Na⁺-Ca²⁺ exchanger modulates Ca²⁺ content in intracellular Ca²⁺ stores in rat osteoblasts

Sang Yong Jung^{1*}, Yong Joo Park^{1*},
Young Jun Park¹, Seok Ho Cha¹,
Myung Za Lee² and Chang Kook Suh^{1,3}

¹Department of Physiology and Biophysics
Center for Advanced Medical Education by BK21 Project
Inha University College of Medicine
Incheon 402-751, Korea

²Department of Radiation Oncology
Hanyang University College of Medicine
Seoul 133-791, Korea

³Corresponding author: Tel, 82-32-890-0921;
Fax, 82-32-884-5997; E-mail, cksuh@inha.ac.kr

*These authors contributed equally to this work.

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Abbreviations: ALP, alkaline phosphatase; AS, antisense oligodeoxynucleotide; NCX, Na⁺-Ca²⁺ exchange; SOCI, store operated calcium influx; [Ca²⁺]_i, intracellular Ca²⁺ concentration

Abstract

Na⁺-Ca²⁺ exchanger (NCX) transports Ca²⁺ coupled with Na⁺ across the plasma membrane in a bi-directional mode. Ca²⁺ flux via NCX mediates osteogenic processes, such as formation of extracellular matrix proteins and bone nodules. However, it is not clearly understood how the NCX regulates cellular Ca²⁺ movements in osteogenic processes. In this study, the role of NCX in modulating Ca²⁺ content of intracellular stores ([Ca²⁺]_{ER}) was investigated by measuring intracellular Ca²⁺ activity in isolated rat osteoblasts. Removal of extracellular Na⁺ elicited a transient increase of intracellular Ca²⁺ concentration ([Ca²⁺]_i). Pretreatment of antisense oligodeoxynucleotide (AS) against NCX depressed this transient Ca²⁺ rise and raised the basal level of [Ca²⁺]_i. In AS-pretreated cells, the expression and activity of alkaline phosphatase (ALP), an osteogenic marker, were decreased. However, the cell viability was not affected by AS-pretreatment. Suppression of NCX activity by the AS-pretreatment decreased ATP-activated Ca²⁺ release from intracellular stores and significantly enhanced Ca²⁺ influx via store operated calcium influx (SOCI), compared to those of S-pretreated or control cells. These results strongly suggest that NCX has a regulatory role in cellular Ca²⁺ pathways

in osteoblasts by modulating intracellular Ca²⁺ content.

Keywords: calcium; osteoblasts; sodium-calcium exchanger

Introduction

Bone is a dynamic organ that undergoes a continuous remodeling by the relative activities of osteoblasts and osteoclasts (Rodan and Martin, 1981). Osteoblasts are highly specialized cells that are responsible for the development of bone, production of bone matrix proteins, maintenance of bone density, and formation of hydroxyapatite mineral (Marks and Hermey, 1996). Osteoblasts employ intracellular Ca²⁺ as a second messenger modulating fluid shear stress or hormonal responses and also a cofactor for bone mineralization (Lieberherr *et al.*, 1993; Heaney, 1996; Hung *et al.*, 1996; Chen *et al.*, 2000; Nesti *et al.*, 2002). When sufficient amounts of calcium and phosphate are supplied, osteoblasts synthesize and secrete a collagenous extracellular matrix that nucleates the formation of hydroxyapatite crystal, in response to diverse extracellular stimuli such as steroid hormones, growth factors, cytokines, biochemical signals and extracellular Ca²⁺ concentration (Yamaguchi *et al.*, 1998; McAllister and Frangos, 1999; De Jong *et al.*, 2002). These stimuli elicit a transient rise of intracellular Ca²⁺ concentration ([Ca²⁺]_i) which is triggered both by Ca²⁺ release from intracellular Ca²⁺ stores and Ca²⁺ entry from extracellular fluid (Lieberherr *et al.*, 1993; Hung *et al.*, 1996; Chen *et al.*, 2000; Nesti *et al.*, 2002).

In osteoblasts, intracellular Ca²⁺ is provided by Ca²⁺ release through inositol-1, 4, 5-phosphate receptor (IP₃R) and ryanodine receptor (RyR) of endoplasmic reticulum (ER), and Ca²⁺ influx via Ca²⁺ channels or store operated calcium influx (SOCI) (Adebanjo *et al.*, 2000; Baldi *et al.*, 2003). Na⁺-Ca²⁺ exchanger (NCX) is a transport protein located in plasma membrane and performs Ca²⁺ extrusion (forward mode) or Ca²⁺ influx (reverse mode) depending upon electrochemical gradients of Na⁺ and Ca²⁺. Recently, the presence of NCX has been reported in rat osteoblast like cell line (Short *et al.*, 1994; Stains and Gay, 1998; Lundquist *et al.*, 2000; Stains and Gay, 2001). And it

has been shown that suppression of NCX activity by inhibitors decreases mineral accumulation in extracellular matrix by blocking the efflux of Ca^{2+} from osteoblasts (Stains and Gay, 2001). In particular, a specific inhibitor of the reverse mode NCX, KB-R7943, has been demonstrated to impair the secretion of bone matrix proteins such as type I collagen and bone sialoprotein (Stains and Gay, 2001). Although it was suggested that Ca^{2+} entry through the reverse mode of NCX is the pathway for activating Ca^{2+} release via ryanodine receptors (RyR) from intracellular Ca^{2+} stores in cardiac myocytes (Viatchenko-Karpinski and Gyorke, 2001), it is not clearly understood whether NCX affects other cellular Ca^{2+} pathways such as IP_3R of intracellular Ca^{2+} stores or SOCl in osteoblasts. Thus, in this study, we investigated whether the NCX located in plasma membranes could modulate the intracellular Ca^{2+} mobilization from the intracellular store in osteoblasts.

Materials and Methods

Materials

PCR primers, dNTP mixture and AMV reverse transcriptase XL were bought from TaKaRa Korea Biomedical Co. (Seoul, Korea). Taq DNA polymerase was purchased from Super-Bio Co., Ltd. (Suwon, Korea). TRI reagent for total RNA isolation was purchased from Sigma-Aldrich (St. Louis, MO). SKF96365 and IP_3 were also from Sigma-Aldrich. Streptomycin, penicillin, DMEM and FBS were bought from Gibco BRL (Grand Island, NY). Antibodies were purchased from Swant (Bellinzona, Switzerland) and Santa Cruz Biotechnology (Santa Cruz, CA). All other chemicals applied in this study were of the highest purity available from Sigma-Aldrich.

Isolation and culture of osteoblast

Primary osteoblasts were isolated from femur and tibia of neonatal rats, as described previously (Nam *et al.*, 2002). Femur and tibia were washed several times with Ca^{2+} -, and Mg^{2+} -free PBS and cut into fragments of 1-3 mm². Bone fragments were incubated in 10 ml of digestion solution (0.137% collagenase type V, 0.05% trypsin, 0.8% NaCl, 0.02% KCl, 0.05% $\text{NaH}_2\text{PO}_4\cdot\text{H}_2\text{O}$) for 45 min and the supernatant was discarded. The remaining precipitate was re-incubated in 10 ml of digestion solution for 30 min and the supernatant was harvested three times at 37°C. Harvested supernatants were spun down in 20 ml of DMEM (GIBCO, Grand Island, NY) at $500 \times g$ for 5 min at

room temperature. Remaining pellets were re-suspended in the DMEM solution and the suspended cells were plated in culture dishes at a density of 1×10^5 cells/dish and cultured in DMEM solution supplemented with 10% FBS (GIBCO), penicillin (100 U/ml) and streptomycin (100 mg/ml) at 37°C under humidified 5% CO_2 atmosphere for 7 d. Once the osteoblasts reached 80% confluence at the sixth day of culture, the AS and S oligodeoxynucleotides were treated. Alkaline phosphatase (ALP) staining was used as a marker of osteoblast differentiation after cultured for 7 d (Lian and Stein, 1992).

Antisense oligodeoxynucleotides (AS)

A tandem pair of chimeric phosphorothioated antisense oligodeoxynucleotides (AS) was designed to target the 3' end region of open reading frame within the NCX cDNA. Synthetic AS and sense (S) oligodeoxynucleotides had the following sequences: AS, 5'-AAAATGGTGAAGAGAGTG-3'; S, 5'-TGAACATGACAAATGTCA3'. AS sequences are complementary to a series of nucleotides totally conserved in all three NCX isoforms. Sequences of all probes were known (Genebank), and were not homologous to any other known sequences.

AS and S oligodeoxynucleotides were dissolved in serum free DMEM containing 0.5% lipofectamine, which did not influence the cell viability and morphology, and incubated at room temperature for 30 min. Liposome-DNA complex was added to each well and incubated at 37°C under humidified 5% CO_2 for 5 h. During the transfection, serum free media was used. After incubation for 5 h, serum was added into the culture medium and incubated for additional 24 h. The final concentrations of AS and S oligodeoxynucleotides were maintained at 1 μM . To induce the biochemical and physiological evidence of NCX knockdown, all experiments in this study were carried out after 24 h treatment with or without AS oligodeoxynucleotides.

RT-PCR

Total cellular RNA was extracted from the cultured osteoblasts by using TRIzol reagent according to the manufacture's instructions. Total RNA (500 ng) was subjected for the first-strand cDNA synthesis by using the cDNA cycle kit with oligo-dT as the primer and reverse transcriptase. We used the following primer pairs for PCR amplification: ALP-forward 5'-ATGCTTCGACTAAGTCTCCACCCA-A-3'; reverse 5'-AAAGATGGGTCATGGGGTTCC-CAA-3'. The PCR products were resolved on 1% agarose gels stained with ethidium bromide, and

photographed (Jung *et al.*, 2006).

Western blot analysis

Cells were washed in PBS and scraped in lysis buffer (50 mM Tris/HCl, 2 mM EDTA, 10 mM EGTA, 5 mM DTT, 250 mM sucrose, 1% Triton X-100 at pH 7.5), containing protease inhibitors (0.5 μ g/ml leupeptin, 5 μ g/ml pepstatin, 1 mM PMSF, 1 mM benzamidin, 2 mM iodoacetamide, 1 μ g/ml aprotinin) and homogenized in lysis buffer by 200 strokes of a glass/Teflon homogenizer. The homogenate was centrifuged at $10,000 \times g$ for 15 min, and the resulting supernatant was centrifuged at $100,000 \times g$ for 60 min. The precipitated was re-suspended in lysis buffer, frozen in liquid nitrogen and stored at -70°C until use. Protein concentrations were determined by Bradford method. The membrane fraction (total protein; 40 μ g) mixed with $5 \times$ SDS loading buffer (100 mM Tris-HCl, 200 mM DTT, 4% SDS, 0.2% bromophenol blue, 20% glycerol at pH 6.8) was boiled for 10 min at 100°C . Protein samples were separated by SDS/PAGE [8 or 10% gel] for 2 h. The separated proteins were electro-transferred at 25 V for 6 h by using PVDF membrane. The blots were blocked with washing buffer (10 mM Tris-HCl, 100 mM NaCl, 0.1% Tween 20) containing 5% (w/v) non-fat milk for 2 h, and probed with mouse anti-NCX 1 monoclonal antibody (R3F1; Swant, Bellinzona, Switzerland) and rabbit anti-IP₃R polyclonal antibody (Merck KGaA, Darmstadt, Germany) diluted at 1:1000. This was followed by incubation in HRP-conjugated Goat anti-mouse IgG (Transduc-

tion laboratory, Lexington, KY) diluted at 1:5000. Blots were developed with ECL (Amersham Pharmacia Biotech AB, Uppsala, Sweden).

Immunofluorescence

Osteoblasts cultured on cover slips were fixed with 4% para-formaldehyde solution in PBS for 15 min, and permeabilized with 0.1% Triton X-100 solution in PBS for 10 min at room temperature. Nonspecific binding sites were blocked with 10% goat serum by incubation in PBS for 30 min at room temperature. R3F1 (1:100) was incubated with fixed cells for 2 h. After washing three times with PBS, cells were stained with FITC-conjugated secondary antibody (1:200, Jackson ImmunoResearch, West Grove, PA) for 1 h. After another washing with PBS, samples were observed at the green wavelengths under confocal microscope (Radiance 2100, Bio-Rad, Hercules, CA) as previously described (Kwak *et al.*, 2005).

Measurements of intracellular Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$) and endoplasmic reticulum Ca^{2+} concentration ($[\text{Ca}^{2+}]_{\text{ER}}$)

Intracellular free Ca^{2+} concentration was measured as described previously (Nam *et al.*, 2002). Cells were washed with PBS, and incubated in 2 ml of buffer (0.05% trypsin and 0.02% EDTA). The cells were then resuspended in Tyrode solution (in mM: 140 NaCl, 4 KCl, 2 CaCl_2 , 1 MgCl_2 , 1 NaH_2PO_4 , 5 HEPES, 5.5 Glucose at pH 7.4) and incubated at 37°C with 3 μM fura-2 AM (Molecular Probe, Eu-

Figure 1. Effects of antisense oligodeoxynucleotides against NCX gene on the expression of NCX and IP₃R. (A) the expression of NCX (70 kD) and IP₃R (260 kD) were detected in control, S- or AS-pretreated cells by immunoblotting. (B) NCX expression was detected by confocal immunofluorescence. NCX was probed with anti-mouse NCX monoclonal antibody (R3F1) and labeled with FITC. When the anti-mouse NCX monoclonal antibody was not used, NCX was probed only with FITC in the AS-pretreated osteoblasts.

gene, OR) for 30 min, and transferred to a recording chamber on an epifluorescence inverted microscope (Nikon Diaphot 300, Tokyo, Japan). Experimental solutions were superfused at a flow rate of 2 ml/min. Fluorescence intensity was measured using a cooled CCD camera (Photometrics PXL37, Tucson, AZ), and processed using the Axon Imaging Workbench v.2.2 (Axon Instrument, Foster city, CA).

To measure the Ca^{2+} concentration in ER ($[\text{Ca}^{2+}]_{\text{ER}}$), cells were seeded on glass cover slips for 1 d, and loaded with 5 μM mag-fura-2-AM (Molecular Probe) dissolved in Tyrode solution and incubated at 37°C for 35–45 min. After a brief rinse in a high K^+ solution (in mM: 125 KCl, 25 NaCl, 0.1 MgCl_2 , 10 Hepes, pH 7.2), the dye-loaded cells

were permeabilized by digitonin (5 $\mu\text{g/ml}$) dissolved in an intracellular buffer (high K^+ solution with 170 nM free $[\text{Ca}^{2+}]$, clamped by $\text{Ca}^{2+}/\text{EGTA}$ buffers and supplemented with 0.375 mM Na_2ATP) for 2–3 min. After plasma membrane was permeabilized, cells were superfused continuously with the intracellular buffer (without digitonin). Intracellular free Ca^{2+} concentration and the Ca^{2+} content in ER were presented as the ratio of fluorescence intensities ($R_{340/380}$) excited by alternating illumination of 340 nm and 380 nm. Data are given as mean $\pm$ SEM, where the n refers to the number of cell measured. Paired t -test was used to evaluate the statistical significance. P values less than 0.05 were considered as statistically significant.

Figure 2. Effects of NCX antisense oligodeoxynucleotide on the NCX activity. (A) Na^+ free solution including 1 μM thapsigargin, 250 μM La^{3+} , and 5 mM caffeine was superfused (left arrow) and then the solution was changed with that containing 140 mM Na^+ (right arrows). (B) The reverse mode of NCX activity was presented as the mean values of ratio measured at the time when ratio was highest. (C) The forward mode of NCX activity was presented as the mean values of Ca^{2+} decline rate ($\Delta R_{340/380}/\Delta t$). (Control: $n=180$, AS: $n=240$, S: $n=210$, * $P < 0.05$ and ** $P < 0.01$).

Assay of alkaline phosphatase (ALP)

After osteoblasts were fixed with 95% ethanol and washed with PBS, ALP expression was measured qualitatively by incubating cells in a staining buffer containing 2.4% fast Violet B salt (Sigma-Aldrich) and 10 μ M naphthol AS-MX phosphate alkaline solution (Sigma-Aldrich) for 30-60 min at 37°C in the darkness.

To determine the ALP activity, enzyme assay solution (8 mM *p*-nitrophenyl phosphate, 12 mM MgCl_2 , and 0.1 mM ZnCl_2 in 0.1 M tris buffer, pH 10.5) was added to the cell lysate and incubated for 30 min at 37°C. The enzyme reaction was stopped by adding 0.1 N NaOH. The amount of *p*-nitrophenol released by ALP reaction was determined by measuring the absorbance at 405 nm. Enzyme activity was expressed in U/mg of protein, where 1 U corresponded to 1 nM *p*-nitrophenol liberated per 30 min at 37°C. Total protein content of the cell lysate was measured as described in materials and methods for the western blotting.

Results

NCX protein expression and activity

AS-dependent suppression of NCX protein expression was confirmed by immunoblotting and immunofluorescence. After the cells were pretreated with 1 μ M AS for 24 h, the expression of NCX protein was significantly reduced in the AS-pretreated cells compared to the cells pretreated with the S oligodeoxynucleotides and the control cells (Figure 1A). Fluorescence intensity was reduced in the AS-

pretreated cells but those of S-pretreated and control cells were much higher. Intensity of fluorescence in control cells that were not reacted with anti-NCX monoclonal antibody was much lower (Figure 1B).

To evaluate the reverse mode and forward mode of physiological NCX activity, Ca^{2+} flux was measured with fura-2 microfluorimetry (Figure 2A). The Ca^{2+} flux via NCX was isolated from other cellular Ca^{2+} pathways by superfusing cells with Na^+ -free solution containing 1 μ M thapsigargin (an inhibitor of ER Ca^{2+} -ATPase), 2.5 mM La^{3+} (an inhibitor of plasma membrane Ca^{2+} -ATPase), and 5 mM caffeine (Figure 2A). The superfusion of cells with Na^+ -free solution promptly induced a transient increase of $[\text{Ca}^{2+}]_i$ in osteoblasts ($> 70\%$). The peak value of $R_{340/380}$ in AS-pretreated cells was 1.54 ± 0.05 , and those of the S-pretreated and control cells were 2.22 ± 0.11 and 2.32 ± 0.08 , respectively (Figure 2B). When 140 mM Na^+ was added into the superfusing solution, $[\text{Ca}^{2+}]_i$ decreased. The values of $\Delta R_{340/380}/\Delta t$ was 0.027 ± 0.01 in the AS-pretreated cells, those of S-pretreated and control cells were 0.47 ± 0.02 , 0.45 ± 0.01 , respectively (Figure 2C).

Ca^{2+} release from endoplasmic reticulum by ATP

When Tyrode solution containing 10 μ M ATP was applied for 10 s, $R_{340/380}$ increased transiently and returned slowly to the basal level (Figure 3A). The difference between the peak and basal values for $R_{340/380}$ in the control cells ($n = 34$) was 2.45 ± 0.27 and those in the AS- ($n = 37$) and S-pretreated cells ($n = 34$) were 1.62 ± 0.11 , 2.0 ± 0.15 , respec-

Figure 3. Effects of NCX inhibition on the ATP-activated Ca^{2+} release from intracellular Ca^{2+} stores. (A) Osteoblasts superfused with normal Tyrode solution were treated with 100 μ M ATP. Arrow means the time when ATP was treated. (B) The difference of response to ATP is represented by the values of peak in respective experimental groups. ** $P < 0.01$.

tively (Figure 3B).

Calcium contents in endoplasmic reticulum ($[\text{Ca}^{2+}]_{\text{ER}}$)

When 3 μM IP_3 was applied to the permeabilized cells, $R_{340/380}$ decreased to a lower steady-state level and additional applications of 10 μM IP_3 decreased the $R_{340/380}$ further (Figure 4A). $R_{340/380}$ in control cells was 1.22 ± 0.02 , and those of AS- and S-treated cells were, 1.11 ± 0.03 , and 1.23 ± 0.01 , respectively (Figure 4B). After Ca^{2+} content in the ER was lowered by the application of IP_3 , 0.1 mM ATP was applied to ensure Ca^{2+} reuptake by the ER. The rate of reuptake in control cells is 91 % and those of AS- and S-treated cells were, 92%, and 87%, respectively. Although the basal levels of

$[\text{Ca}^{2+}]_{\text{ER}}$ were lower, Ca^{2+} release induced by addition of 0.1 mM ATP was depressed in the AS-pretreated cells. However, the IP_3R expression was not affected by AS pretreatments (Figure 1A).

Store operated calcium influx (SOCl)

When Ca^{2+} -free solution containing thapsigargin and EGTA was superfused to empty the intracellular Ca^{2+} store, $R_{340/380}$ declined. When the cells were superfused with 2 mM Ca^{2+} solution to elicit the increase of intracellular Ca^{2+} , $R_{340/380}$ increased (Figure 5A). The increment of $R_{340/380}$ was much greater in the AS-pretreated cells (1.09 ± 0.1 ; $n = 60$) than that of the S-pretreated ($n = 55$) or control cells ($n = 50$), (0.49 ± 0.09 and 0.45 ± 0.07 , res-

Figure 4. Effects of NCX inhibition on $[\text{Ca}^{2+}]_{\text{ER}}$. Osteoblasts loaded 5 μM mag-fura-2 were permeabilized with 0.5 $\mu\text{g}/\text{ml}$ digitonin to remove mag-fura 2 from cytosol for 2-3 min and stabilized with superfusion of high K^+ solution. (A) After being stabilized, osteoblasts were treated with 3 μM (1st arrow) and 10 μM IP_3 (2nd arrow) respectively. (B) Basal level of Ca^{2+} content in intracellular Ca^{2+} stores was presented as the mean values of $R_{340/380}$ before the 1st application of IP_3 . (C) After the application of 10 μM IP_3 , changes of Ca^{2+} content in intracellular Ca^{2+} stores were presented as the mean values of $-\Delta R_{340/380}$. To identify the Ca^{2+} release from the intracellular Ca^{2+} stores, the application of 100 μM ATP raised the Ca^{2+} content in intracellular Ca^{2+} stores. $*P < 0.05$.

Figure 5. Effects of NCX inhibition on SOCI. (A) Ca^{2+} -free Tyrode solution including 1 μM thapsigargin, and 1 mM EGTA was superfused to deplete intracellular Ca^{2+} stores and then Tyrode solution including 2 mM Ca^{2+} was superfused to induce Ca^{2+} influx via SOCI. To confirm the enhancement of Ca^{2+} influx via SOCI in AS-pretreated cells, 10 μM SKF 96365, an inhibitor of SOCI, was applied to cells. (B) The difference of SOCI response is represented by the values of peak in respective experimental groups. ** $P < 0.01$.

pectively) as shown in Figure 5B. When an antagonist of SOCI (SKF96365) was added, the increase of $R_{340/380}$ in the AS-pretreated cells ($n = 61$) was suppressed from 1.09 ± 0.1 to 0.31 ± 0.01 (Figure 5B).

Intracellular Ca^{2+} concentration and ALP expression

To further investigate the physiological effects of AS pretreatment, cell viability, basal intracellular Ca^{2+} concentration, and ALP expression of AS-pretreated osteoblasts were examined. Cells (5×10^5) were seeded in culture dishes and pretreated with AS- or S-oligodeoxynucleotides for 24 h. When the viability of cells were determined by the trypan blue staining, AS-pretreated cells numbered $5.5 \times 10^5 \pm 1.8$ cells ($n = 10$), while the S-pretreated and control cells numbered $5.4 \times 10^5 \pm 0.5$ cells ($n = 11$) and $5.99 \times 10^5 \pm 0.6$ cells ($n = 10$), respectively (Figure 6A).

The effect of AS pretreatment on basal intracellular Ca^{2+} concentration was examined by measuring $R_{340/380}$ of osteoblasts. Resting value of $R_{340/380}$ was 1.35 ± 0.06 in the AS-pretreated cells, while those of S-pretreated and control cells were 1.17 ± 0.09 , 1.14 ± 0.03 , respectively (Figure 6B).

Osteoblastic differentiation was examined by measuring ALP activity of osteoblasts. AS-pretreatment suppressed the expression of ALP mRNA and decreased the staining intensity of ALP, compared to those of control and S-pretreated cells (Figure 6C). Furthermore, AS-pretreatment depressed ALP enzyme activity to 2.15 ± 0.45 U/mg protein, compared to those of control cells and

S-pretreated cells, 3.42 ± 0.3 U/mg protein and 3.65 ± 0.6 U/mg protein (Figure 6D).

Discussion

In the osteogenic processes, intracellular free Ca^{2+} serves as one of the major physiological activators. $[\text{Ca}^{2+}]_i$ is regulated by the activity of Ca^{2+} transporters located in endoplasmic reticulum and also in the plasma membrane of osteoblasts (Hattori *et al.*, 2001; Mathov *et al.*, 2001; Stains *et al.*, 2002). In particular, it was demonstrated that NCX was distributed asymmetrically within the plasma membrane and localized adjacent to extracellular matrix and functions to deliver the intracellular Ca^{2+} to the extracellular matrix and activates bone mineralization (Stains and Gay, 1998; Stains *et al.*, 2002). It was also proposed that the Ca^{2+} transported into the bone matrix via the forward mode of NCX serves as a cofactor leading toward increased formation of hydroxyapatite mineral, while the reverse mode of NCX may be closely related with physiological modulation of intracellular Ca^{2+} involved in signaling process (Stains and Gay, 2001; Stains *et al.*, 2002). However, the physiological roles of Ca^{2+} transported by the reverse mode of NCX are not clearly understood. In several studies, a specific inhibitor for the reverse mode of NCX, KB-R7943, has been employed to suppress NCX activity (Stains and Gay, 2001). However, it was evident that KB-R7943 caused some side effects, such as the inhibition of SOCI, depression of high K^+ -induced increase in $[\text{Ca}^{2+}]_i$,

Figure 6. Effects of antisense oligodeoxynucleotide against NCX on cell viability, basal $[\text{Ca}^{2+}]_i$, and ALP assay. (A) Cell viability was measured using trypan blue exclusion method. (B) Basal $[\text{Ca}^{2+}]_i$ was measured for 5 min with fura-2 AM, $*P < 0.05$. (C) The expression of ALP mRNA was measured with RT-PCR. The expression of GAPDH mRNA was included as an internal control. Violet colored staining is indicative of expression of ALP protein for ALP staining. (D) ALP activity assay. ALP activity was assayed in the control, AS- and S-pretreated cells. $*P < 0.05$.

blockade of high K^+ -evoked release of $[\text{H}]$ nor-adrenaline, and inhibition of neuronal nicotinic receptor activity (Arakawa *et al.*, 2000; Nakamura *et al.*, 2000; Pintado *et al.*, 2000).

In this study, AS technique was employed to suppress the NCX activity in osteoblasts, as used in other cells (Slodziniski *et al.*, 1995; Slodziniski and Blaustein, 1998; Kim *et al.*, 2005). Pretreatments of osteoblasts with AS suppressed the expression of NCX protein, as shown in Figure 1. The anti-NCX monoclonal antibody (R3F1) binds

specifically to the 70 kD NCX protein (Figure 1A). Furthermore, the result obtained by immunofluorescence microscopy revealed that, while the control and S-pretreated cells had intensive immuno-labeling, the AS-pretreated cells had minimal immuno-binding (Figure 1B). The AS-dependent suppression of NCX protein was also supported by measurements of the changes in intracellular Ca^{2+} changes (Figure 2). As described previously, the physiological activity of NCX was evaluated by measuring Na^+ -dependent Ca^{2+} influx

employing an experimental design that allows the NCX to operate in reverse mode (Park *et al.*, 2001; Kim *et al.*, 2005). The Na^+ -dependent Ca^{2+} influx via the reverse mode of NCX was reduced significantly by AS-pretreatment. In addition, the rate of Ca^{2+} efflux via the forward mode of NCX was also depressed by 80% (Figure 2). These data provide experimental evidence that both the NCX protein expression and NCX activity in osteoblasts are decreased by AS-pretreatment.

To evaluate the possibility that NCX interacts with other cellular Ca^{2+} pathways, the effects of down-regulation of the NCX expression on ATP-induced Ca^{2+} release and content of intracellular Ca^{2+} stores were investigated. The ATP-induced Ca^{2+} mobilization from intracellular Ca^{2+} stores was decreased significantly in the osteoblasts pretreated with AS for the inhibition of NCX (Figure 3). However, in NCX-inhibited osteoblasts, the expression of IP_3 receptor remained unaffected (Figure 1A). These results suggest that the AS-derived suppression of NCX expression may deplete the stored Ca^{2+} content in ER as shown in Figure 4. Additional evidence for NCX modulation of intracellular Ca^{2+} stores is provided by the result shown in Figure 5. The Ca^{2+} influx via SOCI was significantly enhanced in AS-pretreated cells compared to that of S-pretreated or control cells. In contrast to our findings, other study suggested that KBR-7943, an inhibitor of the reverse mode of NCX, inhibits the Ca^{2+} influx via SOCI in neuron and astrocytes (Arakawa *et al.*, 2000). However, the result shown in Figure 4, decreased Ca^{2+} content in NCX-inhibited cells, strongly supports the hypothesis that an enhanced Ca^{2+} influx via SOCI, as shown in Figure 5, could be due to the decreased activity of NCX.

Endoplasmic reticulum is a universal signaling organelle, which regulates a wide range of Ca^{2+} signaling process (Berridge, 2002). Depletion or depression of Ca^{2+} content in the ER can signal long-term cellular responses such as gene expression and programmed cell death or apoptosis (Elliott, 2001; Rao *et al.*, 2004) and provides a signal that activates Ca^{2+} transporter in the plasma membrane, such as the store operated calcium influx (SOCI) (Putney *et al.*, 2001). And physiological and functional roles of NCX in SOCI were also reported in neurons, suggesting that interactions between NCX and TRPC3 (cationic transient receptor potential) could serve as a good candidate for SOCI (Rosker *et al.*, 2004).

Suppression of NCX may induce higher resting level of $[\text{Ca}^{2+}]_i$ without affecting the cell viability as shown in Figure 6A and 6B. This may then promote the osteoblastic differentiation as suggested

in other studies (Bancroft *et al.*, 2002; Stains *et al.*, 2002). However, the result shown in Figure 6 implies that expression and activity of ALP could be decreased by inhibition of NCX in osteoblasts. Although $[\text{Ca}^{2+}]_i$ was increased under condition that NCX activity was depressed, Ca^{2+} transport via forward mode of NCX would not increase because the forward mode as well as the reverse mode was depressed, as shown in Figure 2. The increase in osteoblastic differentiation is not expected when NCX activity is depressed by AS pretreatment as shown in this study. It also implies that the ALP activity is related, not in a direct manner, to the increase in $[\text{Ca}^{2+}]_i$. Thus, it is speculated that NCX activity in osteoblasts may interact directly with other cellular metabolisms such as osteoblastic differentiation, regardless of intracellular Ca^{2+} activity. Of other possibility is that the decrease of Ca^{2+} content in the intracellular Ca^{2+} stores rather than the cytoplasmic Ca^{2+} may make a contribution toward suppression of the expression of ALP.

In conclusion, these results suggest that NCX plays an important role in regulating the intracellular Ca^{2+} homeostasis of osteoblasts, either directly or indirectly, by modulating intracellular Ca^{2+} stores.

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