H$_2$O$_2$ Enhances Ca$^{2+}$ Release from Osteoblast Internal Stores

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The physiological activity of osteoblasts is known to be closely related to increased intracellular Ca$^{2+}$ activity ([Ca$^{2+}$]) in osteoblasts. The cellular regulation of [Ca$^{2+}$]$_i$ in osteoblasts is mediated by Ca$^{2+}$ movements associated with Ca$^{2+}$ release from intracellular Ca$^{2+}$ stores, and transmembrane Ca$^{2+}$ influx via Na$^+$/Ca$^{2+}$ exchanger, and Ca$^{2+}$ ATPase. Reactive oxygen species, such as H$_2$O$_2$, play an important role in the regulation of cellular functions, and act as signaling molecules or toxins in cells.

In this study, we investigated the effects of H$_2$O$_2$ on cellular Ca$^{2+}$ regulation in osteoblasts by measuring intracellular Ca$^{2+}$ activities using cellular calcium imaging techniques. Osteoblasts were isolated from the femurs and tibias of neonatal rats, and cultured for 7 days. The cultured osteoblasts were loaded with a Ca$^{2+}$-sensitive fluorescent dye, Fura-2, and fluorescence images were monitored using a cooled CCD camera, and subsequently analyzed using image analyzing software. The results obtained are as follows: (1) The osteoblasts with lower basal Ca$^{2+}$ activities yielded a transient Ca$^{2+}$ increase, a Ca$^{2+}$ spike, while osteoblasts with higher basal Ca$^{2+}$ activities showed a continuous increase in [Ca$^{2+}$]$_i$ leading to cell death. (2) Ca$^{2+}$ spikes, generated after removing Na$^+$ from superfusing solutions, were blocked by H$_2$O$_2$ and this was followed by a sustained increase in Ca$^{2+}$ activity. (3) ATP-induced Ca$^{2+}$ spikes were inhibited by pretreating with H$_2$O$_2$ and this was followed by a continuous increase of [Ca$^{2+}$]. When cells were pretreated with the exogenous nitric oxide (NO) donor S-Nitroso-N-acetylpenicillamine (SNAP, 5 µM), treatments of ATP (1 mM) induced a Ca$^{2+}$ spike-like increase, but [Ca$^{2+}$]$_i$ did not return to the basal level. (4) The expression of inositol-1,4,5-triphosphate receptor (IP$_3$R) was enhanced by H$_2$O$_2$.

Our results suggest that H$_2$O$_2$ modulates intracellular Ca$^{2+}$ activity in osteoblasts by increasing Ca$^{2+}$ release from the intracellular Ca$^{2+}$ stores.

**Key Words:** Ca$^{2+}$ activity, H$_2$O$_2$, Na$^+$-Ca$^{2+}$ exchanger, IP$_3$R, osteoblast, NO

**INTRODUCTION**

Oxidative stress due to reactive oxygen species (ROS) has been related to the regulation of cellular functions in physiological and pathological conditions. ROS such as hydrogen peroxide (H$_2$O$_2$), the superoxide anion (O$_2$•⁻), and the hydroxyl radical (OH•) are produced by the oxidation-reduction processes of oxygen molecule, which is used as energy sources in the body. In addition, ROS production is significantly increased during the inflammatory, and aging processes, and by radiation exposure. The augmentation of ROS production causes the dysfunction of primary ion transport mechanisms and the alteration of second messenger systems, primarily perturbing Ca$^{2+}$ homeostasis.

Intracellular Ca$^{2+}$ is known to regulate neurotransmission, muscle contraction, gene expression, and cell growth and cell death as a secondary messenger. The regulation of Ca$^{2+}$ homeostasis is mediated by transmembrane movements of Ca$^{2+}$ via Ca$^{2+}$ channels and Na$^+$-Ca$^{2+}$ exchanger, and the intracellular movement of Ca$^{2+}$ from intracellular Ca$^{2+}$ stores. For non-excitable cells, such as osteoblasts, Ca$^{2+}$ release from the intracellular Ca$^{2+}$ stores plays a major physiological...
role in Ca\textsuperscript{2+} signaling. It has been established that
inositol-1,4,5-triphosphate receptor (IP\textsubscript{3}R) is
primarily responsible for Ca\textsuperscript{2+} release from the in-
tracellular Ca\textsuperscript{2+} stores of osteoblasts.\textsuperscript{20} When me-
chanical stimuli and agonists, such as ATP reach cell membrane in bone tissue, IP\textsubscript{3} released from the
membrane binds with IP\textsubscript{3}R located in the intracellular Ca\textsuperscript{2+}
stores and activates the release of Ca\textsuperscript{2+} from the intracellular Ca\textsuperscript{2+}
stores.\textsuperscript{21-24} It is
thought that the interaction of Ca\textsuperscript{2+} transport proteins with ROS, and the peroxidation of mem-
bane phospholipids lead to alterations in Ca\textsuperscript{2+}
homeostasis, which further enhances abnormal cellular activity, causing signal transduction
changes, and cell dysfunctions. It has been re-
ported that ROS inhibit the activity of Ca\textsuperscript{2+}-
ATPase in the plasma and the sarcoplasmic reticulum (SR) membranes.\textsuperscript{25,26} and modulate Ca\textsuperscript{2+}
release from the SR, and enhance Ca\textsuperscript{2+} influx via
Na\textsuperscript{+}-Ca\textsuperscript{2+} exchanger in cardiac, smooth, and skele-
tal muscles.\textsuperscript{27,28} It was also reported that H\textsubscript{2}O\textsubscript{2}
causes the loss of intracellular Ca\textsuperscript{2+} homeostasis by
inducing a biphasic rise in intracellular Ca\textsuperscript{2+}
activity ([Ca\textsuperscript{2+}]\textsubscript{i}) in the insulin-secreting cell line
CRI-G1. The early phase, of which, was caused by the
mobilization of intracellular Ca\textsuperscript{2+} and later
phase by Ca\textsuperscript{2+} influx from the extracellular me-
dium.\textsuperscript{12} This study also suggested that ROS, at
least H\textsubscript{2}O\textsubscript{2}, may function as second messenger.\textsuperscript{12}
However, these effects of ROS on [Ca\textsuperscript{2+}]\textsubscript{i} are
conflicting and the effects of H\textsubscript{2}O\textsubscript{2} on [Ca\textsuperscript{2+}]\textsubscript{i}
in non-excitable cells, such as osteoblasts are not
fully understood.

In the present study, the effects of H\textsubscript{2}O\textsubscript{2} on the
[Ca\textsuperscript{2+}]\textsubscript{i} of osteoblasts were investigated by
measuring changes in [Ca\textsuperscript{2+}]\textsubscript{i} and the expression of
IP\textsubscript{3}R in the intracellular Ca\textsuperscript{2+} stores. Our results
indicate that H\textsubscript{2}O\textsubscript{2} enhances Ca\textsuperscript{2+} release from
IP\textsubscript{3}-sensitive intracellular Ca\textsuperscript{2+} stores.

MATERIALS AND METHODS

Cell culture

Femur and tibia of neonatal rats were washed
several times with Ca\textsuperscript{2+}, and Mg\textsuperscript{2+} free phosphate
buffered saline (PBS) and cut into fragments of 1-3
mm\textsuperscript{2}. Bone fragments were incubated in 10mL of
digestion solution [0.137% collagenase type V,
0.05% trypsin, 0.8% NaCl, 0.02% KCl, 0.05% NaH\textsubscript{2}PO\textsubscript{4},H\textsubscript{2}O] for 45 min and the supernatant
was discarded. The remaining precipitate was re-
incubated with 10mL of the same solution for
30 min and the supernatant was harvested three
times. The harvested supernatants were spun
down in 20mL of Dulbecco’s modified Eagle’s medium
(DMEM, GIBCO, Grand Island, NY, USA) at 580 \times g for 5 min. The remaining pellets
were re-suspended in the DMEM solution and the
cells plated in culture dishes at a density of 1 \times
10\textsuperscript{6} cells/dish. The cells were then grown in
DMEM solution supplemented with 10% fetal
bovine serum (GIBCO, Grand Island, NY, USA),
penicillin (100 unit/ml) and streptomycin (100
mg/ml). For the experiments, the cells were
cultured in an incubator at 37°C in a humidified
5% CO\textsubscript{2} atmosphere for 7 days.

Intracellular Ca\textsuperscript{2+} measurements

Intracellular Ca\textsuperscript{2+} activities were measured as
described previously.\textsuperscript{20} Briefly, cells were washed
with PBS and then incubated in 2mL of buffer
(0.05% trypsin and 0.02% EDTA). The cells were
then resuspended in a HEPES buffer, pH 7.4,
containing 140 mM NaCl, 4 mM KCl, 2 mM CaCl\textsubscript{2},
1 mM MgCl\textsubscript{2}, 1 mM NaH\textsubscript{2}PO\textsubscript{4}, 5 mM HEPES,
and 5.5 mM Glucose (Tyrode’s solution). The cell
suspension was then loaded with 3 \mu M fura-2 AM
(Molecular Probe, Eugene, Oregon, USA) for 30
min at 37°C.

After loading, the cells were washed with
Tyrode solution and spun down for 5 min at 580
\times g; Cells re-suspended in the Tyrode solution
were transferred to a recording chamber on an
epifluorescence inverted microscope (Nikon
Diaphot 300, Tokyo, Japan). Experimental solu-
tions were superfused at a flow rate of 2mL/min,
and fluorescence was measured using a cooled
CCD camera (Photometrics PXL37, Tucson, Arizo-
na, USA) and cellular Ca\textsuperscript{2+} imaging was processed
using the Axon Imaging Workbench v.2.2 (Axon
Instrument, Foster city, CA, USA). Intracellular
Ca\textsuperscript{2+} activities are presented as R\textsubscript{405/385} the ratio of
fluorescence intensities excited by alternating illu-
mination of 340nm and 380nm beams.
Western blotting

After cells had grown to 80% of confluency in culture dishes, 0.5 mM H$_2$O$_2$ was added for 5, 10, and 20 min, respectively. The cells were homogenized in 4 ml of a buffer solution containing 50 mM Tris/HCl (pH 7.5), 2 mM EDTA, 10 mM EGTA, 5 mM dithiothreitol (DTT), 250 mM sucrose and 1% Triton X-100 and the protease inhibitors (0.5 μg/ml leupeptin, 5 μg/ml pepstatin, 1 mM PMSF, 1 mM benzamidine, 2 mM iodoacetamide, and 1 μg/ml aprotinin). The homogenate was then centrifuged at 10,000 × g for 15 min, and the resulting supernatant was centrifuged at 100,000 × g for 90 min. The crude microsomal pellet was re-suspended in the same buffer as described above, frozen in liquid nitrogen, and stored at 70°C. Protein concentrations were determined by the Lowry method. Protein samples were separated by SDS/PAGE (8-16% gradient gel) and probed with rabbit anti-mouse IP$_3$R polyclonal antibody (Calbiochem, San Diego, CA, USA). Bands were stained with rabbit anti-mouse IP$_3$R antibodies and developed by enhanced chemiluminescence (ECL, Amersham Pharmacia Biotech AB, Uppsala, Sweden) using horseradish peroxidase-conjugated goat anti-rabbit IgG and ECL. Each lane was loaded with 84 μg of total protein.

Experimental solutions

The Tyrode’s solution used in present study had the following composition: 140 mM NaCl, 2.0 mM CaCl$_2$, 5 mM KCl, 1 mM MgCl$_2$, 1 mM NaH$_2$PO$_4$, 5 mM N-2-hydroxyethyl piperazine-N-2-ethanesulfonic acid (HEPES), and 5.5 mM of glucose, pH 7.4. NaCl was isotonically replaced with N-methyl-D-glutamine (NMG) to remove Na$^+$ from Tyrode’s solution (called Na$^+$ free solution). Ca$^{2+}$ was excluded when Tyrode’s solution was superfused and 1 mM ethylenglyco-bis-aminoethyl ether-N, N, N', N'-tetracetic acid (EGTA) was used as Ca$^{2+}$ chelator. 50 μM S-Nitroso-N-acetylpenicillamine (SNAP) was added as a NO donor. All chemicals were purchased from the Sigma Chemical Co. (St. Louis, MO, USA).

RESULTS

Effects of H$_2$O$_2$ on intracellular Ca$^{2+}$ activity in osteoblasts

When cells were sequentially treated with 0.1, 0.5, and 1.0 mM of H$_2$O$_2$ intracellular Ca$^{2+}$ activities changed in two ways. Cells with lower basal [Ca$^{2+}$]$_i$ (8 of 19 cells tested) showed a Ca$^{2+}$ spike (line 2 of Fig. 1A) and cells with a higher basal [Ca$^{2+}$]$_i$ (11 of 19 cells tested) showed a continuous increase in [Ca$^{2+}$]$_i$ (line 1 of Fig. 1A). The basal levels of [Ca$^{2+}$]$_i$ were 0.95 ± 0.06 and 0.84 ± 0.03, respectively. The membranes of cells that responded in Ca$^{2+}$ spike fashion was less damaged morphologically, and the [Ca$^{2+}$]$_i$ of cells that responded in the other fashion increased continuously, which resulted in a cell membrane that leaked enough for the fluorescent dye to diffuse out (not shown in this paper). For this reason, cells with a Ca$^{2+}$ spike were selected for further study. These observations imply that H$_2$O$_2$ enhances Ca$^{2+}$ release from the intracellular Ca$^{2+}$ stores and leads to changes in Ca$^{2+}$ homeostasis. Thus, in the present study, two experimental protocols were employed to induce Ca$^{2+}$ release from the ER.

To investigate the effects of H$_2$O$_2$ on the expression of IP$_3$R, osteoblasts were treated with 0.5 mM of H$_2$O$_2$. Immunoblotting detected IP$_3$R at 260 KDa and showed that the expression of IP$_3$R was increased in cells exposed to 0.5 mM H$_2$O$_2$ for 5 or 10 min, however, the expression of IP$_3$R was decreased in cells exposed for 20 min (Fig. 1B).

Ca$^{2+}$ spikes generated by the superfusion of Na$^+$ free solution

To examine the effects of H$_2$O$_2$ on Ca$^{2+}$ release from the ER, we first elicited the Ca$^{2+}$ induced Ca$^{2+}$ release (CICR) mechanism from the ER by superfusing with Na$^+$ free solution. After [Ca$^{2+}$]$_i$ had been stabilized with Tyrode’s solution, the cells were superfused with Na$^+$ free solution (Fig. 2A) or Na$^+$, and Ca$^{2+}$ free solution (Fig. 2B), and the Ca$^{2+}$ spikes were induced. This response implies that the intracellular Ca$^{2+}$ increment due to the inhibition of Ca$^{2+}$ efflux via the Na$^+$-Ca$^{2+}$ exchanger or the Ca$^{2+}$ influx via reverse mode of
the Na\textsuperscript+-Ca\textsuperscript{2+} exchanger induces Ca\textsuperscript{2+} release from the ER, that it is a form of CICR mechanism. When 0.5 mM of H\textsubscript{2}O\textsubscript{2} was added to the Na\textsuperscript{+} free solution, Ca\textsuperscript{2+} spikes were not observed, but [Ca\textsuperscript{2+}]; increased slightly compared to its basal level (in 10 of 18 cells tested).

**ATP-induced Ca\textsuperscript{2+} spikes**

In order to investigate the effects of H\textsubscript{2}O\textsubscript{2} on IP\textsubscript{3} activated Ca\textsuperscript{2+} release, purinergic receptor (P\textsubscript{2Y}) activation by ATP was employed to generate IP\textsubscript{3}. When 1 mM of ATP was applied to the osteoblasts three times in succession, Ca\textsuperscript{2+} spikes were induced, and the amplitude of the Ca\textsuperscript{2+} spikes decreased from 1.66 ± 0.22, to 1.17 ± 0.12, and then to 1.10 ± 0.15 (n=9) (Fig. 3A). These results confirm that the Ca\textsuperscript{2+} spikes of osteoblasts generated by 1 mM of ATP were due to Ca\textsuperscript{2+} release by the IP\textsubscript{3} receptors of the ER. After a Ca\textsuperscript{2+} spike was induced by 1 mM ATP, the cells were pretreated with 0.5 mM H\textsubscript{2}O\textsubscript{2} and 1 mM of ATP was applied. Ca\textsuperscript{2+} spikes were not observed but [Ca\textsuperscript{2+}]; responded with a slight but sustained increase (14 of 32 cells tested) (Fig. 3B). Meanwhile, when cells were pretreated with 50 μM of SNAP, 1 mM of ATP induced a spike-like increase in [Ca\textsuperscript{2+}]; followed by a sustained increase, and [Ca\textsuperscript{2+}]; did not return to its basal level when the cells were superfused with Tyrode’s solution (35 of 48 cells tested) (Fig. 3C).

**DISCUSSION**

Oxidative stress by ROS has been implicated as one of the major factors in the decline of physiological functions, and it is known to contribute to the dysfunction of ion transport mechanisms, the alteration of electrical activity and signal transductions in cells. Oxidative stress in cells occurs because of the reactions between ROS and biological macromolecules such as membrane proteins and phospholipids.

The findings of our study suggest that ROS modulates the intracellular Ca\textsuperscript{2+} metabolism in...
osteoblasts. Osteoblasts mediate bone formation in bone tissues, and this physiological osteoblast activity is closely related to Ca\(^{2+}\) homeostasis. Recent evidence shows that H\(_2\)O\(_2\) inhibits the differentiation of osteoblasts.\(^{30}\) However, the biphasic effects of ROS on osteoblast activity have not been studied extensively, and the physiological role of H\(_2\)O\(_2\) in the regulation of intracellular Ca\(^{2+}\) activity is not known. Previous studies have shown that intracellular Ca\(^{2+}\) activity in excitable cells is mediated by cellular processes such as transmembrane Na\(^+\)-Ca\(^{2+}\) exchanger, store-operated Ca\(^{2+}\) influx (SOCI), Ca\(^{2+}\) release from intracellular Ca\(^{2+}\) stores, and Ca\(^{2+}\) induced Ca\(^{2+}\) release (CICR).\(^{33-39}\) Although these regulatory processes are controlled by various biological factors, it was reported that oxidative stress by ROS also modifies the physiological functions of osteoblasts and then causes cell death.\(^{31-32}\) In the present study, the membrane integrity of osteoblasts which responded in a Ca\(^{2+}\) spike fashion was maintained morphologically, while an increasing intracellular Ca\(^{2+}\) activity was associated with the loss of cell membrane integrity, as shown in Fig. 1. These findings suggest that H\(_2\)O\(_2\) may act as a second messenger or cytotoxic factor, and that it alters Na\(^+\)-Ca\(^{2+}\) exchanger activity,\(^{28}\) Ca\(^{2+}\) release from the intracellular Ca\(^{2+}\) stores,\(^{35}\) and membrane Ca\(^{2+}\) permeability.\(^{34}\) In addition, ROS-induced lipid peroxidation causes nonspecific ion flux that leads to the disturbance of Ca\(^{2+}\) homeostasis and further modifies the physiological properties of Na\(^+\)-Ca\(^{2+}\) exchanger, which is sensitive to lipid peroxidation. In this study, we focused on the H\(_2\)O\(_2\) effects that generate Ca\(^{2+}\) spikes. Na\(^+\) free solution or ATP was employed to elicit Ca\(^{2+}\) spikes in osteoblasts. The Na\(^+\) free solution was added to increase intracellular Ca\(^{2+}\) activity via the Na\(^+\)-Ca\(^{2+}\) exchanger and CICR. Meanwhile, ATP stimulates P2Y receptor in the cell membrane and induces Ca\(^{2+}\) release from the ER.\(^{35-38}\) However, P2Y receptors were not identified in the present study. Ca\(^{2+}\) spikes generated by Na\(^+\) free solution and ATP were inhibited by H\(_2\)O\(_2\). ATP induced a spike-like increase in [Ca\(^{2+}\)] followed by a sustained increase, and the [Ca\(^{2+}\)] did not return to the basal level when the cells were superfused with Tyrode’s solution. These findings suggest
that H$_2$O$_2$ may induce Ca$^{2+}$ release from IP$_3$-sensitive intracellular Ca$^{2+}$ stores, and that NO may cause alterations in membrane proteins and lipids. The result of immunoblot suggests that H$_2$O$_2$ can diffuse into the cytosol with ease and then modify the physiological activity of IP$_3$R. Although ROS may cause the loss of cell membrane integrity, the physiological role of H$_2$O$_2$ on intracellular Ca$^{2+}$ activity in osteoblasts involves the modulation of Ca$^{2+}$ release from intracellular Ca$^{2+}$ stores.

These results suggest that H$_2$O$_2$ modulates the intracellular Ca$^{2+}$ activity in osteoblasts by increasing Ca$^{2+}$ release from the intracellular Ca$^{2+}$ stores.

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