N-acetyl cysteine inhibits H$_2$O$_2$-mediated reduction in the mineralization of MC3T3-E1 cells by down-regulating Nrf2/HO-1 pathway

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There are controversial findings regarding the roles of nuclear factor (erythroid-derived 2)-like 2 (Nrf2)/heme oxygenase-1 (HO-1) pathway on bone metabolism under oxidative stress. We investigated how Nrf2/HO-1 pathway affects osteoblast differentiation of MC3T3-E1 cells in response to hydrogen peroxide (H$_2$O$_2$), N-acetyl cysteine (NAC), or both. Exposing the cells to H$_2$O$_2$ decreased the alkaline phosphatase activity, calcium accumulation, and expression of osteoblast markers, such as osteocalcin and runt-related transcription factor-2. In contrast, H$_2$O$_2$ treatment increased the expression of Nrf2 and HO-1 in the cells. Treatment with hemin, a chemical HO-1 inducer, mimicked the inhibitory effect of H$_2$O$_2$ on osteoblast differentiation by increasing the HO-1 expression and decreasing the osteogenic marker genes. Pretreatment with NAC restored all changes induced by H$_2$O$_2$ to near normal levels in the cells. Collectively, our findings suggest that H$_2$O$_2$-mediated activation of Nrf2/HO-1 pathway negatively regulates the osteoblast differentiation, which is inhibited by NAC. [BMB Reports 2015; 48(11): 636-641]

INTRODUCTION

Numerous studies have been performed to clarify the mechanisms by which oxidative stress negatively or positively modulates osteoblast differentiation and mineralization. It is believed that excessive oxidative stress decreases bone formation by down-regulating differentiation and viability of osteoblasts (1, 2). The intracellular accumulation of reactive oxygen species (ROS) in osteoblasts leads to oxidative stress-mediated bone damage (3). It is also believed that ROS accumulation stimulates bone resorption by activating the intracellular signaling involved in osteoclast differentiation, as well as by diminishing the capacity of cellular antioxidant defense systems (4, 5). In contrast, the administration of antioxidant compounds, such as α-tocopherol succinate and *N*-acetyl cysteine (NAC), exerted protective effects on oxidative damages (6, 7). Accordingly, it is suggested that oxidative stress disrupts the differentiation and mineralization of osteoblasts, and this disruption is prevented by antioxidants.

The induction of nuclear factor (erythroid-derived 2)-like 2 (Nrf2) is known to play important roles on the protection of tissues or cells from various oxidative damages (8, 9). Specifically, oxidative stress evokes nuclear Nrf2 translocation, in which the transcription factor leads to the recognition of antioxidant response elements on target genes. Heme oxygenase-1 (HO-1) is one of the main oxidative stress markers which are induced by the activation of Nrf2. The induction of HO-1 regulates numerous cellular responses involved in iron homeostasis, antioxidant defense mechanism, and bone resorption (10-12). Accordingly, it is suggested that the Nrf2/HO-1 signal is sensitively activated by oxidative stress to maintain the intracellular redox balance and the activation of Nrf2/HO-1 pathway tightly affects the processes required for bone homeostasis (13, 14). However, it is important to note that Nrf2 interferes with the transcriptional activation dependent on runt-related transcription factor-2 (Runx2), eventually leading to negative regulation on the differentiation and mineralization of osteoblasts (15). Furthermore, the differentiation and mineralization of osteoblasts was inhibited by upregulating the HO-1 (16). These reports suggest a controversial role of Nrf2/HO-1 signal on osteogenesis.

Here, we examined the effects of oxidative stress on osteoblast differentiation and Nrf2/HO-1 pathway, by exposing the MC3T3-E1 osteoblasts to various concentrations of hydrogen peroxide (H$_2$O$_2$). We also investigated the effects of NAC on osteogenic marker expression and mineralization in H$_2$O$_2$-

exposed osteoblasts. In addition, we explored whether hemin, a chemical inducer of HO-1, mimicked the inhibitory effects of H_2O_2 on osteoblast differentiation and mineralization.

RESULTS

H_2O_2 inhibits dose-dependently ALP activity, calcium accumulation, and viability in MC3T3-E1 cells

Cells were treated with various concentrations (0-400 μM) of H_2O_2 in osteoblast differentiating medium for 7 days and then the cells were evaluated for their differentiation and viability. Exposing the cells to H_2O_2 caused a dose-dependent reduction of alkaline phosphatase (ALP) activity and calcium accumulation, where significant decreases were noted at 100 μM (Fig. 1A). It was also seen that exposing to concentrations higher than 200 μM H_2O_2 decreased viability of the cells (Fig. 1B).

H_2O_2 increases the induction of Nrf2 and HO-1 in osteoblasts

We next examined whether H_2O_2 is the direct mediator to activate Nrf2/HO-1 pathway in osteoblasts. The addition of 100 μM H_2O_2 increased the HO-1 protein and mRNA levels in MC3T3-E1 cells, which were further augmented by treatment with 200 or 400 μM H_2O_2 (Fig. 2A, B). H_2O_2 treatment also increased the nuclear level of Nrf2, with a simultaneous reduction of nuclear Runx2 in the cells (Fig. 2C, D).

NAC inhibits H_2O_2-mediated decrease in osteoblast differentiation by down-regulating the expression of Nrf2 and HO-1

We explored whether the H_2O_2-induced changes in the induction of Nrf2, HO-1, and Runx2 are related to oxidative stress by incubating the cells with 200 μM H_2O_2 in combination with 2.5 or 5 mM NAC. NAC significantly inhibited the H_2O_2-induced increases in HO-1 induction (P < 0.001) and

Nrf2 nuclear translocation (P < 0.05) by restoring the nuclear Runx2 level (Fig. 3A, B). NAC treatment almost completely blocked the H_2O_2-induced reduction in osteocalcin (OCN) mRNA level (Fig. 3C), ALP activity (Fig. 3D), and calcium content (Fig. 3E) in the cells.

HO-1 induction inhibits mineralization and calcium accumulation in MC3T3-E1 cells

We further investigated the regulatory roles of HO-1 on osteoblast differentiation by treating the cells with hemin. In this study, the cells were treated with 50 μM hemin for 24 h and then the osteogenic medium was changed to the medium without hemin. Thereafter, the cells were additionally incubated for various times (0-14 days) according to the experimental purpose. Pretreatment with hemin for 24 h decreased the bone-like nodule formation in MC3T3-E1 cells (Fig. 4A). Hemin at 20 or 50 μM also reduced the ALP activity (Fig. 4B) and calcium accumulation (Fig. 4C) in a dose-dependent manner. Pretreatment with hemin at the same concentrations dose-dependently augmented the expression of HO-1 at 24 h of additional incubation (Fig. 4D), while it decreased the mRNA expression of Runx2 (Fig. 4E) and OCN (Fig. 4F), as well as the nuclear level of Runx2 (Fig. 4G). However, the HO-1 inducer at 10 μM did not change the ALP
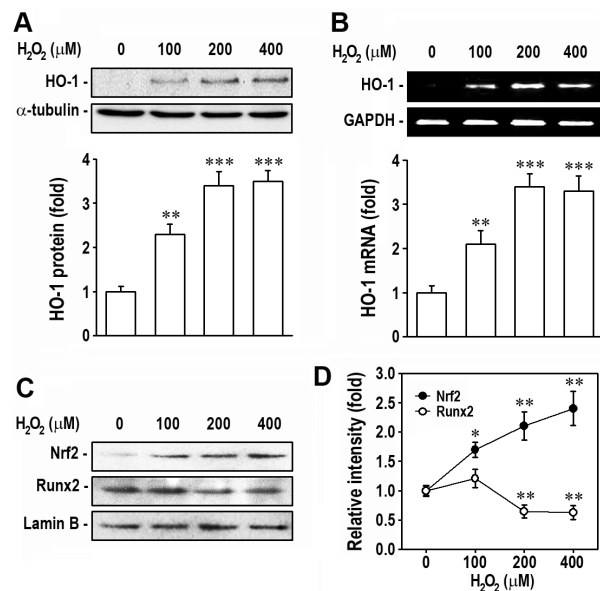
Fig. 2. H_2O_2 upregulates the induction of Nrf2 and HO-1 with attendant corresponding reduction of Runx2 in MC3T3-E1 cells. Cells were exposed to the indicated doses of H_2O_2. The HO-1 protein (A) and mRNA levels (B), and the nuclear protein levels of Nrf2 and Runx2 (C) were determined after 24 h of incubation by immunoblotting or RT-PCR. (D) Relative intensities of Nrf2 and Runx2 proteins from triplicate experiments are shown. *P < 0.05, **P < 0.01, and ***P < 0.001 vs. the control values without H_2O_2.
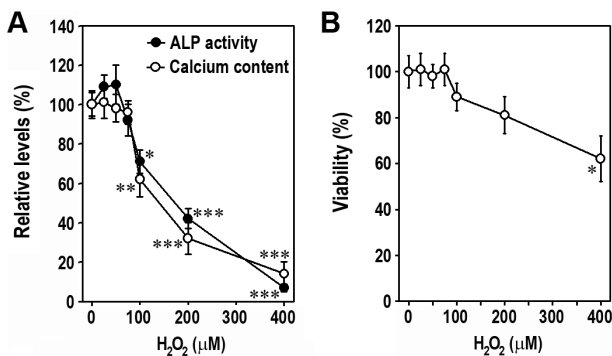
Fig. 1. H_2O_2 inhibits the ALP activity, calcium accumulation, and viability in a dose-dependent manner. Cells were exposed to increasing concentrations (0-400 μM) of H_2O_2. (A) ALP activity and calcium content, and (B) viability of the cells, were measured after 7 days of exposure. *P < 0.05, **P < 0.01, and ***P < 0.001 vs. the control values without H_2O_2.
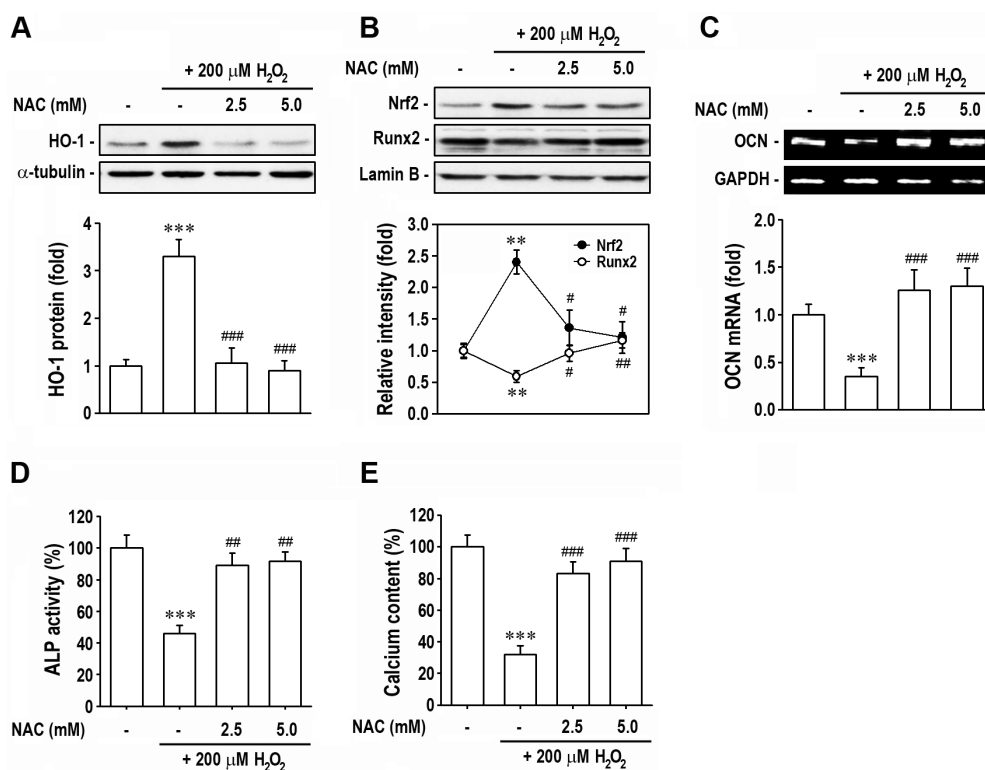
Fig. 3. NAC prevents the H$_2$O$_2$-mediated decreases in OCN expression, ALP activity, and calcium accumulation, by restoring the levels of Nrf2, HO-1, and Runx2 to those of non-treated control values. Cells were incubated with 200 μM H$_2$O$_2$ in the presence and absence of 2.5 mM or 5 mM NAC. (A, B) Protein levels of HO-1, Nrf2, and Runx2 were measured by immunoblotting after 24 h of incubation. (C) OCN mRNA expression, (D) ALP activity, and (E) calcium content, were also determined after 7 days of incubation. **P < 0.01 and ***P < 0.001 vs. the control values without H$_2$O$_2$. #P < 0.05, ##P < 0.01, and ###P < 0.001 vs. 200 μM H$_2$O$_2$ treatment only.

activity and calcium content, or the expression of osteogenic markers in the cells (data not shown). The negative effect of hemin on osteoblast differentiation of MC3T3-E1 cells was not due to cytotoxic effect, in that the treatment with 20 or 50 μM hemin for 1 day did not reduce viability of the cells (Fig. 4H, left panel). In contrast, co-incubation with more than 20 μM hemin for 3 days caused a slight reduction in viability of the cells (Fig. 4H, right panel).

DISCUSSION

The differentiation processes of osteoblasts can be distinguished mainly by two steps, i.e. extracellular matrix maturation and mineralization which are controlled by various osteogenic markers (17). ALP is one of the earliest markers expressed during the osteoblast differentiation process, while OCN, a non-collagenous and vitamin K-dependent protein, is secreted by mature osteoblasts during matrix calcification (18). Our present findings reveal that H$_2$O$_2$ dose-dependently inhibits the maturation and mineralization of MC3T3-E1 cells, along with the decreased expression of osteogenic markers.

There are a number of studies that have shown the beneficial effects of NAC on mineralization from osteogenic (19) and on survival rates in mice exposed to irradiation (5). Consistent with these reports, our data show that NAC at 5 mM reverses the effects of H$_2$O$_2$ to suppress the osteoblastic differentiation of MC3T3-E1 cells. This protective effect of NAC was accompanied by the restoration of bone specific markers ALP and OCN. NAC treatment also inhibited the H$_2$O$_2$-mediated increases of Nrf2 and HO-1 induction in MC3T3-E1 cells. However, NAC treatment alone did not cause any change in the viability, DNA synthesis, and the levels of cellular ROS and HO-1 protein, as well as the HO-1 enzymatic activity, in MC3T3-E1 cells (data not shown). These results strongly support that the decrease in the osteoblast differentiation and mineralization caused by the excessive oxidative stress is prevented by antioxidants.

We previously found that Nrf2-mediated signaling could play dual roles on the viability of cells depending on the condition of stress exposed; under a mild oxidative stress, Nrf2 activation has a protective role by maintaining normal levels of ROS, whereas it leads to cell injury under a persistent oxida-
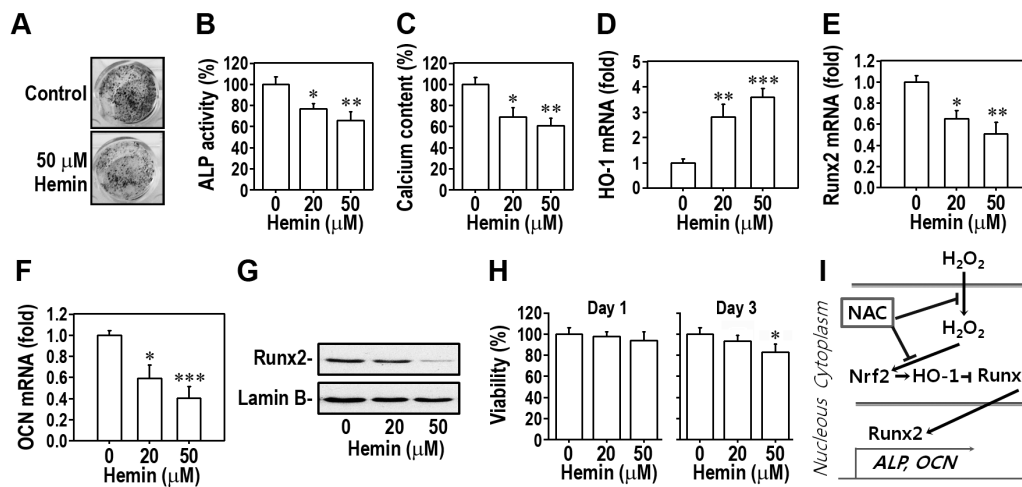
Fig. 4. The increase of HO-1 induction by hemin reduces the expression of osteogenic markers and mineralization of MC3T3-E1 cells. Cells were pretreated with the indicated concentrations of hemin in osteogenic medium for 24 h; the medium was replaced with fresh medium without hemin, followed by additional incubation for various times. Mineralization of the cells was evaluated by alizarin red staining (A) and ALP activity assay (B) at 7 days of incubation, or by calcium content determination (C) at 14 day post-incubation. The mRNA expressions of HO-1 (D), Runx2 (E), and OCN (F), and the nuclear level of Runx2 (G), were determined after 24 h of incubation by real time RT-PCR and Western blot analyses, respectively. Panel H shows viability of the cells exposed to 20 μM and 50 μM hemin for 1 or 3 days in osteogenic medium. (I) A proposed mechanism involved in the H$_2$O$_2$-mediated reduction of osteoblast differentiation, and its inhibition by NAC. *P < 0.05, **P < 0.01, and ***P < 0.001 vs. the control cells without hemin.

tive stress (20). However, the current results highlight that activation of the Nrf2 pathway by H$_2$O$_2$ contributes predominantly to the down-regulation of microenvironmental events required for osteoblast differentiation. There is a report showing that H$_2$O$_2$ exposure within the range of non-toxic concentrations diminishes mineralization of MC3T3-E1 cells, and this is accompanied by reduced osteogenic marker expression and increased Nrf2 induction (21). This is also supported by the current findings which show that exposure only at 400 μM H$_2$O$_2$ significantly decreased viability of the cells. Further studies on the upstream regulators of Nrf2/HO-1 pathway in H$_2$O$_2$-exposed osteoblasts are required. It is also necessary to elucidate how Nrf2 affects Runx2-dependent transcriptional activation in oxidative stress-exposed osteoblasts.

Upregulation of HO-1 by hemin exerts a beneficial effect on wound healing (22). The induction of HO-1 suppressed osteoclastogenesis and bone destruction in an arthritis model (11). It was also reported that HO-1 induction upregulated the osteogenic differentiation in human periodontal ligament cells (23). However, HO-1 induction is also able to negatively regulate osteoblast differentiation, depending on microenvironmental conditions (24). It was also reported that treatment with hemin decreased the mRNA expression of osteogenic markers and calcium deposition in osteoblasts (16). These reports with our present findings suggest that upregulation of HO-1 may inhibit the maturation and mineralization of osteoblasts. It is also likely that H$_2$O$_2$ at relatively high concentrations leads to HO-1 activation, and this is the critical event responsible for the de-

creased osteogenesis in MC3T3-E1 cells.

In conclusion, this study highlights that oxidative stress may reduce the maturation and mineralization in MC3T3-E1 cells via the activation of Nrf2/HO-1 pathway and the decreased expression of bone differentiation markers. The down-regulation of Runx2 and the corresponding reduction of ALP and OCN are the important events related to H$_2$O$_2$-mediated inhibition of osteoblast differentiation and mineralization (Fig. 4I). Our current findings also reveal that NAC restores all the H$_2$O$_2$-induced changes to near levels of untreated control.

MATERIALS AND METHODS

Chemicals and laboratory equipment

Unless otherwise specified, chemicals and laboratory wares were purchased from Sigma-Aldrich Co. LLC. (St. Louis, MO, USA) and Falcon Labware (Becton-Dickinson, Franklin Lakes, NJ, USA), respectively.

Cell cultures

MC3T3-E1 cells (ATCC, CRL-2593) were cultured in α-minimum essential medium (α-MEM) supplemented with 10% fetal bovine serum (FBS; HyClone, Logan, UT, USA) and antibiotics. Culture medium was replaced twice per week. After cells reached 70-80% confluence in 6-well or 96-multiwell culture plates, the medium was replaced with osteoblast differentiating medium (α-MEM supplemented with 10% FBS, 100 nM dexamethasone, 50 μM ascorbic acid, and 5 mM β-glycerophosphate).

H_2O_2 exposure

MC3T3-E1 cells incubated in osteogenic medium were exposed to various concentrations (0-400 μM) of H_2O_2 in the presence and absence of 2.5 mM and 5 mM NAC. After various times (0-14 days) of exposure, the cells were evaluated for their ALP activity, calcium content, viability, Nrf2 and HO-1 induction, bone-specific gene expression, and mineralization.

Measurement of viability

The ability of cells to reduce 3-(4,5-dimethylthiazol-2yl-)-2,5 -diphenyl tetrazolium bromide (MTT), where the reducing activity is proportional to the viability of the cells, was measured according to methods described elsewhere (25).

Determination of mineralization and ALP activity

The degree of mineralization was determined at 7 days after H_2O_2 exposure by staining the cells with alizarin red, according to the methods described previously (26). Intracellular calcium content was measured using a Calcium C kit (Wako Chemical Inc., Osaka, Japan). The calcium content was expressed as the relative percent to the non-treated control level. ALP activity was measured by the methods described previously (27), and the enzyme activity was expressed as the relative percent to the control value.

RNA preparation and polymerase chain reaction

Total RNA was prepared using the SV Total RNA Isolation System (Promega, Madison, WI, USA) and reverse-transcribed using a RNA PCR kit, according to the instruction manuals (Access RT-PCR System, Promega). The quantitative amplifications were performed using a DNA thermal cycler (model PTC-100, MJ Research, Waltham, MA, USA), and the amplified PCR products were detected by ethidium bromide staining after electrophoresis in 1-2% agarose gels. Band intensity was calculated using a gel imaging system (model F1-F2 Fuses type T2A, BIO-RAD, Segrate, Italy). The mRNA expression of HO-1, Runx2, and OCN was also determined by real-time RT-PCR, according to the methods described previously (28). In brief, cDNA synthesis was performed with 1 μg of total RNA using SuperScript Reverse Transcripatase II and oligo$_{12-18}$ primers. Power SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA) was used to detect the accumulation of PCR product during cycling with the ABI 7500 sequence detection system (Applied Biosystems).The PCR primer sequences specific for OCN, Runx2, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) have been described earlier (28). Primer sequences for HO-1 are 5'-aagaggctaagaccgccttc-3' and 5'-gcataaattcccactgccac-3'. All the PCR reactions were performed at least in triplicate, and the expression levels were normalized to GAPDH signal in the same reaction.

Western blot analysis

The nuclear and whole cell proteins were prepared at various intervals after H_2O_2 or hemin exposure, according to the methods described previously (29). Equal amounts of protein samples were separated on a 12% SDS-PAGE, and blotted onto PVDF membranes. Blots were probed with primary antibodies and incubated with horseradish peroxidase-conjugated anti-IgG in a blocking buffer for 1 h. The blots were developed with enhanced chemiluminescence (GE Healthcare, Buckinghamshire, UK) and exposed to X-ray film (Eastman-Kodak, Rochester, NY, USA). The antibodies specific for Runx2 (M-70: sc-10758, rabbit IgG), HO-1 (C-18: sc-1796, goat IgG), Nrf2 (C-20: sc-722, rabbit IgG), lamin B (C-20: sc-6216, goat IgG), and α-tubulin (B-7: sc-5286, mouse IgG$_{2a}$) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

Statistical analysis

Unless specified otherwise, the data are expressed as the mean $\pm$ standard deviations (S.D.) from triplicate experiments (at least three samples per experiment). A one-way analysis of variance (SPSS version 12.0 software) followed by Scheffe's test was applied to determine the significance of differences between groups. A P-value < 0.05 was considered significant.

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