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Activation of AMPK participates hydrogen sulfide-induced cyto-protective effect against dexamethasone in osteoblastic MC3T3-E1 cells

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ABSTRACT

Long-time glucocorticoids (GCs) usage causes osteoporosis. In the present study, we explored the potential role of hydrogen sulfide (H_2S) against dexamethasone (Dex)-induced osteoblast cell damage, and focused on the underlying mechanisms. We showed that two H_2S-producing enzymes, cystathionine β-synthase (CBS) and cystathionine γ-lyase (CSE), were significantly downregulated in human osteonecrosis tissues as well as in Dex-treated osteoblastic MC3T3-E1 cells. H_2S donor NaHS as well as the CBS activator S-adenosyl-L-methionine (SAM) inhibited Dex-induced viability reduction, death and apoptosis in MC3T3-E1 cells. NaHS activated adenosine monophosphate (AMP)-activated protein kinase (AMPK) signaling, which participated its cyto-protective activity. AMPK inhibition by its inhibitor (compound C) or reduction by targeted-shRNA suppressed its pro-survival activity against Dex in MC3T3-E1 cells. Further, we found that NaHS inhibited Dex-mediated reactive oxygen species (ROS) production and ATP depletion. Such effects by NaHS were again inhibited by compound C and AMPKα1-shRNA. In summary, we show that H_2S inhibits Dex-induced osteoblast damage through activation of AMPK signaling. H_2S signaling might be further investigated as a novel target for anti-osteoporosis treatment.

1. Introduction

Glucocorticoids (GCs) are clinically used anti-inflammatory and immuno-suppressive medicine to treat many diseases [1]. However, prolonged and/or overdose GCs (i.e. dexamethasone (Dex)) could cause non-trauma osteonecrosis, and lead to bone fracture [2–4]. Around 30% of patients receiving long-term GCs suffered bone fractures [2]. Osteoblasts play important roles in regulating bone growth and formation, and are main target cells in the pathological process of osteoporosis [5,6].

Hydrogen sulfide (H_2S) has recently been recognized as an important intracellular gas signaling, which regulates many important physiological or pathological cellular functions [7–9]. Endogenous H_2S is formed by cysteine by cystathionine β-synthase (CBS) and cystathionine γ-lyase (CSE) [7–9]. Recent studies have demonstrated the cyto-protective activity of H_2S in a number of systems. For example, H_2S inhibits rotenone-induced neuronal cell apoptosis via preservation of mitochondrial function [10]. In osteoblastic like cells, H_2S protects osteoblastic MC3T3-E1 cells against oxidative stress via inhibition of mitogen-activated protein kinase (MAPK) signaling [11]. However, the potential role of H_2S against GCs (i.e. Dex)-induced osteoblast cell damage has not been studied.

Adenosine monophosphate (AMP)-activated protein kinase (AMPK) plays a vital role in maintaining metabolism and energy balance [12,13]. Recent studies have indentified other consequences following AMPK activation. Activated AMPK regulates nicotinamide adenine dinucleotide phosphate (NADPH) homeostasis to protect cells from oxidative stresses [14]. Further, energy starvation-activated AMPK phosphorylates tuberous sclerosis complex 2 (TSC2) to inhibit mammalian TOR (mTOR) signaling, and eventually promoting cell survival [15]. In cultured osteoblasts, activation of AMPK inhibits H_2O_2-induced cell apoptosis [16,17]. Recent studies have shown that H_2S could activate AMPK [18,19], however its

Abbreviations: AMPK, adenosine monophosphate (AMP)-activated protein kinase; ACC, acetyl-CoA carboxylase; CBS, cystathionine β-synthase; CSE, cystathionine γ-lyase; Dex, dexamethasone; GCs, glucocorticoids; H_2S, hydrogen sulfide; LDH, lactate dehydrogenase; MTT, methyl thiazolyl tetrazolium; ROS, reactive oxygen species; SAM, S-adenosyl-L-methionine.

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role in H_2S-mediated cyto-protective activity is not fully understood.

In the current study, we aim to understand the potential role of H_2S in Dex damages in osteoblastic MC3T3-E1 cells, and to investigative the underlying mechanisms. We found that H_2S protected MC3T3-E1 cells from Dex through activating AMPK signaling, implying a potentially therapeutic value of H_2S for treatment of osteoporosis.

2. Materials and methods

2.1. Chemical and reagents

NaHS, purchased from Sigma Chemicals Co. (St. Louis, MO), was used as the H_2S donor. When NaHS is dissolved in water at pH 7.4, HS- is released and forms H_2S with H^+. This provides a solution of H_2S at a concentration that is about 1/3 of the original concentration of NaHS [11]. Dex and CBS activator S-adenosyl-L-methionine (SAM) were obtained from Sigma Chemicals Co; Compound C was purchased from Calbiochem (Darmstadt, Germany). NaHS, SAM or indicated inhibitor was pre-added, after 30 min, Dex was added, and cells were incubated with medium containing both Dex and co-treatment for different time period. Anti-AMPKα1, acetyl-CoA carboxylase (ACC), Erk1/2, tubulin and rabbit and mouse horseradish peroxidase (HRP)-conjugated IgG antibodies were purchased from Santa Cruz Biotech (Santa Cruz, CA). Antibodies against phospho(p)-AMPKα (Thr 172) and p-ACC (Ser 79) were purchased form Cell Signaling Tech (Denver MA).

2.2. Human femoral head tissue isolation and detection

Necrotic and contralateral healthy femoral head tissues from three informed-consent osteonecrosis patients with long-term GC usage were surgical removed, and were immediately sliced and lysed by tissue lysis buffer (Sigma), followed by Western blot detecting expression of CBS, CSE and Erk1/2 (the loading). The clinical examinations were approved by the institutional review board of authors' institution, and were conducted according to the principles expressed in the Declaration of Helsinki.

2.3. MC3T3-E1 cell culture

The murine calvaria-derived osteoblastic MC3T3-E1 cell line, a gift from Dong's group [17], was seeded at 1×10^5 cells/mL into 75-cm^2 flasks and maintained in α-MEM supplemented with 10% FBS and 1% penicillin/streptomycin. This basic medium was replenished every 3 days. The cultures were then induced to differentiate by transferring cells into the medium supplemented with L-ascorbic acid (50 μg/mL) and β-glycerol phosphate (5 mM) (Sigma).

2.4. MC3T3-E1 cell viability assay

MC3T3-E1 cell viability was evaluated with the methyl thiazolyl tetrazolium (MTT) assay. In brief, after treatment, 20 μL of MTT (5 mg/mL, Sigma) was added to each well for 4 h. Then, culture medium was discarded carefully, and of DMSO (150 μL/well, Sigma) was added. Absorbance was recorded at 570 nm with the Universal Microplate Reader (Bio-Tek instruments, Shanghai, China) using wells without cells as blanks.

2.5. MC3T3-E1 cell apoptosis assay

MC3T3-E1 cell apoptosis was detected by the Annexin V Apoptosis Detection Kit (Biyuntian, Shanghai, China) according to the manufacturer's protocol. Briefly, cells with indicated treatment were stained with FITC-Annexin V and propidium iodide (PI) (Biyuntian, Shanghai, China). Both early (Annexin V^+/PI^-) and late (Annexin V/PI^+) apoptotic cells were sorted by fluorescence-activated cell sorting (FACS) (Beckman Coulter, Inc., Brea, CA). Cell apoptosis intensity was indicated by the Annexin V percentage.

2.6. Lactate dehydrogenase (LDH) release assay

The cell necrosis was determined by LDH release measured by a commercially available two-step enzymatic reaction LDH assay kit (Takara, Tokyo, Japan). The percentage of LDH release was calculated by the following formula: % LDH release = LDH released in conditioned medium/(LDH released in conditioned medium + LDH in cells lysed by Triton-X100 [1%]).

2.7. Western blots

Equal amounts of cell lysates (20 μg) and tissue homogenates (30 μg) were separated by SDS–PAGE and transferred to a nitrocellulose membrane. The membrane was probed with primary antibody overnight at 4 °C. After extensive washing, the membrane was incubated with secondary antibody. Proteins were visualized by using enhanced chemiluminescence reagent (Pierce-Thermo Scientific). The membrane was stripped in 0.5 M NaOH and re-probed with the indicated antibody to assess loading. The total gray of each band was quantified through ImageJ software, and the value was normalized to that of loading control.

2.8. Assay of reactive oxygen species (ROS)

Level of intracellular ROS was quantified to evaluate cellular oxidative stress [20]. Briefly, MC3T3-E1 cells were cultured in 12-well tissue culture plates overnight, and then co-treated with drugs and 2′,7′-dichlorofluorescin diacetate (H2-DCFDA, 1 μM, Molecular Probes, Shanghai, China), a reactive oxygen species-sensitive dye. The nonpolar, nonionic H2-DCFDA crosses cell membranes and is hydrolyzed into non-fluorescent H2-DCF by intracellular esterase. In the presence of ROS, H2-DCF is rapidly oxidized to become highly fluorescent DCF. MC3T3-E1 cells were harvested and suspended in PBS buffer. Relative fluorescent intensities were quantified using a flow cytometer (Becton Dickinson, San Jose, CA), using excitation and emission filters of 488 and 530 nm. DCF OD value, indicator of the ROS level, in treatment group was normalized to that of control group.

2.9. Measurement of intracellular ATP content

After treatment, the intracellular ATP content in MC3T3-E1 cells was measured using the Molecular Probes' ATP Determination Kit (Shanghai, China) according to the manufacturer's directions [21]. This kit is based on the bioluminescence detection of ATP, using recombinant firefly luciferase and its substrate, luciferin. Total chemiluminescence was collected by a luminometer. The amount of ATP from tested MC3T3-E1 cells was quantified by comparison to a calibration curve using ATP as the standard. ATP content was normalized to cell viability. The value in the treatment group was expressed as fold change of control group.

2.10. Stable AMPKα1 knockdown

MC3T3-E1 cells were seeded in a six-well plate with 50–60% confluence in culture medium without antibiotic. The lentiviral particles containing AMPKα1 shRNA (10 μL/mL, Santa Cruz Biotech) were added to the cells for 24 h, afterwards, cell medium was replaced by fresh culture medium for another 24 h. Cells were further cultured in puromycin (0.25 μg/mL)-containing culture

medium for 7–10 days, until resistant colonies were formed. The expression of AMPKα1 in stable cells was tested by Western blot in the resistant colonies. Same amount of scramble-shRNA lentiviral particles (10 μL/mL, Santa Cruz Biotech) was added into control cells.

2.11. Statistical analysis

The results were expressed as means ± SD. Comparisons across more than two groups involved use of one-way ANOVA and then Student–Newman–Keuls test. p values < 0.05 were considered statistically significant. The concentration and duration of drug-treatment were chosen based on literatures and results from pre-experiments.

3. Results

3.1. CBS and CSE are downregulated in human osteonecrosis tissues and in Dex-treated MC3T3-E1 cells

To investigate whether endogenous H_2S is involved in the pathogenesis of osteonecrosis, we examined the expression H_2S-producing enzymes, CBS and CSE, in osteonecrosis femoral head tissues from three patients with long-term Dex usage, and compared them with contralateral healthy femoral head tissues. As shown in Fig. 1A, the CBS and CSE expression was significantly down-regulated in necrotic *vs.* healthy femoral head tissues. CBS and CSE expression level in necrotic femoral l head tissues was about 30–40% of that of healthy femoral head tissues (Fig. 1B). Dex has been added into cultured osteoblasts or osteoblastic like cells to create a cell model of osteonecrosis [17,22]. We thus tested the effect of Dex on CBS and CSE expression, and results showed that Dex dose-dependently downregulated expression of both proteins in osteoblastic like MC3T3-E1 cells (Fig. 1C and D). Thus, CBS and CSE are down-regulated in human osteonecrosis tissues and in Dex-stimulated osteoblastic cells, indicating a potential role of H_2S in osteonecrosis.

3.2. H_2S donor NaHS protects MC3T3-E1 cells from Dex damages

Dex and other GCs were shown to induce both apoptotic and non-apoptotic death of osteoblasts [17,23,24]. Similarly, we found that Dex dose-dependently inhibited MC3T3-E1 cell survival (Fig. 2A, MTT assay). Meanwhile, Dex induced MC3T3-E1 cell apoptosis (Fig. 2B, Annexin V FACS assay) and necrosis (Fig. 2C, LDH release assay). Significantly, pre-treatment with NaHS, the H_2S donor [11,25], dramatically inhibited Dex-induced viability reduction, apoptosis and LDH release in MC3T3-E1 cells (Fig. 2A–C). Interestingly, the CBS activator S-adenosyl-L-methionine (SAM) [26,27], which could also promote H_2S production [26,27], similarly inhibited Dex-induced damages in MC3T3-E1 cells (Fig. 2A–C). These results together indicate that H_2S might exert pro-survival effect against Dex in MC3T3-E1 cells.

3.3. Activation of AMPK is important for NaHS-mediated cytoprotective effect against Dex in MC3T3-E1 cells

Recent studies have indicated that AMPK is an important pro-survival signaling in osteoblasts [16,17]. Above results showed that NaHS protected MC3T3-E1 cells from Dex, thus we tested the

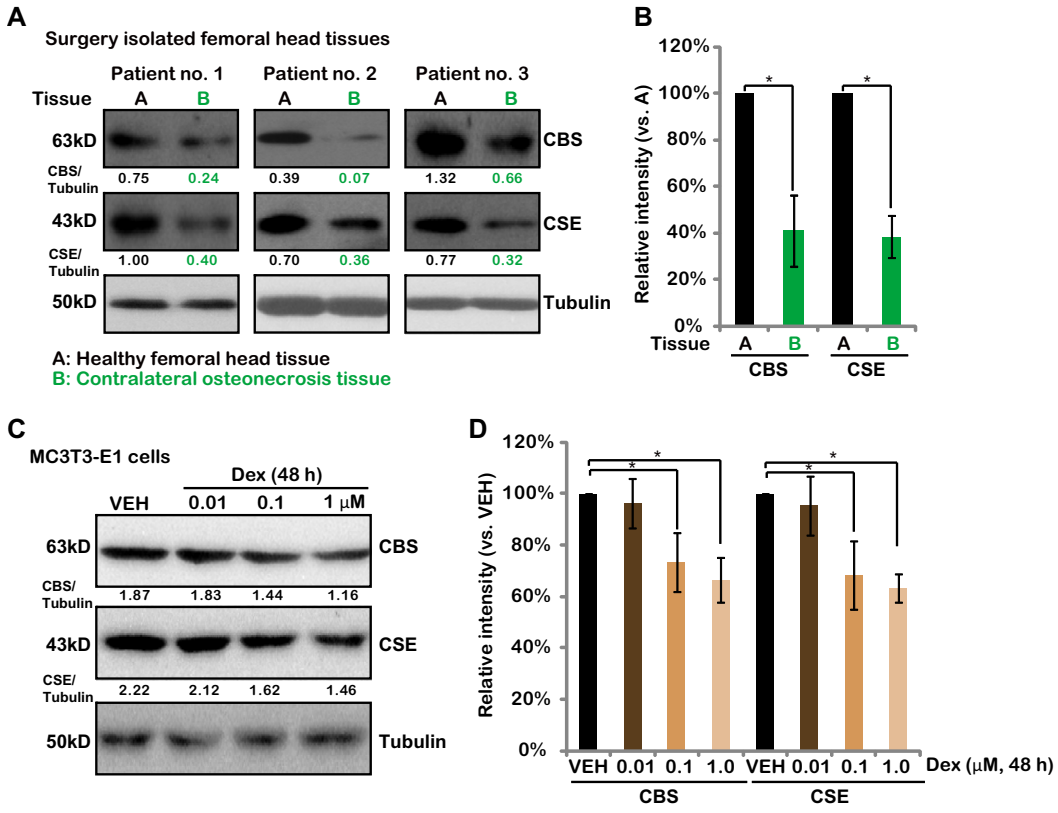

Fig. 1. CBS and CSE are downregulated in human osteonecrosis tissues and in Dex-treated MC3T3-E1 cells-cystathionine β-synthase (CBS), cystathionine γ-lyase (CSE) and tubulin (loading) expressions in necrotic and contralateral healthy femoral head tissues from three osteonecrosis patients were tested Western blots (A). CBS/CSE expression was quantified (B, $n = 3$). MC3T3-E1 cells were treated with indicated concentration of Dex, cells were further cultured for 48 h, CBS, CSE and tubulin expressions were tested (C), CBS/CSE expression was quantified (D, $n = 3$). "VEH" stands for PBS control.*$p < 0.05$.

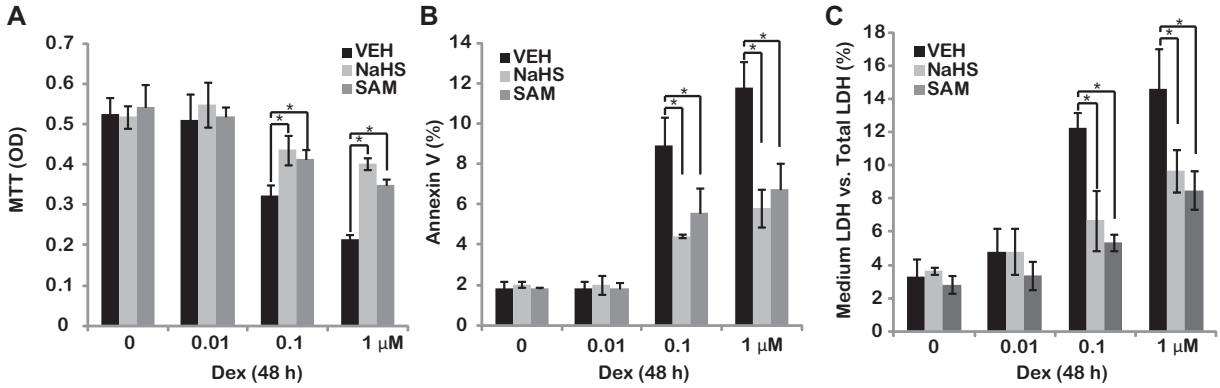

Fig. 2. H_2S donor NaHS protects MC3T3-E1 cells from Dex damages-MC3T3-E1 cells were pre-treated with NaHS (250 μM) or S-adenosyl-L-methionine (SAM, 250 μM) for 30 min, followed by indicated Dex stimulation, cells were further cultured for 48 h, cell viability, cell apoptosis and necrosis were analyzed by MTT assay (A), annexin V FACS assay (B) and LDH release assay (C), respectively. Experiments in this figure were repeated three times, and similar results were obtained. "VEH" stands for PBS control. $^*p < 0.05$.

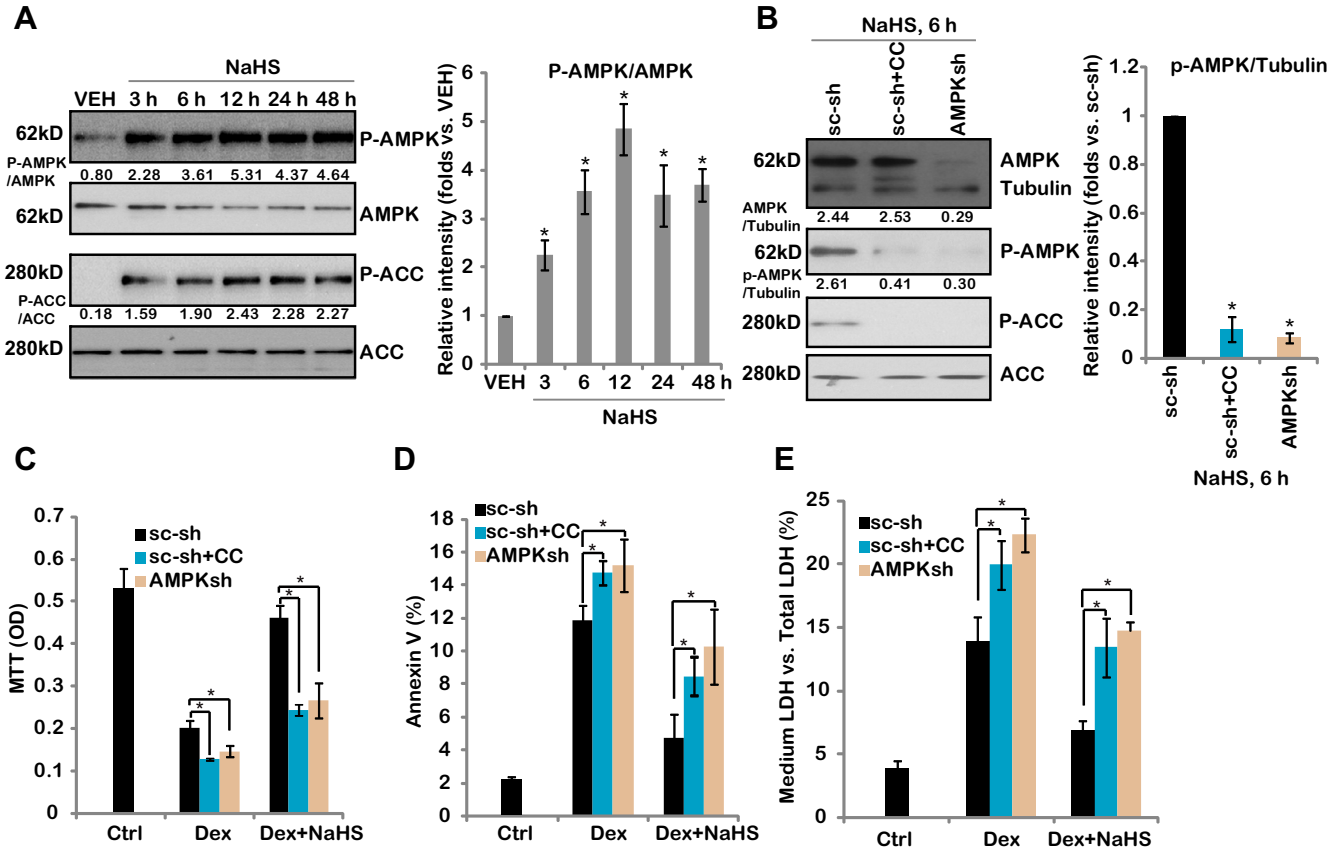

Fig. 3. Activation of AMPK is important for NaHS-mediated cyto-protective effect against Dex in MC3T3-E1 cells-MC3T3-E1 cells were treated with NaHS (250 μM) for indicated time, phosphorylated (p-) and regular AMPK and ACC were tested by Western blots (A, left panel), p-AMPK/AMPK was quantified (A, right panel, $n = 3$). Stable MC3T3-E1 cells with scramble shRNA (sc-sh) or AMPKα shRNA (AMPKsh) were treated with NaHS (250 μM) or plus compound C (CC, 5 μM) for 6 h, p- and regular AMPK/ACC and tubulin were tested (B, left panel), p-AMPK/tubulin was quantified (B, right panel, $n = 3$). Stable MC3T3-E1 cells with scramble shRNA (sc-sh) or AMPKα shRNA (AMPKsh) were pre-treated with NaHS (250 μM) or plus compound C (CC, 5 μM) for 30 min, followed by Dex (1 μM) stimulation, cells were further cultured for 48 h, cell viability, apoptosis and necrosis were analyzed by MTT assay (C), Annexin V FACS assay (D) and LDH release assay (E), respectively. Experiments in this figure were repeated three times, and similar results were obtained. "VEH" stands for PBS control.$^*p < 0.05$.

involvement of AMPK in this process. As shown in Fig. 3A, the H_2S donor NaHS in MC3T3-E1 cells induced significant AMPK activation, which was detected by increased phosphorylation (p-) of AMPKα1 (Thr 172) and its downstream target ACC (Ser 79). AMPK activation by NaHS was sustained for at least 48 h (Fig. 3A). The AMPK inhibitor compound C (CC) and shRNA stable knockdown AMPKα1 prevented NaHS-induced AMPK activation (Fig. 3B). Importantly, compound C or AMPKα1 shRNA dramatically inhibited NaHS-induced cyto-protection against Dex in MC3T3-E1 cells (Fig. 3C–E). Note that compound C or AMPKα1 shRNA alone had no effects on MC3T3-E1 cell death or apoptosis (Data not shown). These findings suggest that the cyto-protective activity of NaHS

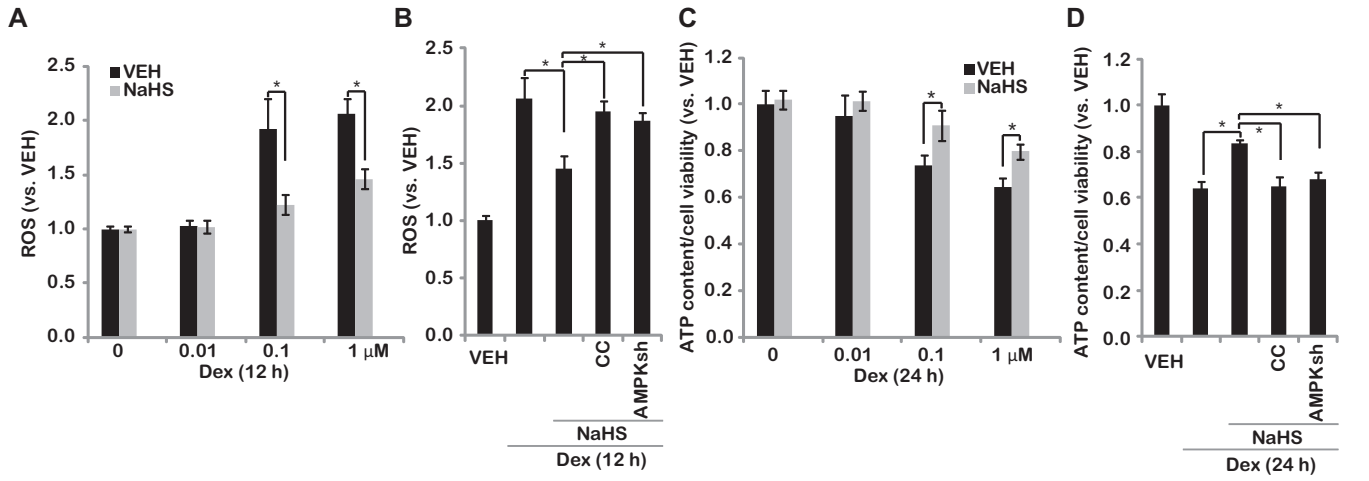

Fig. 4. NaHS inhibits Dex-induced ROS production and ATP depletion in MC3T3-E1 cells-MC3T3-E1 cells were pre-treated with NaHS (250 μM) for 30 min, followed by indicated concentration of Dex stimulation, cells were further cultured for 12 or 24 h, ROS production (A) and ATP content (C) were analyzed. Stable MC3T3-E1 cells with scramble shRNA (sc-sh) or AMPKα shRNA (AMPKsh) were pre-treated with NaHS (250 μM) or plus compound C (CC, 5 μM) for 30 min, followed by Dex (1 μM) stimulation, cells were further cultured for 12 or 24 h, ROS production (B) and ATP content (D) were analyzed. Experiments in this figure were repeated three times, and similar results were obtained. "VEH" stands for PBS control. $^{*}p < 0.05$.

in Dex-treated MC3T3-E1 cells might be mediated by activating AMPK signaling.

3.4. NaHS inhibits Dex-induced ROS production and ATP depletion in MC3T3-E1 cells

A recent study demonstrated that AMPK activation promoted cell survival through inhibiting oxidative stress and maintaining ATP homeostasis [14]. Meanwhile, H_2S displayed anti-oxidant activity in a number of studies [8,11]. Dex was shown to induce ROS production, which was required for cell apoptosis in many systems [28–30]. Here, we found that ROS level was also increased after Dex stimulation in MC3T3-E1 cells (Fig. 4A). NaHS significantly suppressed Dex-induced ROS production (Fig. 4A). Inhibition of AMPK by compound C or by targeted-shRNA dramatically inhibited the anti-oxidant activity of NaHS (Fig. 4B), suggesting an important role of AMPK activation in the process. Further, as shown in Fig. 4C, Dex decreased ATP content in MC3T3-E1 cells, which was also inhibited by NaHS. The effect of NaHS on ATP content was again alleviated by AMPK inhibitor or shRNA-depletion (Fig. 4D). Note that ROS and ATP content were detected 12/24 h after Dex stimulation, when no significant cell death or apoptosis was detected (Data not shown). Together, these results suggest that NaHS inhibits Dex-induced ROS production and ATP depletion, activation of AMPK signaling plays a vital role in the process.

4. Discussion

Many patients with long-term GCs therapy suffer osteoporosis [2]. The histological images of their bone showed reduced number of osteoblasts and increased levels of osteoblast apoptosis [2,3]. GCs administration (i.e. Dex) could directly induce osteoblast cell death and apoptosis [24,30–32]. Although it has been long considered that H_2S is a noxious gas, there is now accumulating evidence that it is an endogenously produced gaseous signaling messenger [9]. H_2S regulates heart contractility, and protects hearts against ischemia injury [9]. Further, H_2S protects astrocytes against H_2O_2-induced neural injury via enhancing glutamate uptake [25]. Meanwhile, it inhibits rotenone-induced apoptosis via preservation of mitochondrial functions in neurons [10]. In cultured osteoblasts, H_2S protects MC3T3-E1 cells against H_2O_2-induced oxidative damage [11]. Thus, H_2S is a novel signaling molecule and an important cyto-protectant [9].

In this study, we explored the potential role of H_2S in Dex-induced osteoblast damage. We found that both CBS and CSE, two key H_2S-producing enzymes, were significantly downregulated in human osteoporosis tissues as well as in Dex-stimulated MC3T3-E1 cells, indicating a potential role of H_2S in osteoporosis. H_2S donor NaHS activated AMPK signaling and inhibited Dex-induced MC3T3-E1 cell damage. While inhibiting AMPK prevented NaHS-mediated cyto-protective effects.

Recent studies have demonstrated how activation of AMPK promotes cell survival. AMPK attenuates oxidative stress-mediated cell damage through promoting NADPH synthesis and limiting its consumption [14]. At the same time, activated AMPK regulates ATP homeostasis [14]. Thus, the AMPK/ACC signaling axis works as an anti-oxidant to maintain ATP/NADPH homeostasis, and to rescue cells from oxidative stresses [14]. She et al. demonstrated that AMPK activation could inhibit H_2O_2-induced osteoblast apoptosis through activating autophagy [17]. Similarly, Zhu et al. showed that A-769662, the AMPK activator, protects osteoblasts from H_2O_2-induced apoptosis [16]. Importantly, a number of recent studies displayed that H_2S could activate AMPK signaling [18,19]. In this study, we found that H_2S-mediated cyto-protective activity in MC3T3-E1 cells was also dependent on AMPK activation. NaHS activated AMPK to inhibit Dex-induced ROS production and ATP depletion. Thus, AMPK signaling might be the key signaling molecular activated by H_2S to mediate its cyto-protective effect against Dex in MC3T3-E1 cell.

In summary, H_2S inhibits Dex-induced MC3T3-E1 cell damage through activating AMPK signaling. H_2S signaling might be further investigated as a novel target for anti-osteoporosis treatment.

Competing interests

The authors declare no conflict of interest.

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