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Original Article

β-aminoisobutyric acid accelerates the proliferation and differentiation of MC3T3-E1 cells via moderate activation of ROS signaling

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Abstract

Backgroud: Osteoporosis is one of the bone-metabolic diseases associated with decreased bone renewal and bone mineral density. β-aminoisobutyric acid (BAIBA), a natural thymine catabolite, can reduce inflammation in skeletal muscle and alleviate hepatic endoplasmic reticulum stress. However, the roles of BAIBA in osteoblast proliferation and differentiation remain largely unknown.

Methods: The cultured MC3T3-E1 cells received various treatments in this study, including BAIBA alone, H_2O_2 alone, BAIBA plus *N*-acetyl-L-cysteine and BAIBA plus apocynin. Cell proliferation was determined by CCK-8 assay and ^{3}H-Thymidine incorporation. Cell differentiation was evaluated by determining mRNA level of differentiation makers and ALP, and ALP activity. Reactive oxygen species (ROS) were determined by DHE staining while superoxide anion level and NAD(P)H oxidase activity were determined by the lucigenin-derived chemiluminescence method. The content of hydrogen peroxide (H_2O_2) was detected using a commercial kit. The level of NOX1, NOX2 and NOX4 was determined by Western-blot or qRT-PCR.

Results: We show that treatment of BAIBA stimulated the proliferation of MC3T3-E1 osteoprogenitor cells and enhanced the gene expression of osteoblast differentiation markers. Incubation of MC3T3-E1 cells with BAIBA evoked increases in NAD(P)H oxidase-derived reactive oxygen species (ROS). Scavenging of reactive oxygen species (*N*-acetyl-L-cysteine) or inhibition of NAD(P)H oxidase (apocynin) abolished the BAIBA-elicited proliferation and differentiation of MC3T3-E1 cells.

Conclusion: Our results provide the first evidence that BAIBA stimulates proliferation and differentiation of osteoprogenitor cells via activation of NAD(P)H oxidase/ROS signaling.

Keywords: β-aminoisobutyric acid; Cell proliferation and differentiation; Osteoprogenitors; ROS signaling

1. Introduction

The skeleton is renewed by 10% annually.[1] Osteoporosis is a bone metabolic disease characterized by low bone mass and decreased bone strength, and it is a common disorder among older, postmenopausal, or estrogen-deficient women.[2] Osteoporosis poses a great burden on the elderly.[3] It is well accepted that the imbalance in bone resorption and bone formation may be critically involved in the pathophysiology of osteoporosis.[4] The combination of increased bone resorption with decreased bone formation result in significant reductions in bone mass, contributing to net bone loss or osteoporosis.[5] Osteoblasts are crucial to bone formation.[6] Therefore, promoting the proliferation and differentiation of osteoblast precursor cells may be a promising target for developing osteoporosis treatment strategies.

It is generally accepted that excess reactive oxygen species (ROS) can induce multiple disorders, including bone loss, while moderate levels of ROS exhibit a physiological

Conflicts of interest: The authors declare that they have no conflicts of interest related to the subject matter or materials discussed in this article.

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intracellular signaling role, leading to cell proliferation and differentiation.[7–9] Recently, researchers have focused on the roles of NAD(P)H oxidase-derived ROS in regulating bone formation and resorption. However, results from different group are inconsistent. Loss of functional NOX2 reportedly protects against alcohol-induced bone resorption in female mice.[10,11] However, a host of evidences suggest that NAD(P)H oxidases may play a protective role in promoting bone formation. It has been demonstrated that NOX2 knockout mice exhibit spontaneous bone destruction and aging-dependent development of arthritis.[12] Bone morphogenetic protein 2 (BMP2)-induced osteoblast differentiation was shown to depend on NOX4-derived ROS.[13] Another study corroborated the view that ROS-dependent signaling plays a role in osteoblast differentiation.[14] However, the effects of NAD(P)H oxidases-derived ROS on bone formation remain controversial. We hypothesized that the absolute levels of ROS production determine their effects, with moderately elevated ROS having beneficial effects on bone formation.

β-aminoisobutyric acid (BAIBA) is a natural catabolite of the branched-chain amino acid thymine[15]. BAIBA was recently found to reduce inflammation in skeletal muscle via the AMP-activated protein kinase (AMPK)-peroxisome proliferator-activated receptor (PPAR)δ signaling pathway.[16] BAIBA reportedly enhances browning of white fat and hepatic β-oxidation via PPARα.[15] BAIBA alleviates hepatic endoplasmic reticular stress, and improves glucose/lipid metabolism in diabetes.[17] Further, BAIBA ameliorates fasting blood glucose levels, insulin tolerance, and glucose tolerance in diabetic mice.[17] We hypothesized that BAIBA may promote proliferation and differentiation of osteoblasts. Therefore, this study investigated whether and how BAIBA promote proliferation and differentiation of osteoblasts.

2. Methods

2.1. Cell culture and differentiation

MC3T3-E1 cells were purchased from the American Type Culture Collection (ATCC, Manassas, USA). The cells were maintained in α-MEM supplemented with 10% heat-inactivated fetal bovine serum and 1% penicillin in 5% CO_2–95% air at 37 °C in a humidified incubator. MC3T3-E1 cells were cultured in plates or dishes with medium containing 50 μg/ml L-ascorbic acid along with 5 mM β-glycerophosphate to induce differentiation.

2.2. Cell proliferation assay

The proliferation of MC3T3-E1 cells was assessed with the cell counting kit-8 (CCK-8) (Dojindo Molecular Technologies, Inc., Kumamoto, Japan) in accordance with the manufacturer's suggestions. Cells were seeded at an initial density of 2×10^4 cells/mL in 96-well plates. The absorbance at 450 nm was determined to measure cell viability. ^{3}H-Thymidine incorporation was employed to determine cell proliferation rate as previous report.[18]

2.3. Alkaline phosphatase (ALP) activity assay

ALP activity was measured as described previously.[19] In brief, MC3T3-E1 cells were seeded into 6-well plates in osteogenic medium for 7 days, and then incubated in culture medium containing various concentrations of BAIBA for the indicated times. After treatment, the harvested cells were assessed using a quantitative colorimetric assay (Varioskan1 Flash; Thermo Fisher Scientific, Rockford, IL, USA). The results were expressed relative to the no-treatment control.

2.4. RNA isolation and quantitative reverse-transcription (qRT-)PCR

Total RNA was isolated from frozen specimens by using TRIzol (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. The concentration and purity of RNA were determined by measuring the absorbance at 260 and 280 nm with the aid of NanoDrop 1000 spectrophotometer (Thermo Scientific, Wilmington, DE, USA). The RNA was reverse-transcribed using PrimeScript RT-PCR kit (Takara, Otsu, Shiga, Japan). qRT-PCR was conducted using SYBR Premix Ex Taq (Takara, Otsu, Shiga, Japan) on an ABI 7500 Real Time PCR system (Applied Biosystems, Foster City, CA, USA). Relative target gene expression was quantified by normalizing the target gene level to that of an internal control by the ΔΔCt method.[20,21] The primers used are listed in Table 1.

2.5. Measurement of ROS generation in vitro

Dihydroethidium (DHE) was applied to measure intracellular ROS in MC3T3-E1 cells as previously described.[22] The stimulated MC3T3-E1 cells were fixed and loaded with DHE (10 μM) for 30 min in a light-protected humidified chamber. The cells were photographed with the aid of a fluorescence microscope. The fluorescence intensity was calculated using IMAGE-PRO PLUS 6.0 using standard parameter settings. The intensity in regions of interest was measured as previously described.[23–25] In addition, the content of hydrogen peroxide (H_2O_2) was detected using a commercial kit (Beyotime, Shanghai, China) following the manufacturer's instructions, as previously described.[26–30]

2.6. Measurement of superoxide anions and NAD(P)H oxidase activity

Superoxide anion levels and NAD(P)H oxidase activity were measured by a lucigenin-derived chemiluminescence method using a luminometer (20/20n, Turner, BioSystems, Sunnyvale, USA), as previously reported.[31]

2.7. Chemicals

N-acetyl-L-cysteine (NAC) and dihydroethidium were obtained from Beyotime Biotechnology (Shanghai, China). Apocynin and dimethyl sulfoxide (DMSO) were obtained from

Table 1
The paired primers for PCR.

Genes	Forward (5′-3′)	Reverse (5′-3′)
RUNX2	5′–CCGTGGCCTTCAAGGTTGT-3′	5′-TTCATAACAGCGGAGGCATTT-3′
OSX	5′–CCCTTCTCAAGCACCAATGG-3′	5′-AAGGGTGGGTAGTCATTTGCATA-3′
ALP	5′-TGACCTTCTCTCCTCCATCC-3′	5′-CTTCCTGGGAGTCTCATCCT-3′
OC	5′-CTTGAAGACCGCCTACAAAC-3′	5′-GCTGCTGTGACATCCATAC-3′
OPG	5′-GTGGAATAGATGTCACCCTGTGT-3′	5′-TTTGGTCCCAGGCAAACTGT-3′
OPN	5′-TGCACCCAGATCCTATAGCC-3′	5′-CTCCATCGTCATCATCATCG-3′
Col I 1α	5′-GCTCCTCTTAGGGGCCACT-3′	5′–CCACGTCTCACCATTGGGG-3′
NOX1	5′-TCCTTCGCTTTTATCGCTCC-3′	5′-TCGCTTCCTCATCTGCAATTC-3′
NOX2	5′-TCCTATGTTCCTGTACCTTTGTG-3′	5′-GTCCCACCTCCATCTTGAATC-3′
NOX4	5′-GGAAGCCCATTTGAGGAGTCAC-3′	5′-CTGAGGTACAGCTGGATGTTCA-3′
GAPDH	5′-TGCACCACCAACTGCTTAG-3′	5′-GGATGCAGGGATGATGTTC-3′

RUNX2 = Runt-related transcription factor-2; Osx = Osterix; Col1α 1 = Collagen type 1 alpha-1; OC = Osteocalcin; OPN = Osteopontin; OPG = Osteoprotegerin.

Sigma Chemical (St. Louis, MO, USA). Cell culture supplies were purchased from Costar (Corning, Cypress, CA, USA).

2.8. Statistical analysis

Data are presented as the mean ± standard deviation (SD). SPSS 19.0 software (SPSS Inc., Chicago, IL, USA) was used for statistical analysis. The *t*-test was used to evaluate the relationship between two groups. One-way or two-way ANOVA followed by Bonferroni's post-hoc test was used when multiple comparisons were made. A two-sided *p*-value of <0.05 was considered statistically significant.

3. Results

3.1. Effect of BAIBA on the proliferation of MC3T3-E1 cells

BAIBA dose- and time-dependently promoted the proliferation of MC3T3-E1 cells, and the maximum effect was

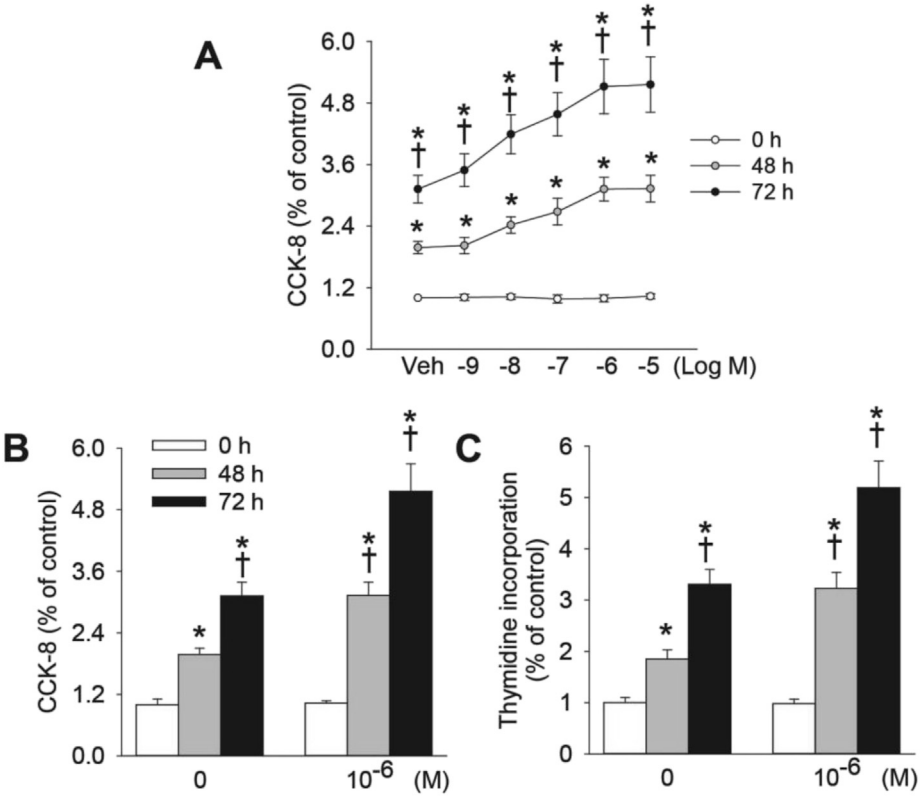

Fig. 1. Effects of β-aminoisobutyric acid (BAIBA) on the proliferation of MC3T3-E1 cells. (A) Cells were treated with various doses of BAIBA (10^{-9},10^{-8},10^{-7},10^{-6}, 10^{-5} M) for 0, 48, 72 h, and proliferation was determined with CCK-8. (B) Cells were treated with of BAIBA (0 or 10^{-6} M) for 0, 48, 72 h, and proliferation was determined by CCK-8 assay. (C) Incorporation of ^{3}H-thymidine into MC3T3-E1 cells at indicated time points. Values are mean ± SD. *$p < 0.05$ vs. 0 h or 0 M; †$p < 0.05$ vs. 48 h.

observed at the dose of 10^{-6} M for 48 h (Fig. 1A and B). ^{3}H-Thymidine incorporation confirmed that BAIBA promoted the proliferation of MC3T3-E1 cells (Fig. 1C).

3.2. Effect of BAIBA on the differentiation of MC3T3-E1 cells

qPCR results demonstrated that BAIBA clearly upregulated the mRNA expression of osteoblast transcription regulators, including Runt-related transcription factor-2 (RUNX2) and osterix (Osx), as well as the differentiation markers collagen type 1 alpha-1 (Col1α-1), osteocalcin (OC), osteopontin (OPN), and osteoprotegerin (OPG), in a dose-dependent manner (Fig. 2). In addition, both the activity (Fig. 3A) and mRNA level (Fig. 3B) of ALP were dose-dependently increased in MC3T3-E1 cells upon BAIBA stimulation. These data indicated that BAIBA dramatically accelerated osteoblast differentiation.

3.3. NAD(P)H-derived ROS mediates the effects of BAIBA

Treatment of MC3T3-E1 cells with BAIBA obviously led to a significant increase in ROS production as evidenced by DHE fluorescence images (Fig. 4A). Incubation of MC3T3-E1 cells with BAIBA resulted in increased superoxide anion levels and NAD(P)H oxidase activity (Fig. 4C and D). Moreover, the levels of H_2O_2 were enhanced in BAIBA-challenged MC3T3-E1 cells (Fig. 4E). NAD(P)H oxidases are major sources of superoxide anions, and NAD(P)H oxidase-derived ROS are critically involved in the pathogenesis of osteoporosis.[23] In this study, we found that NOX4 protein was dramatically up-regulated in MC3T3-E1 cells in response to BAIBA, without affecting both NOX1 and NOX2 protein expression (Fig. 4E and F), which provided direct evidence that NOX4 induced by BAIBA might contribute to the reversion of osteopenia and osteoporosis.

Based on previous reports, we hypothesized that moderate ROS levels may promote bone information, while excess ROS would cause bone loss.[13] Treatment with H_2O_2 (100 μM) dramatically increased ROS levels by 7-fold, while BAIBA at low dose caused a slight increase in ROS generation in MC3T3-E1 cells. Intriguingly, BAIBA at high dose (10 μM) substantially stimulated ROS production in MC3T3-E1 cells, comparable to H_2O_2 treatment (Fig. 5A). Additionally, the mRNA levels of NOX4 were significantly enhanced by high dose of BAIBA and H_2O_2 treatment, to levels far above those induced by low dose of BAIBA. However, the mRNA levels of NOX1 and NOX-2 were not affected in MC3T3-E1 cells (Fig. 5B–D). It is well known that H_2O_2 (100 μM) can disrupt bone information by excessive oxidative damage.[23] These results suggested that BAIBA has a beneficial influence on osteoprogenitor cells at appropriate concentration.

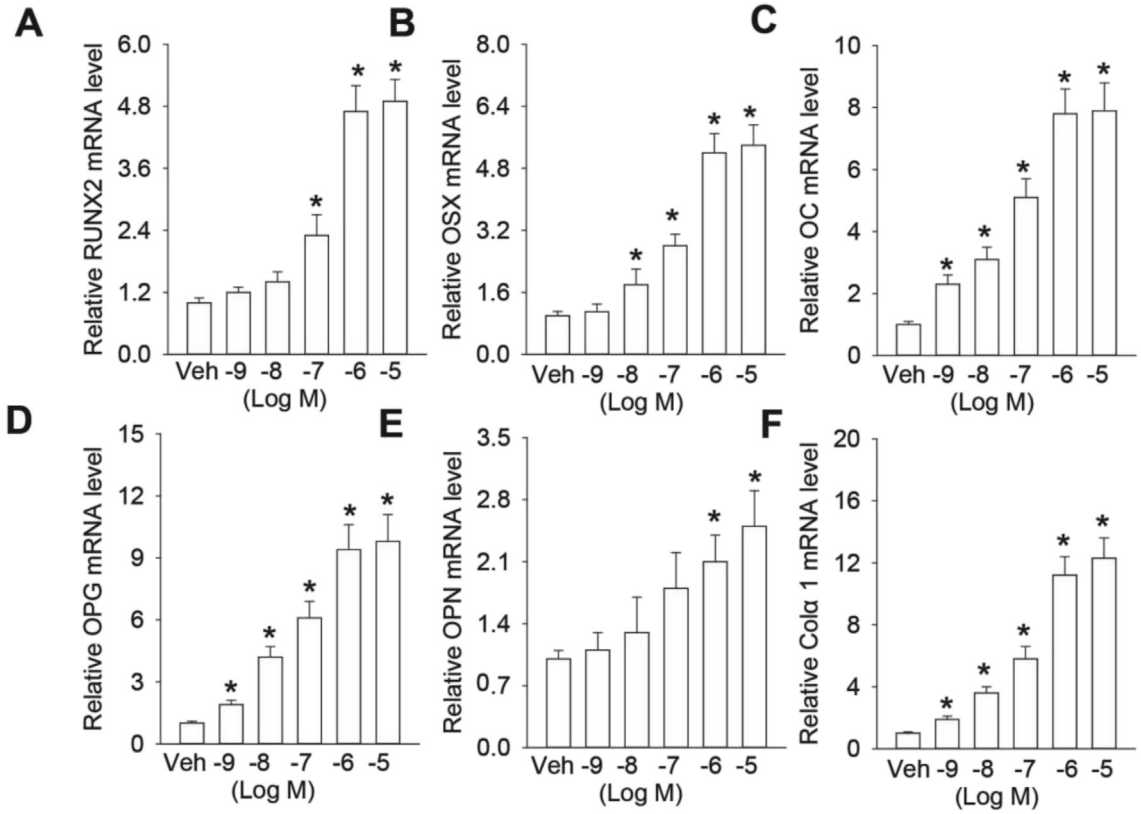

Fig. 2. Effect of β-aminoisobutyric acid (BAIBA) on the mRNA expression of the differentiation markers (RUNX2, OSX, OC, OPG, OPN, and Colα 1) in MC3T3-E1 cells. Cells were treated with various doses of BAIBA (0, 10^{-9}, 10^{-8}, 10^{-7}, 10^{-6}, 10^{-5} M) for 3 days and assayed by qRT-PCR. Values are mean ± SD. *$p < 0.05$ vs. 0 M. n = 6 for each group.

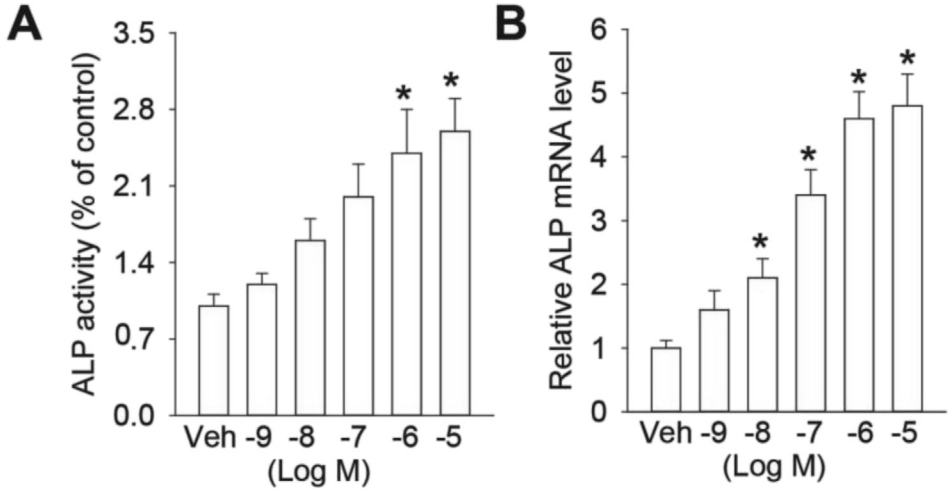

Fig. 3. Effect of β-aminoisobutyric acid (BAIBA) on the activity and mRNA levels alkaline phosphatase (ALP) in MC3T3-E1 cells. (A) Cells were treated with various doses of BAIBA (0, 10^{-9}, 10^{-8}, 10^{-7}, 10^{-6}, 10^{-5} M) for 3 days and ALP activity was determined with a BCIP/NBT alkaline phosphatase color development kit. (B) mRNA levels of ALP were determined by qRT-PCR. Values are mean ± SD. *$p < 0.05$ vs. 0 M. n = 6 for each group.

3.4. ROS signaling is involved in the effects of BAIBA

The BAIBA-induced proliferation and differentiation of MC3T3-E1 cells as reflected by increases in optical density, mRNA expression of RUNX2, Osx, Col1α-1, OC, OPN, OPG, and ALP, and ALP activity were effectively prevented by the ROS scavenger NAC and the NADPH oxidase inhibitor apocynin (Apo) (Fig. 6).

4. Discussion

Osteoporosis is a common comorbidity for sarcopenia associated with lowered density and strength of skeleton.[32] BAIBA was initially developed as an anti-obesity agent that acts via increasing fatty acid oxidation (FAO) in the liver and decreasing body fat mass in mice.[33] In the present study, treatment of MC3T3-E1 cells with BAIBA obviously

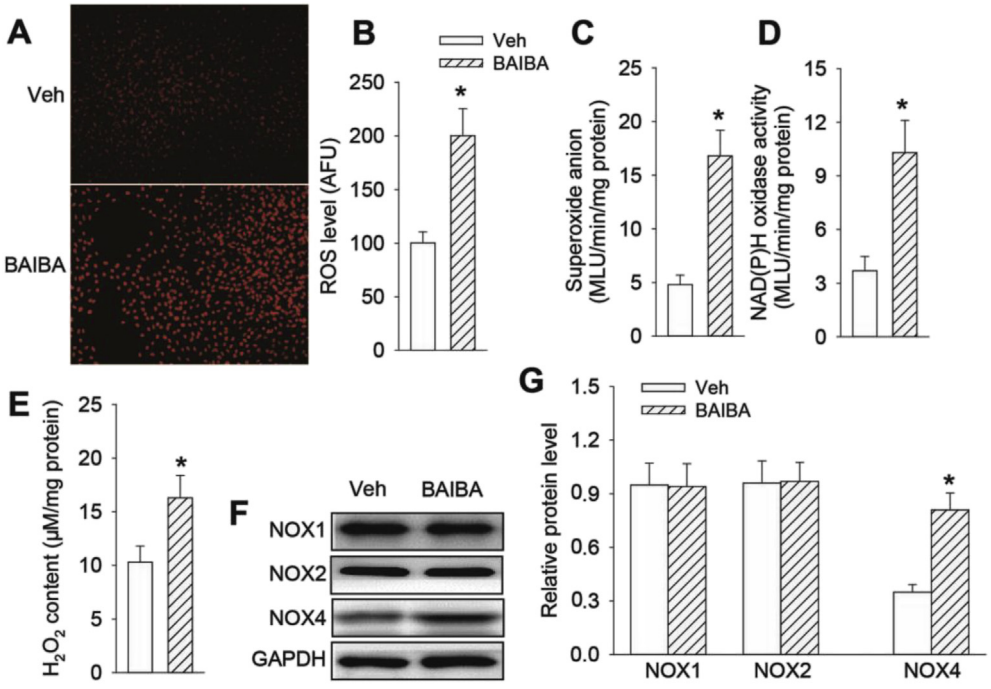

Fig. 4. Effect of β-aminoisobutyric acid (BAIBA) on reactive oxygen species (ROS) production in MC3T3-E1 cells. (A,B) Cells were treated with BAIBA (10^{-6} M) for 3 days, and the level of ROS was detected by DHE staining, while superoxide anion level (C) and NAD(P)H oxidase activity (D) were determined by the lucigenin-derived chemiluminescence method. (E) H_2O_2 content. (F) Representative western blots showing the protein levels of NOX1, NOX2, and NOX4. (G) Quantification of the protein levels of NOX1, NOX2, and NOX4. Values are mean ± SD. *$p < 0.05$ vs. Veh. †$p < 0.05$ vs. BAIBA. n = 6 for each group.

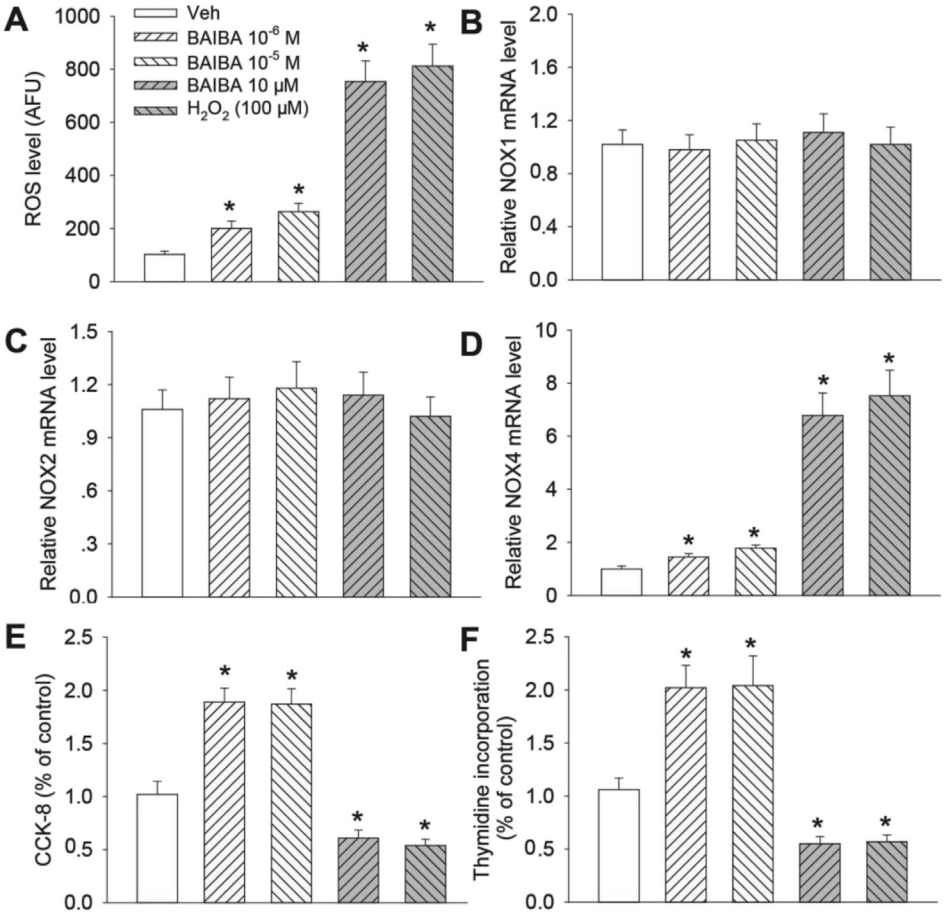

Fig. 5. Effects of β-aminoisobutyric acid (BAIBA) and H_2O_2 on reactive oxygen species (ROS) production in MC3T3-E1 cells. Cells were treated with various doses of BAIBA (0, 10^{-6}, 10^{-5} M, 10 μM) or H_2O_2 (100 μM) for 3 days. (A) ROS level. (B) Relative NOX1 mRNA level. (C) Relative NOX2 mRNA level. (D) Relative NOX4 mRNA level. (E) Cell proliferation as determined with CCK-8. (F) Cell proliferation as determined by ^{3}H-thymidine incorporation. Values are mean ± SD. *$p < 0.05$ vs. Veh. n = 6 for each group.

accelerated the proliferation and differentiation of osteoblasts, and the underlying mechanisms were related to the activation of NADPH oxidase/ROS signaling.

The homeostasis between bone formation and bone resorption is requisite for maintaining bone growth.[34] Prevention of bone loss or osteoclast activity inhibition may be recommended as therapeutics for osteoporosis. However, as osteoporosis patients generally have already lost bone mass before treatment, promotion of bone formation is a better approach to treat osteoporosis.[35] Osteoblasts are fundamental components in the formation of new bone tissues.[36] Promoting the proliferation and differentiation of osteoblasts is a pivotal event in bone formation.[36] In the present study, we showed that BAIBA dose- and time-dependently promoted the proliferation of MC3T3-E1 cells. qPCR results demonstrated that BAIBA obviously upregulated the mRNA expression of the osteoblast transcription regulators RUNX2 and Osx, as well as the differentiation markers Col1α-1, OC, OPN, and OPG. In addition, we showed that both the activity and mRNA expression of ALP were dose-relatedly increased in MC3T3-E1 cells exposed to BAIBA stimulation. These data indicate that BAIBA dramatically accelerates osteoblasts proliferation and differentiation.

ROS are suggested to be crucial in pre-osteoblast proliferation induced by low-level laser therapy[37] and are thought to be key secondary messengers in osteoblast proliferation and differentiation.[14,38] In this study, we showed that treatment of MC3T3-E1 cells with BAIBA led to a significant increase in ROS production as evidenced by DHE fluorescence and increased superoxide anion level, NAD(P)H oxidase activity, and H_2O_2 content. In addition, the ROS scavenger NAC as well as the NADPH oxidase inhibitor apocynin significantly abolished the proliferation and differentiation of osteoblasts challenged by BAIBA. These results suggested that the ROS signaling pathway is involved in BAIBA-evoked osteoblast proliferation and differentiation. It is worth mentioning that subtype NAD(P)H oxidases such as NOX1, NOX2, and NOX4 are abundantly expressed in osteoblasts.[23] We showed that the protein levels of NOX4 are dramatically upregulated in MC3T3-E1 cells in response to BAIBA, which provides direct evidence that NOX4 may largely contribute to the BAIBA-induced ROS production in MC3T3-E1 cells.

In conclusion, the results indicate that BAIBA promotes the proliferation and differentiation of osteoblasts via activation of the ROS signaling pathway, which may help to clarify the mechanism of BAIBA-induced bone formation. It is

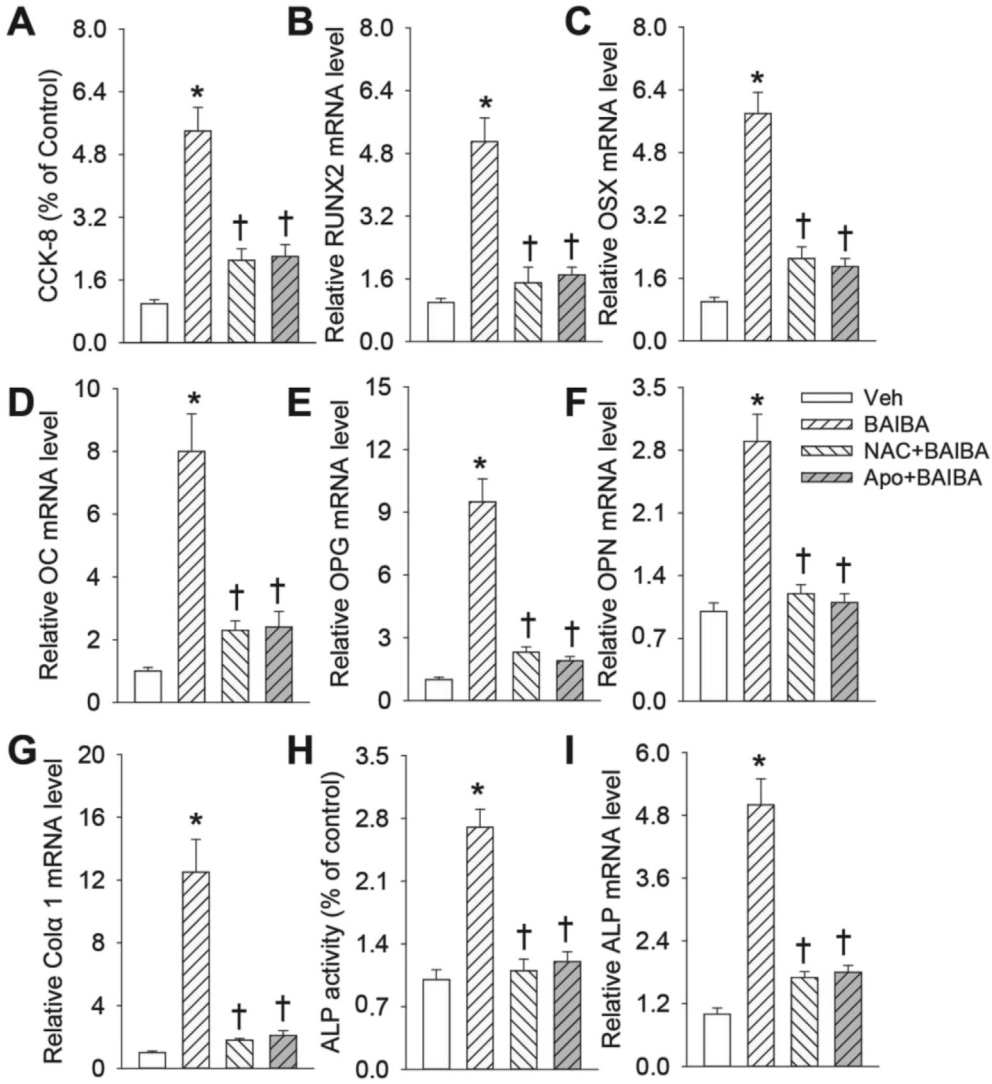

Fig. 6. Role of ROS signaling in BAIBA-induced MC3T3-E1 cell proliferation and differentiation. Cells were pre-incubated with NAC (1 mM) or apocynin (Apo, 100 μM) for 6 h and then treated with BAIBA (10^{-6} M) for 3 days. The proliferation of MC3T3-E1 cells was determined by CCK-8 assay (A). The RUNX2 (B), OSX (C), OC (D), OPG (E), OPN (F), Colα 1(G), ALP activity (H), and ALP levels (I) were quantified. Values are mean ± SD. *$p < 0.05$ vs. Veh. †$p < 0.05$ vs. BAIBA. n = 6 for each group.

particularly worth noting that excess ROS cause bone loss. We showed that high dose of BAIBA has the same effect on excessive ROS production as H_2O_2 in osteoblasts cells, while low dose of BAIBA in association with moderate ROS production plays a protective role in osteoblast proliferation and differentiation. We will evaluate the potential of BAIBA as a bone-forming drug in a future study. The suitable dose of BAIBA in animal studies remains unclear and requires further research.

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