Uncarboxylated osteocalcin inhibits high glucose-induced ROS production and stimulates osteoblastic differentiation by preventing the activation of PI3K/Akt in MC3T3-E1 cells

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Abstract. Uncarboxylated osteocalcin, an osteoblast-derived protein, plays an important role in the regulation of glucose metabolism. It has previously been demonstrated that high glucose levels inhibit osteoblast proliferation and differentiation. However, the mechanisms through which uncarboxylated osteocalcin regulates osteoblast proliferation and differentiation under high glucose conditions remain unclear. Thus, in the present study, we aimed to examine the effects of uncarboxylated osteocalcin on the proliferation and differentiation of MC3T3-E1 cells under high glucose conditions. We demonstrated that high glucose levels induced the production of reactive oxygen species (ROS) in MC3T3-E1 cells, and this production was inhibited by treatment with uncarboxylated osteocalcin and N-acetyl-L-cysteine (NAC), a ROS scavenger. In addition, we found that uncarboxylated osteocalcin reduced high glucose-induced oxidative stress and increased the mRNA expression of the osteogenic markers, runt-related transcription factor 2 (Runx2), osterix and osteocalcin, as well as the formation of mineralized nodules; it also inhibited adipogenic differentiation, as shown by a decrease in the mRNA expression of the adipogenic markers, peroxisome proliferator-activated receptor γ (PPARγ), adipocyte fatty acid-binding protein (adipocyte protein 2; aP2) and fatty acid synthase (FAS), and reduced lipid drop accumulation. Furthermore, we found that uncarboxylated osteocalcin inhibited PI3K/Akt signaling which was induced by ROS and facilitated the osteogenic differentiation of MC3T3-E1 cells under high glucose conditions. Taken together and to the best of ou knowledge, our results demonstrate for the first time that uncarboxylated osteocalcin inhibits high glucose-induced ROS production and stimulates osteoblastic differentiation by inhibiting the activation of PI3K/Akt in MC3T3-E1 cells. Therefore, we suggest that uncarboxylated osteocalcin may be a potential therapeutic agent for diabetes-related osteoporosis.

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Introduction

Osteocalcin is a non-collagenous vitamin K-dependent protein, which is secreted particularly by osteoblasts, and its forms include carboxylated osteocalcin and uncarboxylated osteocalcin (1). The role played by osteocalcin in the skeleton has not yet been fully elucidated. Hoang *et al* indicated that carboxylated osteocalcin bound with high affinity to the mineral component of bone, hydroxyapatite, and regulated mineralization (2). Certain studies have also suggested that carboxylated osteocalcin has no effect on mineralization (3-5). Others have provided evidence that carboxylated osteocalcin may play a role in bone turnover (6,7). Moreover, serum concentrations of osteocalcin have been established as the main marker of bone turnover (8). However, it remains unknown as to whether uncarboxylated osteocalcin influences osteoblast proliferation and differentiation.

Previous research has demonstrated that uncarboxylated osteocalcin is involved in energy metabolism. Fukumoto and Martin revealed that bone, as an endocrine organ, used the osteoblast-specific secreted molecule, osteocalcin, to promote glucose homeostasis (9). Subsequently, Ferron *et al* provided evidence that daily injections of uncarboxylated osteocalcin improved glucose handling and prevented the development of type 2 diabetes in mice (10). *In vitro* studies have also demonstrated that uncarboxylated osteocalcin regulates glucose homeostasis by increasing insulin secretion and sensitivity (11,12). In another study, Pi *et al* reported that uncarboxylated osteocalcin mediated insulin secretion by β-cells through G protein-coupled receptor, class C, group 6, member A (GPRC6A) and regulated glucose metabolism (12). GPRC6A, a G protein-coupled receptor, is an osteocalcin receptor which exists in osteoblasts (13,14). In addition, GPRC6A is widely expressed in brain and peripheral tissues with highest levels being noted in the kidneys, skeletal muscle, testes and leucocytes (14).

Hyperglycemia, as a characteristic of diabetes mellitus, has been suggested to be a potential contributor to diabetes-related osteoporosis (15,16). Epidemiological studies have theorized that patients with diabetes have an increased risk of developing osteoporosis or osteoporotic fractures (17,18).

Evidence from rodent models and clinical research has indicated that dysfunctions in glucose metabolism are associated with a higher risk of bone loss and fractures (19). In addition, hyperglycemia impairs bone formation and function in both types 1 (20) and 2 diabetes (21). The results of our previous studies demonstrated that high glucose levels inhibited osteogenic differentiation and promoted adipogenic differentiation in primary osteoblasts (22) and MG-63 cells (23). In clinical studies, it was noted that the serum osteocalcin concentrations were reduced in patients with diabetes (24,25). However, to the best of our knowledge, whether uncarboxylated osteocalcin promotes the proliferation and differentiation of osteoblasts under high glucose conditions has not been investigated to date.

In the present study, we examined the effects of uncarboxylated osteocalcin on the proliferation and differentiation of osteoblasts under high glucose conditions. Our results revealed that uncarboxylated osteocalcin prevented the activation of PI3K/Akt signaling, inhibited high glucose induced-reactive oxygen species (ROS) production and stimulated the osteoblastic differentiation of MC3T3-E1 cells. However, we found that uncarboxylated osteocalcin had no effect on osteoblastic differentiation under normal conditions.

Materials and methods

Preparation of recombinant osteocalcin. The bacterial expression and purification of recombinant mouse uncarboxylated osteocalcin were performed as previously described (26). Briefly, His-tagged osteocalcin (GE Healthcare, Uppsala, Sweden) fusion protein was bacterially produced and purified on His-sepharose (GE Healthcare) according to standard procedures. The purity (>95%) of the osteocalcin preparation was assessed by 15% SDS-PAGE with Coomassie blue staining. The concentration of the recombinant osteocalcin protein was precisely determined using mouse osteocalcin enzyme-linked immunosorbent assay (ELISA) according to manufacturer's instructions (Immunotopics, San Clemente, CA, USA).

Cell culture. MC3T3-E1 cells were purchased from The Cell Bank of Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China). The MC3T3-E1 cells were cultured in α-MEM (HyClone, Logan, UT, USA) supplemented with 10% fetal bovine serum (FBS; Gibco, Grand Island, NY, USA) and 1% penicillin-streptomycin in 5% CO_2 at 37˚C.

Cell proliferation assay. In the present study, a Cell Counting Kit-8 (CCK-8) was used to examine the effects of uncarboxylated osteocalcin on the proliferation of osteoblasts under high glucose conditions. Briefly, the cells were plated in 96-well plates (3,000 cells/well) in medium containing 10% serum for 24 h and then replaced with 4% serum medium containing normal (5.5 mM) or high levels of glucose (25.5 mM) and treated with various concentrations of uncarboxylated osteocalcin (0.3, 3, 10 and 30 ng/ml) for 1, 2 or 3 days. The absorbance values were read at a 450 nm wavelength using an automated microplate reader (Bio-Rad Laboratories, Inc., Hercules, CA, USA).

Measurement of ROS production. The production of ROS was quantified using the peroxide-dependent oxidation of 2',7'-dichlorofluorescein diacetate (DCFH-DA) (Beyotime Institute of Biotechnology, China). Intracellular esterases hydrolyze DCFH-DA and form non-fluorescent DCFH, which is further oxidized by ROS to form the fluorescent compound, DCF. The MC3T3-E1 cells were plated in 24-well plates with 10% FBS. After the cells had attached, the medium was replaced with new medium containing 4% FBS and normal (5.5 mM) or high (25.5 mM) amounts of glucose, as well as with or without the antioxidant, N-acetyl-L-cysteine (NAC), and uncarboxylated osteocalcin (3 ng/ml) for 48 h. Subsequently, DCFH-DA (final concentration of 10 mM) was added to the cells followed by incubation at 37˚C for 30 min. Finally, the cells were digested for the detection of ROS levels using a BioTek Synergy HT microplate reader with the excitation set at 485 nm and the emission at 528 nm (BioTek Instruments Inc., Winooski, VT, USA.

Assay of mineralization. The MC3T3-E1 cells were seeded at a density of $1x10^6$ cells/well in 6-well plates with α-MEM containing 10% FBS and grown until they reached confluence. The medium was then replaced with mineralizion medium (α-MEM, 4% FBS, 50 μg/ml ascorbic acid, 10 mM β-glycerophosphate phosphate and $1x10^{-8}$ M dexamethasone; Sigma, St. Louis, MO, USA) containing normal (5.5 mM) or high (25.5 mM) amounts of glucose and either with or without the antioxidant, NAC, and uncarboxylated osteocalcin (3 ng/ml). The mineralizing medium was replaced every day. Calcified nodule formation was detected by Alizarin red S staining on day 21. Briefly, the cells were washed twice with PBS and were then fixed with 10% formaldehyde for 30 min at room temperature. The cells were then stained with 2% Alizarin Red S (pH 4.2; Sigma) for 20 min at room temperature and thoroughly rinsed with water. For quantification, the bound stain was eluted with 10% wt/vol) cetylpyridinium chloride, and the absorbance of supernatants was measured at 550 nm using a microplate reader (Bio-Rad Laboratories, Inc.).

Lipid droplet assay. Oil Red O staining (Sigma) was used to analyze the formation of lipid droplets. The MC3T3-E1 cells were plated at a density of $1x10^6$ cells in 6-well plates with α-MEM containing 10% FBS and then treated with adipogenic differentiation medium (α-MEM, 4% FBS, 10 μg/ml insulin and $1x10^{-7}$ M dexamethasone) containing normal (5.5 mM) or high (25.5 mM) levels of glucose, and either with or without the antioxidant, NAC, and uncarboxylated osteocalcin (3 ng/ml). Fourteen days later, the formation of lipid droplets was determined by staining with freshly diluted Oil Red O solution (0.1% Oil Red O:water, 3:2) for 10 min. Images were observed under an inverted phase contrast microscope (IMT-2-21; Olympus, Tokyo, Japan). The optical density at 510 nm was measured to quantify lipid accumulation in osteoblasts.

Alkaline phosphatase (ALP) assay. The MC3T3-E1 cells were cultured in α-MEM with 10% FBS and the medium was then changed to new medium with 4% FBS, which contained normal (5.5 mM) or high (25.5 mM) levels of glucose and inhibitors [LY294002 (Sigma); PI3K inhibitor], as well as with or without the antioxidant, NAC, and uncarboxylated osteocalcin (3 ng/ml). ALP activity in the culture supernatants was measured using an ALP assay kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) according to the manufacturer's instructions.

Table I. Primer sequences used for RT-qPCR.

Gene	Forward primers (5'→3')	Reverse primers (5'→3')
Runx2	GCCTTCAAGGTGGTAGCCC	CGTTACCCGCCATGACAGTA
Osx	ACTGGCTAGGTGGTGGTCAG	GGTAGGGAGCTGGGTTAAGG
OC	GCAATAAGGTAGTGAACAGACTCC	AGCAGGGTTAAGCTCACACTG
PPARγ	GCATGGTGCCTTCGCTGA	TGGCATCTCTGTGTCAACCATG
aP2	ACACCGAGATTTCCTTCAAACTG	CCATCTAGGGTTATGATGCTCTTC
FAS	GGCTGCAGTGAATGAATTTG	TTCGTACCTCCTTGGCAAAC
β-actin	GCTCTTTTCCAGCCTTCCTT	AGGTCTTTACGGATGTCAACG

Runx2, runt-related transcription factor 2; PPARγ, peroxisome proliferator-activated receptor γ; aP2, adipocyte protein 2; FAS, fatty acid synthase.

Enzyme-linked immunosorbent assay for type I collagen secretion. The cultured cells were treated as above, and collagen I (Col I) secretion was quantified using commercial ELISA kits (Boster Biological Technology, Wuhan, China) according to the manufacturer's instructions.

Reverse transcription-quantitative PCR (RT-qPCR). To detect the expression of osteogenic [runt-related transcription factor 2 (Runx2), osterix and osteocalcin] and adipogenic markers [peroxisome proliferator-activated receptor γ (PPARγ), adipocyte fatty acid-binding protein (adipocyte Protein 2; aP2) and fatty acid synthase (FAS)], total RNA was extracted using an RNeasy mini spin column (Qiagen, Inc., Valencia, CA, USA), and 2 μg RNA was reverse transcribed into cDNA using the SuperScript First-Stand Synthesis system for RT-PCR (Invitrogen, Carlsbad, CA, USA). Quantitative PCR (qPCR) was performed using 2 μl of cDNA in a 10 μl reaction volume with an ABI Gene Amp 5700 Sequence Detection System and QuantiTect SYBR-Green PCR Master mix (Qiagen, Inc.). The forward and reverse primer sequences are listed in Table I. The relative mRNA levels were normalized using β-actin as a housekeeping gene. The cycling conditions included incubation at 95˚C for 5 min, 40 cycles of denaturation at 94˚C for 30 sec, annealing at 56˚C for 45 sec and a final extension at 72˚C for 30 sec. We compared each gene sample level using ABI Gene Amp 5700 SDS software.

Western blot analysis. The cells were washed twice with ice-cold PBS and scraped on ice into RIPA lysis buffer. The lysates were cleared by centrifugation (12,000 rpm) at 4˚C for 15 min. An equivalent amount of protein was electrophoresed on a 10% SDS-PAGE gel and transferred onto PVDF membranes (Millipore Corp., Bedford, MA, USA). The membranes were blocked in TBS buffer containing 5% skim milk for 1 h at room temperature, and the membranes were then incubated overnight at 4˚C with a primary monoclonal antibody against p-Akt (Ser473) or Akt (1:1,000 dilution) (Cell Signaling Technology, Beverly, MA, USA), followed by a horseradish peroxidase (HRP) conjugated secondary antibody (1:5,000 dilution) (Cell Signaling Technology) for 1 h at room temperature after being washed 3 times with TBST for 10 min each. Subsequently, the membranes were washed 3 times with TBST, and the immunoreactive bands were visualized using an enhanced chemiluminescence (ECL) kit (Biomiga, Inc., San Diego, CA, USA). The intensity of the protein bands was quantified by densitometric scanning using ImageJ software and normalized to β-actin.

Statistical analysis. All experiments were performed at least 3 times. Data are presented as the means ± SD. The results were analyzed by one way analysis of variance (ANOVA) followed by Bonferroni's multiple comparison tests using statistical software SPSS 13.0. A p-value <0.05 was considered to indicate a statistically significant difference.

Results

High glucose inhibits osteoblast proliferation but this effect is reversed by treatment with uncarboxylated osteocalcin in MC3T3-E1 cells. In order to determine whether uncarboxylated osteocalcin influences the proliferation of MC3T3-E1 cells under high glucose conditions, various concentrations of uncarboxylated osteocalcin (0.3, 3, 10 and 30 ng/ml) were added to the culture medium containing high levels of glucose (25 mM). CCK-8 assay was used to examine cell viability. As shown in Fig. 1A, high glucose levels inhibited the growth of osteoblasts, whereas treatment with various concentrations of uncarboxylated osteocalcin reversed this effect. Our results indicated that uncarboxylated osteocalcin attenuated the high glucose-induced inhibition of osteoblast proliferation. In addition, we selected the concentration of 3 ng/ml uncarboxylated osteocalcin as the treatment dose for subsequent experiments.

Uncarboxylated osteocalcin inhibits high glucose-induced ROS production and stimulates the osteoblast differentiation, but inhibits the adipogenic differentiation of MC3T3-E1 cells

Uncarboxylated osteocalcin inhibits high glucose-induced ROS production. It has previously been noted that high glucose-induced ROS production inhibits the osteogenic differentiation and promotes the adipogenic differentiation of primary osteoblasts (22). In this study, in order to determine whether uncarboxylated osteocalcin reverses the effects of ROS (induced by high glucose) on osteoblast differentiation, we measured intracellular ROS levels by DCFH-DA staining. Our results revealed that the intracellular ROS level was significantly reduced by treatment with uncarboxylated osteocalcin (3 ng/

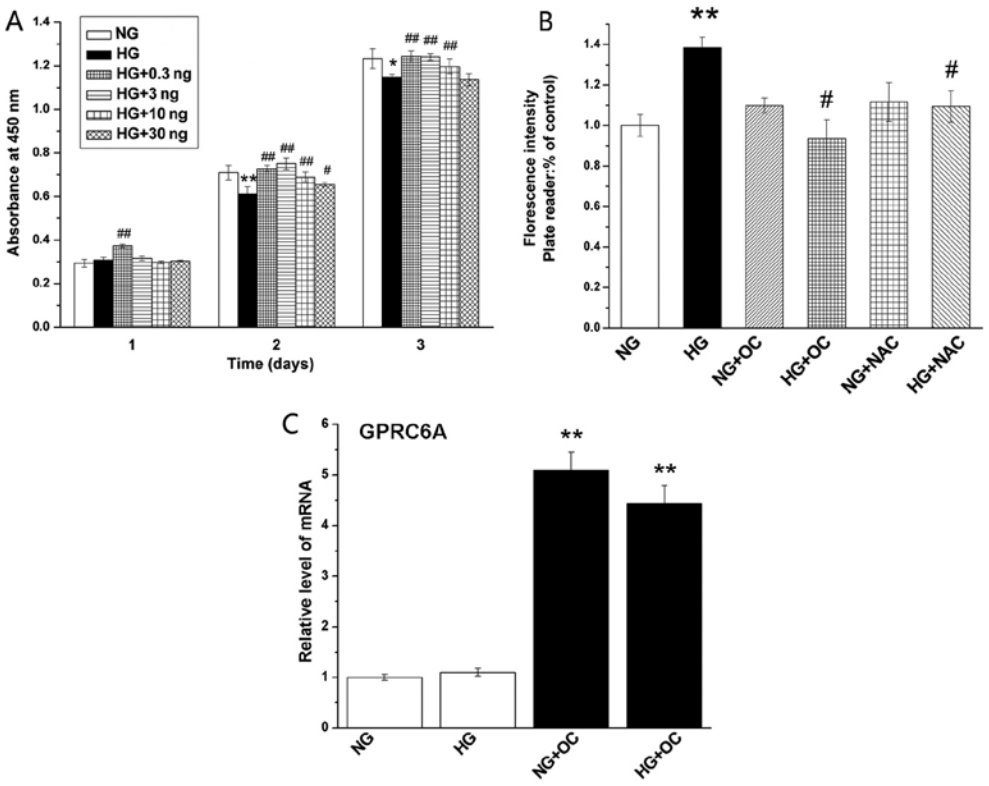

Figure 1. Uncarboxylated osteocalcin increases osteoblast proliferation and reduces high glucose-induced reactive oxygen species (ROS) production in MC3T3-E1 cells. (A) Uncarboxylated osteocalcin increased the proliferation of MC3T3-E1 cells under high glucose conditions. Cells were exposed to normal glucose (5.5 mM) or high glucose (25.5 mM) medium containing various concentrations of uncarboxylated osteocalcin (0.3, 3, 10 and 30 ng/ml) for 1, 2 or 3 days, and cell growth was measured by CCK-8 assay. (B) Cells were cultured in the presence of 5.5 mM (normal glucose, NG), 25.5 mM glucose (high glucose, HG) as well as with or without the antioxidant, N-acetyl-L-cysteine (NAC), and uncarboxylated osteocalcin (3 ng/ml), and ROS levels were detected by DCFH-DA staining. (C) Uncarboxylated osteocalcin stimulates the expression of G protein-coupled receptor, class C, group 6, member A (GPRC6A). Results are presented as the means ± SD, n=3 experiments, $^{*}p<0.05$, $^{**}p<0.01$, vs. NG and $^{\#}p<0.05$, $^{\#\#}p<0.01$ vs. HG.

ml) or NAC (ROS scavenger) in the cells exposed to high glucose (25.5 mM) compared to the cells treated only with high glucose (Fig. 1B). These results suggest that uncarboxylated osteocalcin reduces ROS levels induced by high glucose.

Uncarboxylated osteocalcin increases the expression of GPRC6A. Osteoblasts contain the osteocalcin receptor GPRC6A. To determine the expression of GPRC6A, RT-qPCR was performed. The results revealed that the expression level of GPRC6A was significantly enhanced by uncarboxylated osteocalcin (Fig. 1C).

High glucose-induced ROS production inhibits mineralization and facilitates lipid droplet formation, but these effects are reversed by treatment with uncarboxylated osteocalcin. Both the images and the quantification of the Alizarin red staining demonstrated that high glucose-induced ROS production significantly inhibited the formation of mineralized nodules in MC3T3-E1 cells under high glucose conditions. However, these effects were reversed by treatment with NAC, as well as uncarboxylated osteocalcin (Fig. 2A and C). In addition, Oil Red O staining demonstrated that lipid droplet accumulation was significantly increased by high glucose-induced ROS production, and this effect was also reversed by treatment with NAC or uncarboxylated osteocalcin (Fig. 2B and D). However, we also noted that uncarboxylated osteocalcin had no effect on cells cultured under normal glucose conditions. These results demonstrated that uncarboxylated osteocalcin promoted the formation of mineralized nodules and decreased lipid droplet accumulation in MC3T3-E1 cells under high glucose conditions.

High glucose-induced ROS production inhibits the expression of osteogenic genes and promotes the expression of adipogenic genes, but these effects are reversed by treatment with uncarboxylated osteocalcin. RT-qPCR was performed to further characterize the gene expression levels of the osteogenic markers, Runx2, osterix and osteocalcin, and the gene expression of the adipogenic markers, PPARγ, aP2, and FAS in MC3T3-E1 cells under high glucose conditions. The results revealed that high glucose-induced ROS production increased the mRNA expression of adipogenic markers and inhibited the expression of osteogenic markers in MC3T3-E1 cells under high glucose conditions; these effects were reversed by treatment with NAC and uncarboxylated osteocalcin. However, uncarboxylated osteocalcin did not have such a marked effect on cells cultured under normal glucose conditions (Fig. 3). The above results indicated that uncarboxylated osteocalcin promoted the expression of osteogenic genes and inhibited the expression of adipogenic genes.

High glucose-induced ROS production decreases (Col I) protein levels and increases ALP activity, but these effects are reversed by treatment with uncarboxylated osteocalcin. Subsequently, we examined the effects of uncarboxylated osteocalcin on the Col I protein levels and ALP activity in MC3T3-E1 cells cultured under high glucose conditions. The results

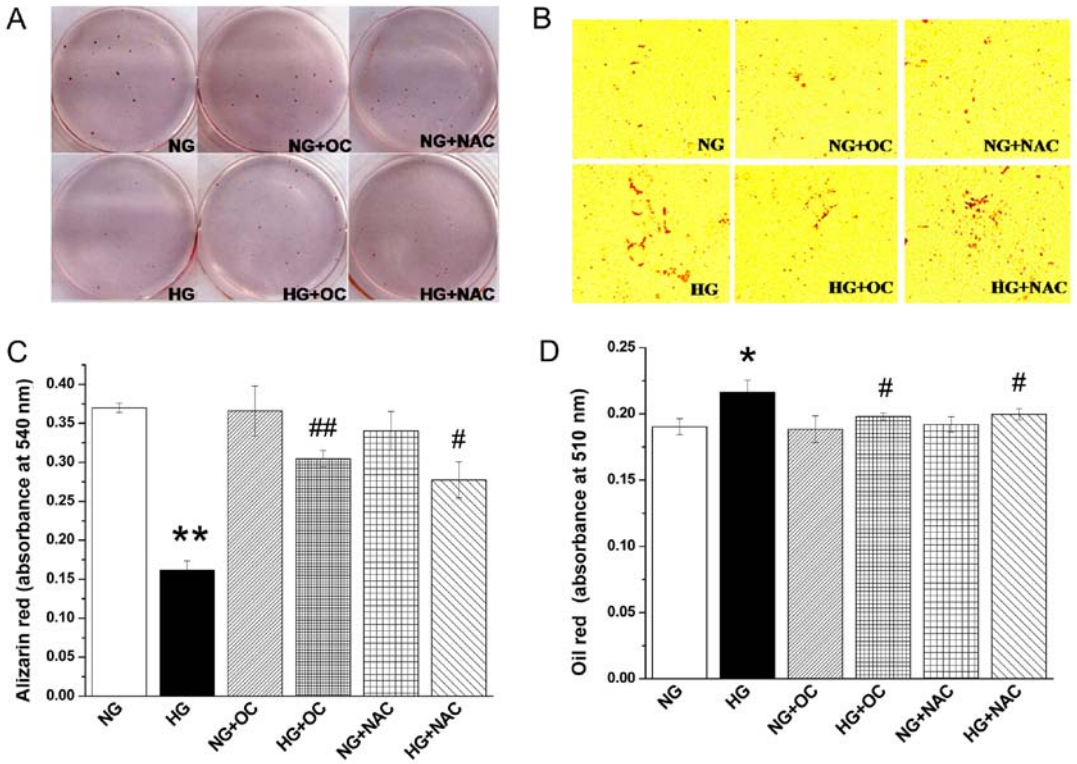

Figure 2. Uncarboxylated osteocalcin reduces high glucose-induced reactive oxygen species (ROS) production to increase mineralization and inhibit lipid droplet accumulation in MC3T3-E1 cells. Cells were cultured in inductive medium containing normal levels of glucose (5.5 mM, NG) or high glucose (25.5 mM, HG), with or without the antioxidant, N-acetyl-L-cysteine (NAC), and uncarboxylated osteocalcin (3 ng/ml). (A) Alizarin red staining and (C) quantification showed that high glucose inhibited mineralized bone nodules. (B) Oil Red O staining and (D) quantification showed that high glucose-induced ROS promoted lipid droplet formation. Results are presented as the means ± SD, n=3 experiments, *p<0.05, **p<0.01, vs. NG and $^{\#}$p<0.05, $^{\#\#}$p<0.01, vs. HG.

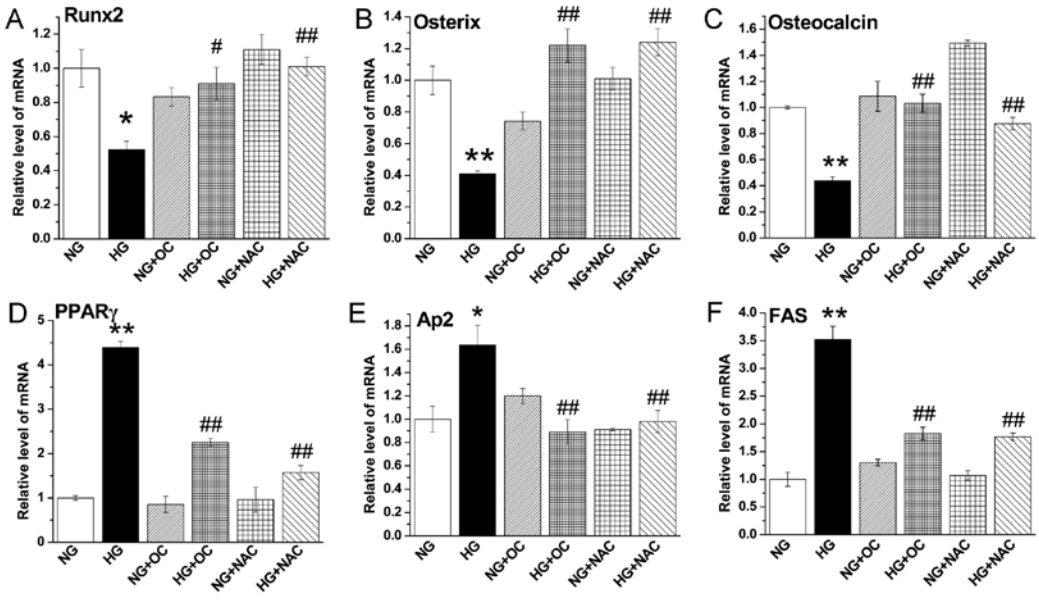

Figure 3. Uncarboxylated osteocalcin reduces high glucose-induced reactive oxygen species (ROS) production to increase the expression of osteogenic genes and inhibit the expression of adipogenic genes. RT-qPCR was performed to quantify the expression of the osteogenic genes, (A) runt-related transcription factor 2 (Runx2), (B) osterix and (C) osteocalcin, and the adipogenic genes, (D) peroxisome proliferator-activated receptor γ (PPARγ), (E) adipocyte protein 2 (aP2) and (F) fatty acid synthase (FAS). The expression of target genes was normalized to β-actin gene expression. Results are presented as the means ± SD, n=3 experiments, *p<0.05, **p<0.01 vs. normal glucose (NG), and $^{\#}$p<0.05, $^{\#\#}$p<0.01 vs. high glucose (HG).

revealed that high glucose levels increased ALP activity and decreased the protein levels of Col I, and treatment with NAC or uncarboxylated osteocalcin also reversed these effects (Fig. 4). No such phenomenon was observed in the MC3T3-E1 cells cultured under normal glucose conditions. Taken together, our results indicated that uncarboxylated osteocalcin reduced ROS

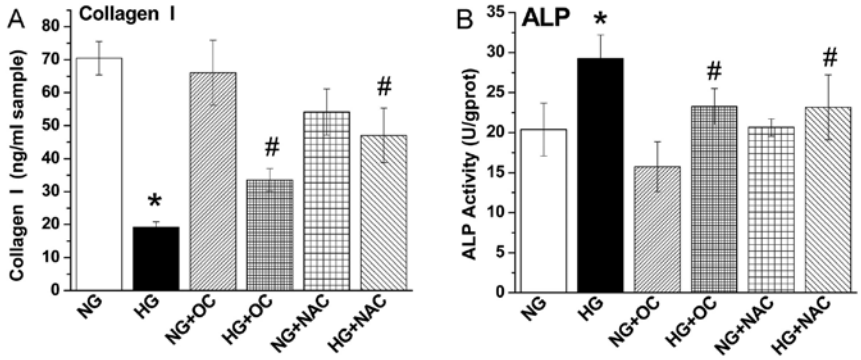

Figure 4. Uncarboxylated osteocalcin reduces high glucose-induced reactive oxygen species (ROS) production to promote collagen I (Col I) protein expression and decrease alkaline phosphatase (ALP) activity in MC3T3-E1 cells under high glucose conditions. Cells were cultured in the presence of 5.5 mM (normal glucose, NG), 25.5 mM glucose (high glucose, HG) as well as with or without the antioxidant, N-acetyl-L-cysteine (NAC), and uncarboxylated osteocalcin (3 ng/ml) for 7 days. (A) ALP activity was determined with an ALP kit and (B) Col I expression was measured by ELISA. Results are presented as the means ± SD, n=3 experiments, $^{*}p<0.05$ vs. NG, and $^{\#}p<0.05$ vs. HG.

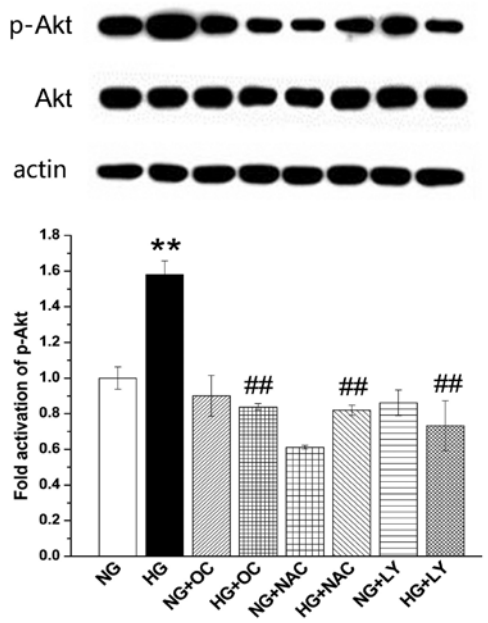

Figure 5. Uncarboxylated osteocalcin reduces the phosphorylation levels of PI3K/Akt stimulated by high glucose-induced reactive oxygen species (ROS) production. Cells were cultured in normal and high glucose medium with or without the specific PI3K inhibitor, LY294002, and N-acetyl-L-cysteine (NAC), as well as uncarboxylated osteocalcin (3 ng/ml). p-Akt and Akt were detected by western blot analysis using specific antibody and densitometry quantifications of activated p-Akt. Results are presented as the means ± SD, n=3 experiments, $^{**}p<0.01$ vs. normal glucose (NG), and $^{\#\#}p<0.01$ vs. high glucose (HG).

levels and promoted the osteogenic differentiation and inhibited the adipogenic differentiation of MC3T3-E1 cells under high glucose conditions.

High glucose-induced ROS production inhibits osteoblast differentiation and increases adipogenic differentiation via the activation of the PI3K/Akt signal pathway, but these effects are reversed by treatment with uncarboxylated osteocalcin in MC3T3-E1 cells

Uncarboxylated osteocalcin inhibits the activation of the PI3K/Akt signaling pathway by high glucose-induced ROS production. In order to further determine whether uncarboxylated osteocalcin inhibits ROS-induced PI3K/Akt signaling, we measured the phosphorylation level of PI3K/Akt with or without LY-294002 (a PI3K/Akt specific inhibitor), NAC, as well as uncarboxylated osteocalcin. As shown in Fig. 5, the intracellular p-Akt level increased significantly under high glucose conditions but was reduced by the addition of LY294002, NAC and uncarboxylated osteocalcin. These results suggest that uncarboxylated osteocalcin inhibits PI3K/Akt signaling by reducing ROS levels.

Uncarboxylated osteocalcin inhibits the PI3K/Akt signaling pathway to promote the expression of osteogenic genes and inhibit the expression of adipogenic genes. We measured the expression levels of osteogenic differentiation- and adipogenic differentiation-related genes. Our results demonstrated that LY294002 or uncarboxylated osteocalcin also reversed the effects of high glucose on the expression of marker genes of osteogenic and adipogenic differentiation in MC3T3-E1 cells. We did not observe the same phenomenon in the cells cultured under normal glucose conditions (Fig. 6).

Uncarboxylated osteocalcin inhibits the PI3K/Akt signaling pathway to increase Col I protein levels and decrease ALP activity. Subsequently, we examined ALP activity and Col I levels. The results revealed that the increase in ALP activity and the decrease in Col I levels in the cells cultured with high concentrations of glucose (25.5 mM) were reversed by the addition of LY294002 or uncarboxylated osteocalcin. By contrast, this did not occur in the cells cultured with normal levels of glucose (5.5 mM) (Fig. 7). These results suggest that uncarboxylated osteocalcin facilitates osteogenic differentiation and inhibits adipogenic differentiation by blocking ROS-stimulated PI3K/Akt signaling in MC3T3-E1 cells under high glucose conditions.

Discussion

Previous research in the field of skeletal biology has revealed that the differentiation and functions of the two bone-specific cell types, osteoblasts and osteoclasts, are determined by secreted molecules that can either be cytokines acting locally, or hormones acting systemically (27,28). In the present study, we demonstrated that uncarboxylated osteocalcin promoted

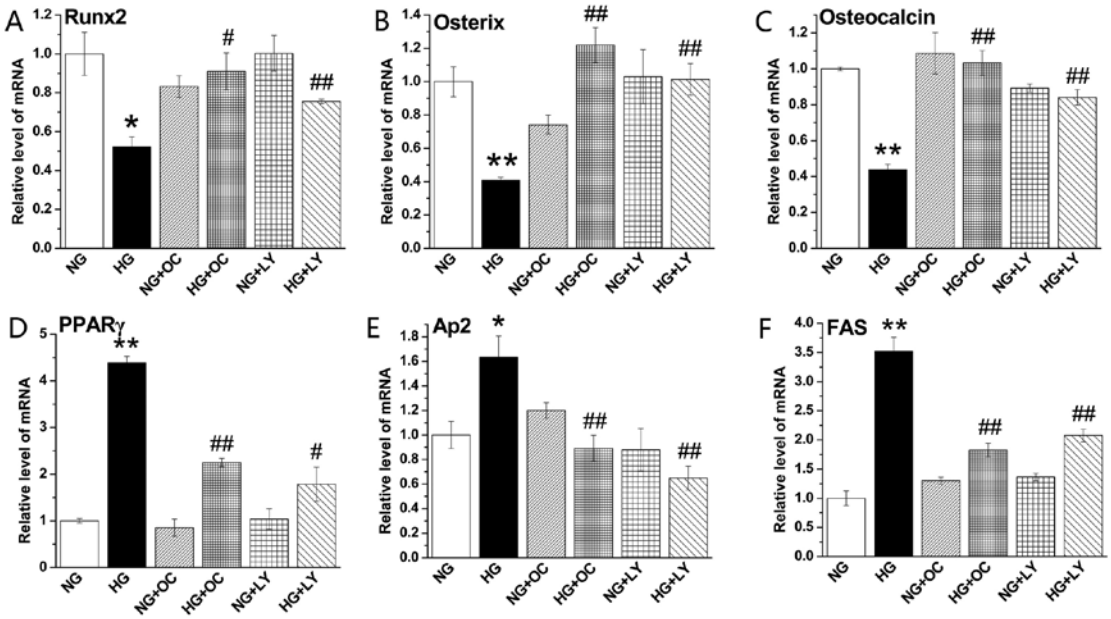

Figure 6. Uncarboxylated osteocalcin increases the expression of osteogenic genes and inhibits the expression of adipogenic genes by blocking the activation of the PI3K/Akt pathway induced by reactive oxygen species (ROS) production in MC3T3-E1 cells under high glucose conditions. Cells were cultured in normal and high glucose medium with or without the specific PI3K inhibitor, LY294002, and N-acetyl-L-cysteine (NAC) as well as uncarboxylated osteocalcin (3 ng/ml). RT-qPCR was performed to quantify the expression of the osteogenic genes, (A) runt-related transcription factor 2 (Runx2), (B) osterix and (C) osteocalcin, and the adipogenic genes, (D) peroxisome proliferator-activated receptor γ (PPARγ), (E) adipocyte protein 2 (aP2) and (F) fatty acid synthase (FAS). The expression of target genes was normalized to β-actin gene expression. Results are presented as the means ± SD, n=3 experiments, $^{*}p<0.05$, $^{**}p<0.01$ vs. normal glucose (NG), and $^{\#}p<0.05$, $^{\#\#}p<0.01$ vs. high glucose (HG).

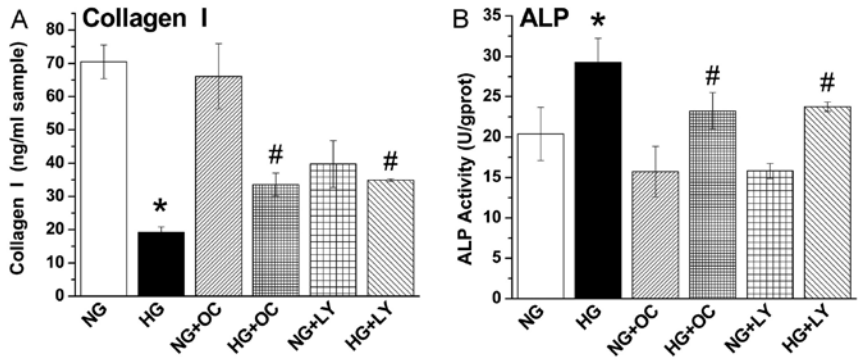

Figure 7. Uncarboxylated osteocalcin promotes collagen I (Col I) protein expression and decreases alkaline phosphatase (ALP) activity by blocking the activation of the PI3K/Akt pathway induced by reactive oxygen species (ROS) production in MC3T3-E1 cells under high glucose conditions. Cells were cultured in normal and high glucose medium with or without the specific PI3K inhibitor, LY294002, and N-acetyl-L-cysteine (NAC) as well as uncarboxylated osteocalcin (3 ng/ml) for 7 days. (A) ALP activity was determined with an ALP kit and (B) Col I expression was measured by ELISA. Results are presented as the means ± SD, n=3 experiments, $^{*}p<0.05$ vs. normal glucose (NG) and $^{\#}p<0.05$ vs. high glucose (HG).

osteogenic differentiation and inhibited adipogenic differentiation by blocking the ROS-induced activation of the PI3K/Akt pathway in MC3T3-E1 cells under high glucose conditions, manifested by an increased expression of the osteogenic markers, Runx2, osterix and osteocalcin, and the formation of mineralized nodules, as well as the decreased mRNA expression of the adipogenic markers, PPARγ, aP2 and FAS, and decreased lipid drop accumulation.

Uncarboxylated osteocalcin and carboxylated osteocalcin are two different forms of osteocalcin. Previous research has demonstrated that in osteocalcin-deficient mice, lower carbonate:phosphate ratios were noted in variectomized knockout cortices than wild-type ones, and crystallite size and perfection resembled that in wild-type trabeculae, providing evidence that osteocalcin regulates bone mineralization (29). Recently, Poundarik *et al* confirmed that osteocalcin$^{-/-}$ mice had lower crystal thickness than wild-type mice, in a small angle X-ray scattering (SAXS) study (30). However, as shown in our study in Figs. 1-4, our results proved that uncarboxylated osteocalcin did not influence the proliferation, differentiation and mineralization of MC3T3-E1 cells cultured under normal glucose conditions.

Previous research has demonstrated that high glucose not only inhibits the proliferation and function (31), but also decreases the osteogenic differentiation and mineralization (32) of osteoblasts. Our previous studies also found that high

amounts of glucose inhibited the osteogenic differentiation of MG-63 cells (23) and high glucose-induced ROS production inhibited the osteogenic, but promoted the adipogenic differentiation of rat primary osteoblasts (22). Oxidative stress has been acknowledged as a major regulator of mineral tissue homeostasis and the promotion of bone resorption (33-36). It has previously been suggested that oxidative stress leads to bone pathogenesis, including osteoporosis, bone tumor development and diabetes-induced bone complications (37). In addition, Liu *et al* demonstrated that the presence of metallothionein, a ROS inhibitor, restored cell differentiation, suggesting that oxidative stress plays a crucial role in the inhibition of osteoblastic differentiation (38). In the present study, our results revealed that uncarboxylated osteocalcin reduced high glucose-induced ROS production and increased the osteogenic differentiation, but decreased the adipogenic differentiation of MC3T3-E1 cells under high glucose conditions.

However, the molecular mechanisms responsible for the effects of uncarboxylated osteocalcin on the differentiation of osteoblasts under high glucose conditions have yet to be elucidated. It has previously been shown that high glucose increases PPARγ expression and PI3K activity and the phosphorylation of its downstream effector Akt during adipogenesis (39). In this study, we also proved that high glucose-induced ROS production accelerated the adipogenic and weakened the osteogenic differentiation of primary osteoblasts through the PI3K-Akt pathway. As shown in Fig. 5, high glucose increased the phosphorylation level of PI3K/Akt, which was reversed by uncarboxylated osteocalcin, LY-294002, as well as NAC in MC3T3-E1 cells under high glucose conditions. These results suggested that uncarboxylated osteocalcin blocked PI3K-Akt signaling induced by ROS and upregulated the expression of osteogenic markers, increased Col I protein levels, as well as mineralization, but downregulated the expression of adipogenic markers and lipid droplet formation.

Overall, the findings of our study demonstrated that uncarboxylated osteocalcin inhibited high glucose induced-ROS production and stimulated osteoblast differentiation by preventing the activation of PI3K/Akt in MC3T3-E1 cells. These results provide new insight into the molecular mechanisms involved in the regulation of glucose metabolism by uncarboxylated osteocalcin. These findings suggest that uncarboxylated osteocalcin may be a potential therapeutic agent for use in the treatment of diabetes-related osteoporosis.

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