2003). Using individual MAPK inhibitors, we examined the possible involvement of serine/threonine protein kinases in NaF-induced c-Fos protein expression. We also examined osteoprotegerin (OPG)
expression, which is induced by osteoblasts and inhibits osteoclastogenesis (Baud’huin et al., 2013; Reid & Holen, 2009), in NaF-exposed MC3T3-E1 cells lacking c-Fos expression.

Materials and methods

Chemicals

NaF was obtained from Sigma-Aldrich (St. Louis, MO). U0126, SP600125 and SB203580 were purchased from Calbiochem (La Jolla, CA). BIX02189 was purchased from Selleck Chemicals (Houston, TX). c-Fos (4), Fra-1 (R-20), Fra-2 (L-15) and actin (I-19) antibodies were obtained from Santa Cruz Biotechnology and Cell Signaling Technology. (HRP)-conjugated secondary antibodies were obtained from Cell Signaling Technology, Inc. (Beverly, MA). Horseradish peroxidase (HRP)-conjugated secondary antibodies were obtained from Santa Cruz Biotechnology and Cell Signaling Technology.

Cell culture and treatments

MC3T3-E1 cells (Riken Cell Bank, Ibaraki, Japan) were grown in α-MEM medium supplemented with 10% heat-inactivated fetal bovine serum, 100 U/ml penicillin and 100 μg/ml streptomycin (GIBCO, Invitrogen Corp., Carlsbad, CA) in a humidified atmosphere of 5% CO₂ at 37°C. Exponentially growing MC3T3-E1 cells were seeded at 2×10⁵ cells/well in six-well culture plates in α-MEM. Equal volumes of these two solutions were mixed and allowed to incubate for 1 d before each experiment. Cells were incubated in serum-free media containing the appropriate concentration of NaF for 15 min to 24 h at 37°C. U0126, BIX02189, SP600125 and SB203580 were dissolved in dimethyl sulfoxide (DMSO). Cells were incubated in serum-free media with 0.1% DMSO as a control or 10⁻⁵ M of each compound for 30 min before treating with 5 mM NaF for an additional 2 h.

Western blotting

After treating with NaF, cells were washed with phosphate-buffered saline (PBS) and lysed with sodium dodecyl sulfate (SDS)–polyacrylamide gel Laemmli sample buffer. Cell lysates were collected, sonicated and boiled for 5 min. Then, protein concentrations were determined using the RC DC Protein Assay (Bio-Rad Laboratories, Inc., Hercules, CA). Equal amounts of protein (15 μg) were subjected to SDS–10% polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane (Hybond-ECL, Amersham Pharmacia Biotech, Buckinghamshire, UK). The membrane was blocked with 5% nonfat milk in Tris-buffered saline containing 0.1% Tween-20 for 1 h at room temperature. The membrane was then incubated overnight at 4°C with the primary antibody, and protein was detected with a Phototope-HRP western blot detection kit (Cell signaling Technology, Inc.). The bands on the developed film were quantified with ImageJ 1.48 (National Institutes of Health, Bethesda, MD). The density of each band was normalized to that of actin or total MAPK.

Quantitative real-time PCR

Total RNA was purified using RNeasy® Plus Mini Kit (Qiagen Gmbh, Hilden, Germany). Aliquots of total RNA (0.5 μg) were reverse-transcribed into cDNA in the presence of random primers with the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA) according to the manufacturer’s instructions. cDNA was amplified using the Universal SYBR® Select Master Mix (Life Technologies Japan Ltd., Tokyo, Japan) in the Applied Biosystems StepOne Real-Time PCR System. The primer sequences were as follows: c-fos, 5’-GGGGACACGGCTTTCC TACTA-3’ (forward) and 5’-GGGGATAAAAGTTGCCACT A-3’ (reverse) (Soves et al., 2014); OPG, 5‘-GTGGAATAGA TGTCACCCCTTGTT-3’ (forward) and 5’-TTTGGTTCGACGG CAAAAGTT-3’ (reverse) (Lin et al., 2014); and GAPDH, 5’- AACCTGGCATGTGAAGG-3’ (forward) and 5’-TG TGG ATGCAGGATGATC-3’ (reverse). The cycling conditions were as follows: 2 min at 95°C, followed by 40 cycles of a 15-s denaturation step at 95°C and a 60-s annealing/extension step at 60°C. The expression levels of c-fos and OPG genes were normalized to the level of GAPDH expression as described previously (Iwatsuki et al., 2011).

DNA-binding activity of c-Fos protein

After collecting the nuclear fractions using Nuclear Extract Kit (Active Motif), c-Fos DNA-binding activity was quantified using the TransAM® AP-1 c-Fos Transcription Factor Assay Kit (Active Motif). Briefly, 1 μg of nuclear extracts were added to a 96-well plate on which has been immobilized an oligonucleotide that contains a 12-O-tetradecanoylphorbol-13-acetate-response element (TRE; 5’-TGAGTCA-3’). The primary antibody against c-Fos was added, followed by a secondary HRP-conjugated antibody and HRP substrate. The absorbance was measured at 450 nm with a reference at 655 nm at the end of colometric change.

Gene knockdown of c-Fos by siRNA

Lipofectamine RNAiMAX® (Invitrogen Corp.) was used to transfect the siRNA targeting mouse c-fos gene (Stealth siRNA™ MSS274405) and the negative control siRNA (Stealth RNAi™ siRNA Negative Control Med GC Duplex #2, Life Technologies Japan Ltd.) into MC3T3-E1 cells according to the manufacturer’s instructions. The siRNAs were dissolved in nuclease-free water and diluted to 50 pmol with 250 μl Opti-MEM® (Invitrogen Corp.). Lipofectamine RNAiMAX (2.5 μl) was also diluted 100-fold with Opti-MEM. Equal volumes of these two solutions were mixed gently and incubated for 20 min at room temperature. MC3T3-E1 cells were seeded at 2×10⁵ cells/well in six-well culture plates in α-MEM medium without antibiotics and treated with 505 μl siRNA-Lipofectamine RNAiMAX complexes. After incubating for 24 h, cells were washed with PBS and used for the experiments.

Statistical analysis

The experiments were repeated three times. Data were expressed as means ± standard deviation (SD). Statistical
significance was determined with one-way analysis of variance test followed by the Dunnett’s multiple-comparison test. A value of $p < 0.05$ was considered statistically significant.

**Results**

**c-Fos expression induced from fluoride exposure**

The c-Fos protein of MC3T3-E1 cells exposed to 5 mM NaF increased after 1 h, peaked at 2 h and then decreased substantially, although it remained elevated at 8 h (Figure 1A, top panel). c-Fos protein phosphorylated at Ser32 also accumulated with a peak at 2 h after 5 mM NaF exposure (Supplementary Figure S1A). Fra-1 protein increased after 6 h and was further enhanced at 8 h (Figure 1A, third panel), whereas FosB and Fra-2 proteins showed no significant changes from 1 to 8 h (Figure 1A, second and fourth panels). When MC3T3-E1 cells were incubated with 0.1–10 mM NaF for 2 h, c-Fos protein increased in cells treated with 5 mM NaF and higher concentration (Figure 1B). Real-time PCR analysis further showed that 5 mM NaF induced $c$-fos gene expression in MC3T3-E1 cells. c-fos mRNA began to increase after 1 h, peaked at 2 h, and then gradually declined (Figure 1C). c-Fos DNA-binding activity was determined in the nuclear extracts of MC3T3-E1 cells treated with 5 mM NaF. DNA-binding activity increased after 2 h, peaked at 4 h, followed by a moderate decrease, although it remained elevated above the control level during the 8-h incubation period (Figure 1D).

**MAPKs phosphorylation after fluoride exposure**

In MC3T3-E1 cells treated with 5 mM NaF, phosphorylated ERK1/2 (ERK2/p42 and ERK1/p44) peaked at 30 min and then returned to the control level at 4 h (Figure 2, top panel). Phosphorylated ERK5 increased from 15 min to 1 h of exposure before decreasing substantially (Figure 2, third panel). Phosphorylated JNK (p46 and p54) and p38 also increased after 1 h and remained elevated at 4 h (Figure 2, fifth and seventh panels). In contrast, total (phosphorylation state-independent) ERK1/2, ERK5, JNK and p38 proteins did not change during the 4-h incubation period (Figure 2, second, fourth, sixth and eighth panels).

**MAPK involvement in fluoride-induced c-Fos protein expression**

ERK1/2 is activated by MAPK/ERK kinase 1/2 (MEK1/2) and ERK5 by MEK5; both are members of MAPK kinase (MAPKK) family (Cargnello & Roux, 2011). NaF-induced expression of c-Fos protein and its phosphorylation at Ser32 were almost completely eliminated by U0126, an inhibitor of both activated and non-activated forms of MEK1/2, and BIX02189, an inhibitor of MEK5 (Figure 3, top and middle panels, lanes 7 and 8). Conversely, treating with the JNK
inhibitor, SP600125 and the p38 inhibitor, SB203580, reduced NaF-induced c-Fos expression by 51.4% and 38.1%, respectively (Figure 3, top panel, lanes 9 and 10), and phosphorylation of c-Fos at Ser32 by 46.6% and 52.0%, respectively (Figure 3, middle panel, lanes 9 and 10). Treating with each MAPK inhibitor alone did not affect c-Fos expression and its phosphorylation at Ser32 (Figure 3, top and middle panels, lanes 2-5).

Effects of c-Fos knockdown on OPG expression in cells exposed to fluoride

Transfecting with siRNA targeting against the mouse c-fos gene almost completely eliminated NaF-induced c-Fos protein expression in MC3T3-E1 cells (Figure 4A). Knockdown of c-Fos expression markedly suppressed the elevated DNA-binding activity of c-Fos induced by NaF (Figure 4B). Treating with 5 mM NaF for 24 h increased the mRNA level of the OPG gene in MC3T3-E1 cells (8.5-fold increase) (Figure 4C). This elevation was significantly enhanced in c-Fos deficient cells (15.3-fold increase), suggesting that c-Fos regulated OPG expression negatively. Knockdown of c-Fos expression consistently increased OPG mRNA in cells not exposed to NaF (2.6-fold increase) (Figure 4C).

Discussion

In MC3T3-E1 cells exposed to 5 mM NaF, both c-fos mRNA and c-Fos protein increased after 1 h and peaked at 2 h (27.4-fold and 36.7-fold increase, respectively) before decreasing. Treating with actinomycin D almost completely eliminated NaF-induced elevation of c-fos mRNA (Supplementary Figure S2), suggesting that c-Fos expression was dependent on transcription activation. In addition, Fra-1 protein increased in MC3T3-E1 cells treated with 5 mM NaF for longer than 6 h (2.7-fold increase at 8 h). In contrast, FosB and Fra-2 proteins did not show significant changes during the 8-h observation period. These findings suggest that each member of the Fos family responds differently to cellular stress caused by NaF exposure, with c-Fos expression being the most sensitive in MC3T3-E1 cells. Similar results were observed in rat oral epithelial ROE2 cells (Tabuchi et al., 2014), human embryo hepatocytes L-02 cells (Niu et al., 2012), rat testes (Wang et al., 2014) and MC3T3-E1 cells (Duan et al., 2014), where NaF induced c-Fos expression. Treating MC3T3-E1 cells with 5 mM NaF increased c-Fos DNA-binding activity and another AP-1 protein c-Jun (Supplementary Figure S1B), suggesting that AP-1 transactivation activity might be increased by NaF exposure in osteoblastic cells. As concentrations lower than 1 mM NaF did not affect c-Fos protein level in MC3T3-E1 cells, the pathological consequences of c-Fos expression and AP-1 activation may be implicated in the osteotoxic effects of fluorides.

MAPKs are evolutionarily conserved enzymes that transmit extracellular signals to critical intracellular regulatory targets (Chang & Karin, 2001). Activating MAPKs requires dual phosphorylation of threonine and tyrosine residues by specific MAPKK in the catalytic domain. Consistent with our previous experiments using MDPC-23 mouse
ERK1/2 phosphorylates and activates Elk-1, a transcription factor and the ternary complex factor Elk-1 (Whitmarsh & Davis, 1996). ERK1/2 phosphorylation and activation at Ser32 were markedly suppressed by treating with the MEK1/2 inhibitor, U0126 and the MEK5 inhibitor, BIX02189, and partially suppressed by the JNK inhibitor, SP600125 and the p38 inhibitor, SB203580. One major transcriptional control element in the c-fos promoter is the serum response element (SRE) that is bound by a transcription factor complex, including the dimeric serum response factor and the ternary complex factor Elk-1 (Whitmarsh & Davis, 1996). ERK1/2 phosphorylates and activates Elk-1, resulting in enhanced SRE-dependent c-fos transcription (O’Donnell et al., 2012; Whitmarsh & Davis, 1996).

In addition, phosphorylation of the c-Fos protein at Ser374 by ERK1/2 (Chen et al., 1993; Murphy et al., 2002) and at Ser32 by ERK5 (Sasaki et al., 2006) stabilizes the c-Fos protein. Collectively, these findings suggest that, at least in MC3T3-E1 cells exposed to NaF, ERK1/2 and ERK5 pathways are primary responsible for c-fos expression, c-Fos accumulation or both. It remains to be clarified whether oxidative stress generated by fluoride (Barbier et al., 2010) triggers these MAPK signaling cascades and subsequent c-Fos expression in osteoblastic cells.

Bone function is tightly controlled by balancing osteoblast and osteoclast activity (Trouvin & Goe°b, 2010). The key signaling molecules that regulate osteoclastogenesis are the receptor activator of nuclear factor-κB ligand (RANKL) and its decoy receptor OPG. OPG produced by osteoblasts prevents RANKL from binding to its receptor RANK on the surface of osteoclasts and osteoclast precursors and inhibits bone resorption. OPG is primarily produced by osteoblasts and osteoblast progenitors, which are activated by fluoride (Karube et al., 2009). NaF-induced c-Fos expression and its phosphorylation at Ser374 were markedly suppressed by treating with the MEK1/2 inhibitor, U0126 and the MEK5 inhibitor, BIX02189, and partially suppressed by the JNK inhibitor, SP600125 and the p38 inhibitor, SB203580. One major transcriptional control element in the c-fos promoter is the serum response element (SRE) that is bound by a transcription factor complex, including the dimeric serum response factor and the ternary complex factor Elk-1 (Whitmarsh & Davis, 1996). ERK1/2 phosphorylates and activates Elk-1, resulting in enhanced SRE-dependent c-fos transcription (O’Donnell et al., 2012; Whitmarsh & Davis, 1996).

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osteoclast activation and subsequent bone resorption (Reid & Holen, 2009). It has been reported that NaF exposure induces OPG expression in MC3T3-E1 cells (Ren et al., 2011), OS732 human osteoblast-like cells (Lü et al., 2014; Xu et al., 2010) and rat bones (Wang et al., 2015). We found that NaF-induced elevation of OPG mRNA was further enhanced by c-Fos knockdown in MC3T3-E1 cells. Inducing the dominant-negative form of c-fos increased the basal level of OPG mRNA and reduced the suppression of OPG expression by parathyroid hormone in UAMS-32 stromal/osteoblastic cells (Fu et al., 2002). The AP-1 inhibitor, curcumin, augmented interleukin-1α-induced OPG production in periodontal ligament cells (Suda et al., 2009). All these results suggest that c-Fos (and AP-1 activity) may suppress OPG expression in some types of cells, including osteoblastic cells, whereas the underlying mechanisms remain unknown. Thus, while fluoride suppresses osteoclast activation by inducing OPG expression, the concomitant-induced c-Fos expression might function to balance bone formation and resorption.

In addition to regulating RANK–RANKL interactions in bone metabolism, OPG can stimulate cell survival by acting as a receptor for the tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) (Reid & Holen, 2009). It has been reported that OPG siRNA reduced the cell viability of MC3T3-E1 cells treated with 1 or 10 mM NaF for 24 h (Ren et al., 2011). However, c-Fos knockdown, accompanied with a marked elevation of OPG mRNA, failed to prevent decreased cell growth of MC3T3-E1 cells treated with 5 mM NaF for 24 and 48 h (Supplementary Figure S3). Additional experiments will be needed to clarify the functional roles of c-Fos expression and its negative regulation of OPG expression in the pathophysiological consequences of osteoblastic cells exposed to fluorides.

In summary, this study has shown that treating MC3T3-E1 osteoblastic cells with NaF induced c-Fos expression and increased DNA-binding of c-Fos protein. In addition, Fra-1 protein, and not FosB or Fra-2, was elevated in response to fluoride exposure. Although all members of the MAPK family were activated by NaF exposure, ERK1/2 and ERK5 were the major signaling pathways that led to c-fos expression and/or c-Fos accumulation. Inducing c-Fos expression might negatively regulate fluoride-induced expression of OPG, a decoy receptor for RANKL. Investigations on c-Fos expression and its downstream target molecules may help understand the toxicological mechanisms of skeletal fluorosis.

Declaration of interest

The authors declare that there are no conflicts of interest.

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Supplementary material available online

Supplementary Figures 1–3