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Original Article

H_2O_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 ($[Ca^{2+}]_i$) 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_2O_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_2O_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_2O_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_2O_2 and this was followed by a continuous increase of $[Ca^{2+}]_i$. When cells were pretreated with the exogenous nitric oxide (NO) donor S-Nitroso-N-acetylpenicilance (SNAP, 50 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_3R) was enhanced by H_2O_2.

Our results suggest that H_2O_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_2O_2, Na^+-Ca^{2+} exchanger, IP_3R, 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.[1-7] ROS such as hydrogen peroxide (H_2O_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.[8] 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.[9]

Intracellular Ca^{2+} is known to regulate neurotransmission, muscle contraction, gene expression, and cell growth and cell death as a secondary messenger.[10-12] 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.[13-19] For non-excitable cells, such as osteoblasts, Ca^{2+} release from the intracellular Ca^{2+} stores plays a major physiological

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role in Ca^{2+} signaling. It has been established that inositol-1,4,5-triphosphate receptor (IP_3R) is primarily responsible for Ca^{2+} release from the intracellular Ca^{2+} stores of osteoblasts.[20] When mechanical stimuli and agonists, such as ATP reach cell membrane in bone tissue, IP_3 released from the cell membrane binds with IP_3R located in the intracellular Ca^{2+} stores and activates the release of Ca^{2+} from the intracellular Ca^{2+} stores.[21-24] It is thought that the interaction of Ca^{2+} transport proteins with ROS, and the peroxidation of membrane phospholipids lead to alterations in Ca^{2+} homeostasis, which further enhances abnormal cellular activity, causing signal transduction changes, and cell dysfunctions. It has been reported that ROS inhibit the activity of Ca^{2+}-ATPase in the plasma and the sarcoplasmic reticulum (SR) membranes.[25,26] and modulate Ca^{2+} release from the SR, and enhance Ca^{2+} influx via Na^{+}-Ca^{2+} exchanger in cardiac, smooth, and skeletal muscles.[27,28] It was also reported that H_2O_2 causes the loss of intracellular Ca^{2+} homeostasis by inducing a biphasic rise in intracellular Ca^{2+} activity ($[Ca^{2+}]_i$) in the insulin-secreting cell line CRI-G1. The early phase, of which, was caused by the mobilization of intracellular Ca^{2+} and later phase by Ca^{2+} influx from the extracellular medium.[12] This study also suggested that ROS, at least H_2O_2, may function as second messenger.[12] However, these effects of ROS on $[Ca^{2+}]_i$ are conflicting and the effects of H_2O_2 on $[Ca^{2+}]_i$ in non-excitable cells, such as osteoblasts are not fully understood.

In the present study, the effects of H_2O_2 on the $[Ca^{2+}]_i$ of osteoblasts were investigated by measuring changes in $[Ca^{2+}]_i$ and the expression of IP_3R in the intracellular Ca^{2+} stores. Our results indicate that H_2O_2 enhances Ca^{2+} release from IP_3-sensitive intracellular Ca^{2+} stores.

MATERIALS AND METHODS

Cell culture

Femur and tibia of neonatal rats were washed several times with Ca^{2+}, and Mg^{2+} free phosphate buffered saline (PBS) and cut into fragments of 1-3 mm^2. 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% $NaH_2PO_4H_2O$] for 45 min and the supernatant was discarded. The remaining precipitate was re-incubated with 10 ml of the same solution for 30 min and the supernatant was harvested three times. The harvested supernatants were spun down in 20 ml of Dulbecco's modified Eagles's medium (DMEM, GIBCO, Grand Island, NY, USA) at 580 × 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 1×10^5 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℃ in a humidified 5% CO_2 atmosphere for 7 days.

Intracellular Ca^{2+} measurements

Intracellular Ca_{2+} activities were measured as described previously.[29] Briefly, cells were washed with PBS and then incubated in 2 ml 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 $CaCl_2$, 1 mM $MgCl_2$, 1 mM NaH_2PO_4, 5 mM HEPES, and 5.5 mM Glucose (Tyrode's solution). The cell suspension was then loaded with 3 μM fura-2 AM (Molecular Probe, Eugene, Oregon, USA) for 30 min at 37℃.

After loading, the cells were washed with Tyrode solution and spun down for 5 min at 580 × 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 solutions were superfused at a flow rate of 2 ml/min, and fluorescence was measured using a cooled CCD camera (Photometrics PXL37, Tucson, Arizona, USA) and cellular Ca^{2+} imaging was processed using the Axon Imaging Workbench v. 2.2 (Axon Instrument, Foster city, CA, USA). Intracellular Ca^{2+} activities are presented as $R_{340/380}$, the ratio of fluorescence intensities excited by alternating illumination of 340 nm and 380 nm beams.

Western blotting

After cells had grown to 80% of confluency in culture dishes, 0.5 mM H_2O_2 was added for 5, 10, and 20 min, respectively. The cells were the 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℃. 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_3R polyclonal antibody (Calbiochem, San Diego, CA, USA). Bands were stained with rabbit anti-mouse IP_3R 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_2PO_4, 5 mM N-2-hydroxyethyl piperazine-N-2-ethanosulfonic 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 ethylenglycol-bis-aminoethyl ether-N, N, N', N'-tetraacetic acid (EGTA) was used as Ca^{2+} chelator. 50 μM S-Nitroso-N-acetylpenicilance (SNAP) was added as a NO donor. All chemicals were purchased from the Sigma Chemical Co. (St. Louis, MO, USA).

RESULTS

Effects of H_2O_2 on intracellular Ca^{2+} activity in osteoblasts

When cells were sequentially treated with 0.1, 0.5, and 1.0 mM of H_2O_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_2O_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_2O_2 on the expression of IP_3R, osteoblasts were treated with 0.5 mM of H_2O_2. Immunoblotting detected IP_3R at 260 KDa and showed that the expression of IP_3R was increased in cells exposed to 0.5 mM H_2O_2 for 5 or 10 min, however, the expression of IP_3R 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_2O_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

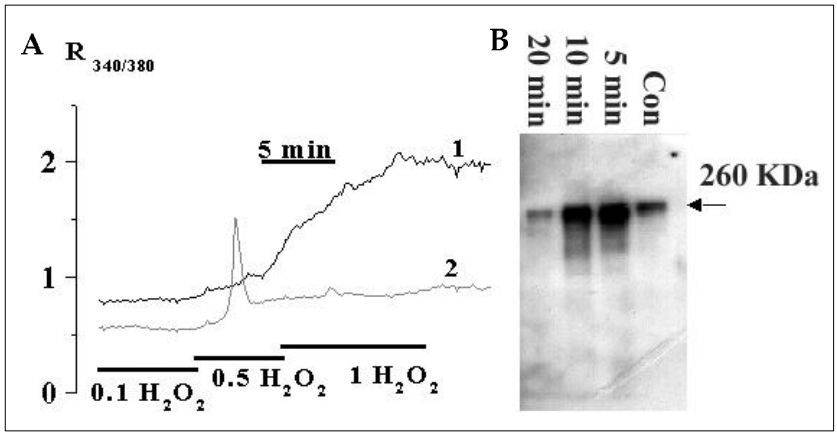

Fig. 1. Effects of H_2O_2 on intracellular Ca^{2+} activity and the expression of IP_3R in osteoblasts. A. When 0.1, 0.5, and 1.0 mM of H_2O_2 were added to Tyrode's solution, the cells responded in two ways. Cells with lower basal levels of $[Ca^{2+}]_i$ showed a Ca^{2+} spike (line 2) and cells with a higher basal level of $[Ca^{2+}]_i$ showed a continuous increase in $[Ca^{2+}]_i$ (line 1). B. The cells were exposed to 0.5 mM H_2O_2 for 5, 10, and 20 min, and stained with rabbit anti-mouse IP_3R antibody for western blot.

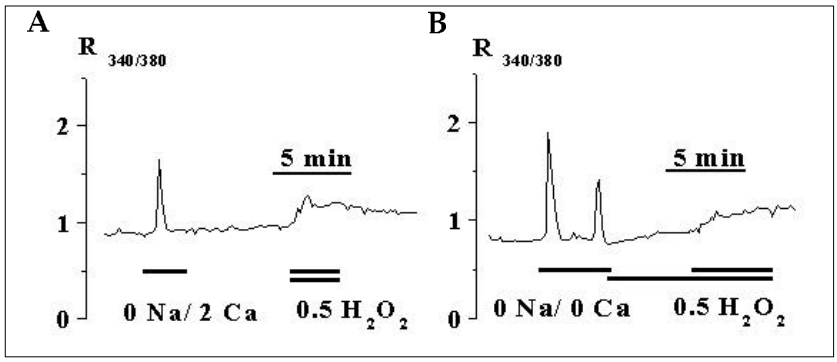

Fig. 2. Effects of H_2O_2 on Ca^{2+} spikes induced by Na^+ removal. A. After the stable basal $[Ca^{2+}]_i$ was determined in Tyrode's solution, the cells were superfused with the Na^+ free solution to elicit Ca^{2+} spikes. Cells were treated with 0.5 mM H_2O_2 and then superfused with the Na^+ free solution. B. After stable basal $[Ca^{2+}]_i$ had been in Tyrode's solution, the cells were superfused with the Na^+ free solution without Ca^{2+}. The cells were then pretreated with 0.5 mM H_2O_2 and superfused with Na^+ free solution without Ca^{2+}.

the Na^+-Ca^{2+} exchanger induces Ca^{2+} release from the ER, that it is a form of CICR mechanism. When 0.5 mM of H_2O_2 was added to the Na^+ free solution, Ca^{2+} spikes were not observed, but $[Ca^{2+}]_i$ increased slightly compared to its basal level (in 10 of 18 cells tested).

ATP-induced Ca^{2+} spikes

In order to investigate the effects of H_2O_2 on IP_3 activated Ca^{2+} release, purinergic receptor (P_2Y) activation by ATP was employed to generate IP_3. When 1 mM of ATP was applied to the osteoblasts three times in succession, Ca^{2+} spikes were induced, and the amplitude of the Ca^{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^{2+} spikes of osteoblasts generated by 1 mM of ATP were due to Ca^{2+} release by the IP_3 receptors of the ER. After a Ca^{2+} spike was induced by 1 mM ATP, the cells were pretreated with 0.5 mM H_2O_2 and 1 mM of ATP was applied. Ca^{2+} spikes were not observed but $[Ca^{2+}]_i$ 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 $[Ca^{2+}]_i$ followed by a sustained increase, and $[Ca^{2+}]_i$ 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.[9]

The findings of our study suggest that ROS modulates the intracellular Ca^{2+} metabolism in

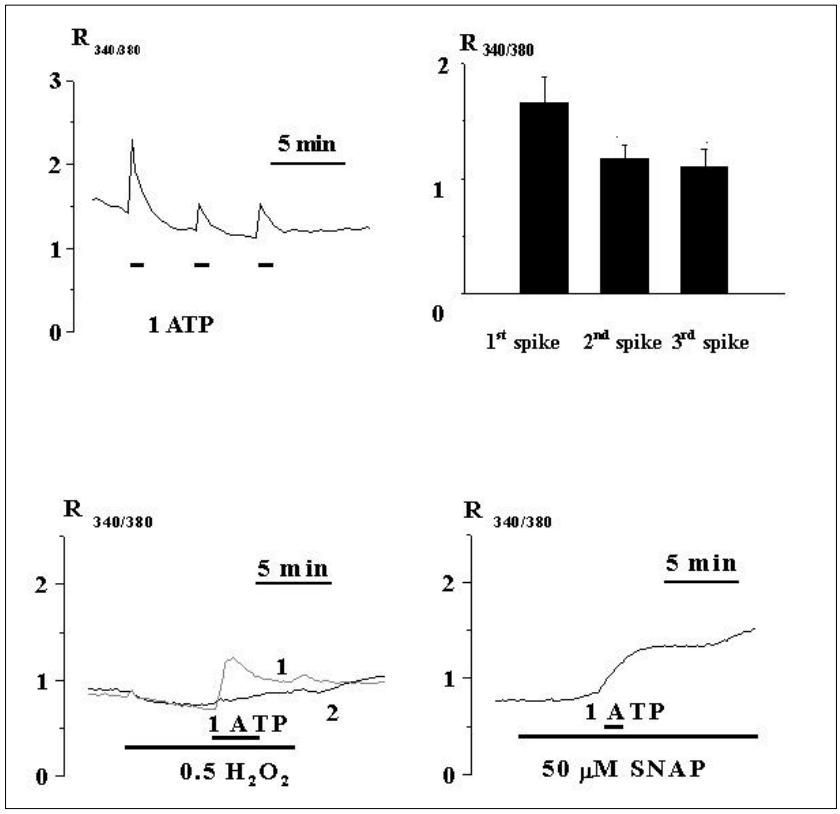

Fig. 3. Effects of H_2O_2 and NO on ATP-induced Ca^{2+} spikes. A. Tyrode solution was superfused and then 1 mM of ATP was added three times. Additions of 1 mM of ATP stimulated the IP3R in the ER and generated ATP-induced Ca^{2+} spikes. Values shown are means ± SEM (inset). B. To investigate the effects of H_2O_2 on ATP-induced Ca^{2+} spikes, cells were pretreated with Tyrode's solution containing 0.5 mM of H_2O_2, and 1 mM ATP was applied. C. To investigate the effects of NO on ATP-induced Ca^{2+} spikes, the cells were pretreated with Tyrode's solution containing 50 μM of SNAP and 1 mM of ATP was then applied.

osteoblasts. Osteoblasts mediate bone formation in bone tissues, and this physiological osteoblast acti vity is closely related to Ca^{2+} homeostasis. Recent evidence shows that H_2O_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_2O_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).[13-19] Although these regulatory processes are controlled by various biological factors, it was reported that oxidative stress by ROS also modifies the physiological functions of ostoblasts 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_2O_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,[33] 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_2O_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 applied to increase intracellular Ca^{2+} activity via the Na^+-Ca^{2+} exchanger and CICR. Meanwhile, ATP stimulates P_2Y receptor in the cell membrane and induces Ca^{2+} release from the ER.[35-36] However, P_2Y receptors were not identified in the present study. Ca^{2+} spikes generated by Na^+ free solution and ATP were inhibited by H_2O_2. ATP induced a spike-like increase in $[Ca^{2+}]$ followed by a sustained increase, and the $[Ca^{2+}]_i$ did not return to the basal level when the cells was superfused with Tyrode's solution. These findings suggest

that H_2O_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_2O_2 can diffuse into the cytosol with ease and then may modify the physiological activity of IP_3R. Although ROS may cause the loss of cell membrane integrity, the physiological role of H_2O_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_2O_2 modulates the intracellular Ca^{2+} activity in osteoblasts by increasing Ca^{2+} release from the intracellular Ca^{2+} stores.

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S.N. Nam and S.Y. Jung contributed equally to this work.

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