ARTICLE
Journal of Cellular Biochemistry 114:2595–2602 (2013)

Journal of Cellular Biochemistry

Activation of the PI3K/Akt Pathway by Oxidative Stress Mediates High Glucose-Induced Increase of Adipogenic Differentiation in Primary Rat Osteoblasts

Yu Zhang and Jian-Hong Yang*

College of Life Science, University of Chinese Academy of Sciences, 19A Yuquan Road, Beijing, 100049, People's Republic of China

ABSTRACT

Diabetes mellitus is associated with increased risk of osteopenia and bone fracture that may be related to hyperglycemia. However, the mechanisms accounting for diabetic bone disorder are unclear. Here, we showed that high glucose significantly promoted the production of reactive oxygen species (ROS) in rat primary osteoblasts. Most importantly, we reported for the first time that ROS induced by high glucose increased alkaline phosphatase activity, inhibited type I collagen (collagen I) protein level and cell mineralization, as well as gene expression of osteogenic markers including runt-related transcription factor 2 (Runx2), collagen I, and osteocalcin, but promoted lipid droplet formation and gene expression of adipogenic markers including peroxisome proliferator-activated receptor gamma, adipocyte fatty acid binding protein (aP2), and adipsin, which were restored by pretreatment with N-acetyl-L-cysteine (NAC), a ROS scavenger. Moreover, high glucose-induced oxidative stress activated PI3K/Akt pathway to inhibited osteogenic differentiation but stimulated adipogenic differentiation. In contrast, NAC and a PI3K inhibitor, LY-294002, reversed the down-regulation of osteogenic markers and the up-regulation of adipogenic markers as well as the activation of Akt under high glucose. These results indicated that oxidative stress played a key role in high glucose-induced increase of adipogenic differentiation, which contributed to the inhibition of osteogenic differentiation. This process was mediated by PI3K/Akt pathway in rat primary osteoblasts. Hence, suppression of oxidative stress could be a potential therapeutic approach for diabetic osteopenia. J. Cell. Biochem. 114: 2595–2602, 2013.

KEY WORDS: HIGH GLUCOSE; OXIDATIVE STRESS; OSTEOGENIC DIFFERENTIATION; ADIPOGENIC DIFFERENTIATION; PI3K/Akt

Diabetes mellitus (DM) describes a metabolic disorder of multiple etiology characterized by chronic hyperglycemia resulting from defects in insulin secretion, insulin action, or both [Hamada et al., 2009a]. Osteoporosis is one of the chronic complications of diabetic mellitus. Evidence showed that hyperglycemia may be implicated in the pathogenesis of the diabetic bone loss [García-Hernández et al., 2011]. In general, people with type 1 diabetes have lower bone mineral density (BMD) under high glucose concentration. However, either type 1 or type 2 diabetes experience a higher incidence of bone fracture than the general population. In the streptozotocin induced mouse model of insulin-dependent DM, Botolin and McCabe [2006] demonstrated that chronic hyperglycemia contributed to bone loss by modulating osteoblast gene expression, function, and bone formation. In a rat model of type 2 DM, low bone mass and loss bone strength were due to delayed bone regeneration [Hamann et al., 2013] and impaired osteoblast function [Hamann et al., 2011]. In vitro studies showed that high glucose not only inhibited proliferation and function of primary rat osteoblasts [Zhen et al., 2010] but also decreased osteogenic differentiation and mineralization of rat's mandibular bone marrow stromal cells [Li et al., 2012].

Decreased osteoblast differentiation and increased adipocyte differentiation may contribute to bone loss in diabetic mice [Botolin et al., 2005; Botolin and McCabe, 2007]. Both osteogenic and adipogenic differentiation are regulated by a number of pathways and transcription factors. Osteogenesis is regulated by runt-related transcription factor-2 (Runx-2), while adipogenesis by peroxisome proliferator-activated receptor gamma (PPARγ). Akune et al. [2004] demonstrated that homozygous PPARγ-deficient ES cells failed to differentiate into adipocytes, but spontaneously differentiated into

Grant sponsor: Scientific Research Foundation of University of Chinese Academy of Sciences (UCAS); Grant number: 055101FM03.

*Correspondence to: Dr. Jian-Hong Yang, College of Life Science, University of Chinese Academy of Sciences, 19A Yuquan Road, Beijing 100049, People's Republic of China. E-mail: yangjhong@yahoo.com.cn

Manuscript Received: 20 March 2013; Manuscript Accepted: 31 May 2013

Accepted manuscript online in Wiley Online Library (wileyonlinelibrary.com): 11 June 2013

DOI 10.1002/jcb.24607 • © 2013 Wiley Periodicals, Inc.

osteoblasts, and heterozygous PPARγ-deficient mice exhibited high bone mass with increased osteoblastogenesis. However, osteoblast-targeted overexpression of PPARγ inhibited bone mass gain in male mice and exaggerated ovariectomy (ovx)-induced bone loss in mice [Cho et al., 2011]. Chuang et al. [2007] proved that hyperglycemia enhanced PPARγ-dependent adipogenic induction in bone marrow stromal cells (MSCs). Additionally, our previous research demonstrated that high glucose stimulated adipogenic and inhibited osteogenic differentiation in human osteoblastic MG-63 cells through cAMP/protein kinase A (PKA)/extracellular signal-regulated kinase (ERK) pathway [Wang et al., 2010]. Further studies will need to elucidate the mechanisms involved in the increased adipogenic and reduced osteogenic differentiation stimulated by high glucose.

Oxidative stress plays an important role in the pathogenesis of diabetic osteopenia and osteoporosis [Hamada et al., 2009b; Zhen et al., 2010]. Oxidative stress impaired osteoblast formation and function as well as mineralization under the diabetic condition. In contrast, suppression of increased oxidative stress by TRX (thioredoxin) overexpression attenuated diabetic osteopenia by restoring BMD and stimulating bone formation in streptozotocin-induced diabetic mice [Hamada et al., 2009b]. While the relationship between oxidative stress and diabetic osteopenia or osteoporosis were well documented, the mechanism by which reactive oxygen species (ROS) inhibits osteoblast differentiation in rat primary osteoblasts under high glucose has not been reported.

In this study, we investigated the effects of oxidative stress evoked by high glucose on differentiation of primary rat calvaria osteoblasts and the involved signal pathways. Our results showed that it was the enhanced adipogenic differentiation induced by oxidative stress under high glucose that inhibited osteogenic differentiation of primary osteoblasts. This process was mediated by oxidative stress via PI3K/Akt pathway.

MATERIALS AND METHODS

CELL ISOLATION AND CULTURE

Primary rat osteoblasts were isolated from calvaria of neonatal Wistar rats (purchased from the Laboratory Animal Center of the Academy of Military Medical Sciences, certificate number of the rats: SCXK 2007-0004, Beijing, China). Calvaria were gently digested at 37°C for 10 min four times with 0.1% collagenase and 0.25% trypsin, and the last two fractionated cells were collected. Cells were cultured in α-MEM (Gibco-BRL, Gaithersburg, MD) supplemented with 10% fetal bovine serum (FBS, HyClone, Logan, UT), 100 U/ml penicillin and 100 mg/L streptomycin and kept at 37°C in a humidified atmosphere of 95% air and 5% CO_2. Culture medium was replaced every 3 days thereafter. Cells between the second and the fourth passages were used in all experiments.

CELL PROLIFERATION ASSAY

MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) colorimetric assay was used to evaluate the effect of different concentration of glucose on the cell proliferation. Briefly, cells were plated in 96-well plates (4,000 cells/well) for 24 h in the medium containing 10% serum and then replaced with 4% serum medium containing normal (5.5 mM) or high glucose (15.5, 25.5, or 35.5 mM) for 0, 1, or 2 days. Next media were changed with phenol red-free DMEM containing 0.5 mg/ml MTT. After 4 h incubation at 37°C, dimethylsulfoxide (DMSO) (150 μl) was added to each well to dissolve the formed formazen crystals. The osteoblast proliferation was determined by absorbance measurement at 492 nm using an automated microplate reader (Bio-Rad, Laboratories, Richmond, CA).

MEASUREMENT OF ROS

Intracellular ROS amount was measured using peroxide-dependent oxidation of 2′-7′-dichlorofluorescein-diacetate (DCFH-DA) (Beyotime, Jiangsu, China). DCFH-DA is hydrolyzed by intracellular esterases to form non-fluorescent DCFH, which is further oxidized by ROS to form the fluorescent compound DCF. Cells were plated in 24-well plates (1×10^5 cells/well) and incubated in the medium containing normal (5.5 mM) or high (25.5 mM) glucose and with or without antioxidant N-acetyl-L-cysteine (NAC) for 2 days. Thirty minutes before the termination of incubation period, DCFH-DA (final concentration of 10 mM) was added to the cells and incubated for the 30 min at 37°C. Then, the cells were harvested for the detection of ROS accumulation with excitation at 485 and emission at 528 nm using BioTek Synergy HT microplate reader (BioTek Instruments, Inc., Winooski, VT).

MINERALIZATION ASSAY

Cells were seeded in 6-well plates at a density of 5×10^5 cells/well or in 24-well plates at a density of 1×10^5 cells/ml/well with α-MEM containing 10% FBS. When cells reached confluent (Day 0), the medium was replaced with mineralizing media (α-MEM, 4% FBS, 1% P/S, 100 μg/ml ascorbic acid, 10 mM β-glycerophosphate phosphate, and 1×10^{-8} M dexamethasone) containing normal (5.5 mM) or high (25.5 mM) glucose and with or without NAC. The formation of mineralized nodule was detected at Day 25, using Alizarin Red-S staining. Briefly, cells were rinsed twice with phosphate-buffered saline (PBS) followed by fixation with 4% paraformaldehyde for 30 min on ice. Then cells were stained with 2% Alizarin Red-S (pH 4.2) for 20 min at room temperature and extensively rinsed with distilled water. Images were captured with a digital camera. For quantification, the bound staining was eluted with 10% (wt/vol) cetylpyridinium chloride and the absorbance of supernatants was measured by the automated microplate reader (Bio-Rad) at 540 nm.

OIL RED O (ORO) STAINING AND ASSAY

Lipid accumulation in osteoblasts was determined by ORO staining. Cells were seeded in 6-well plates at 5×10^5 cells/well and then treated with the adipogenic differentiation medium (a-MEM, 4% FBS, 1% P/S, 10 μg/ml insulin, and 1×10^{-7} M dexamethasone) containing normal (5.5 mM) or high (25.5 mM) glucose and with or without NAC. Thirty days after induction, cells were washed three times with PBS, fixed with 4% formaldehyde at room temperature for 20 min and stained with freshly diluted ORO solution (three parts of 0.1% ORO in isopropyl alcohol and two parts of water) for 15 min. Cells were then washed twice with distilled water. Images were observed under the inverted phase contrast microscope. For quantitative analysis, ORO staining was dissolved with isopropyl alcohol and the optical density

was measured at 510 nm by microplate reader. All experiments were performed in triplicate.

ALKALINE PHOSPHATASE (ALP) ASSAY

To evaluate osteoblastic differentiation, ALP activity was measured. Cells (2×10^5 cells/well) were pre-treated with or without 10 μM LY-294002 (Promega, Madison, WI), a specific inhibitor of PI3K, for 1 h and with or without NAC for 1 h, then treated with or without high glucose for 5 days. Cells were harvested using cell scrapper by adding 200 μl cold PBS to each well and lysed by ultrasonification. ALP activity was measured by ALP assay kit (Nanjing Jian Cheng, Nanjing, China) according to the manufacturer's instructions. The protein concentration of the cell lysate was determined using a micro-BCA assay kit (Beyotime), and ALP activity was normalized to the total protein concentration.

ENZYME-LINKED IMMUNOSORBENT ASSAY FOR TYPE I COLLAGEN SECRETION

Cultured cells were treated as above and collagen I secretion was quantified using commercial ELISA kits (Boster Bio-Tech, Wuhan, Hubei, China) according to the manufacturer's instructions.

ANALYSIS OF MRNA EXPRESSION BY REAL-TIME POLYMERASE CHAIN REACTION

Total RNA was extracted from cells by RNeasy mini spin column (Qiagen, Inc., Valencia, CA) and cDNA was synthesized from 5 μl of total RNA using the SuperScript First-Stand Synthesis System for RT-PCR (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Real-time PCR was performed using 2 μl of 10 times diluted cDNA in a 25 μl reaction volume with an ABI GeneAmp 5700 Sequence Detection System and QuantiTect SYBR Green PCR Master Mix (Qiagen). The forward and reverse primer sequences are listed in Table I. The relative RNA levels were normalized by using β-actin as a housekeeping gene. The cycling conditions were as follows: incubation at 95°C for 5 min, 40 cycles of denaturation at 94°C for 30 s, annealing at 56°C for 30 s and extension at 72°C for 30 s. We compared each gene sample level by using ABI GeneAmp 5700 SDS software.

TABLE I. Primer Sequences Used for Real-Time RT-PCR

Gene	Sequence 5′–3′	Gen Bank ID
Runx2	F: TCCTGGTCACAATGGGATACC R: ATCTCCTGGGTCACCCTTAGG	NM_053470.2
OC	F: TGCAAAGCCCAGCGACTCT R: AGTCCATTGTTGAGGTAGCG	NM_013414.1
Col I	F: TTGACCCTAACCAAGGATGC R: CACCCCTTCTGCGTTGTATT	XM_003749691
PPARγ	F: CGCTGATGCACTGCCTATGA R: GGGCCAGAATGGCATCTCT	NM_013124.3
Adipsin	F: TGGTGGATGAGCAGTGGGT R: AGGGTTCAGGACTGGACAGG	M92059.1
aP2	F: GCGTAGAAGGGGACTTGGTC R: TTCCTGTCATCTGGGGTGATT	NM_053365.1
β-actin	F: GCCCTAGACTTCGAGC R: CTTTACGGATGTCAACGT	NM_031144.3

F, forward; R, reverse.

WESTERN BLOTTING ANALYSIS

Cells were rinsed twice by 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. Approximately 20 mg protein samples ran on a 10% SDS–PAGE gel and proteins were transferred to PVDF membranes (Millipore, Bedford, MA). After blocking non-specific binding sites with 5% non-fat milk for 60 min at room temperature, the membranes were incubated overnight at 4°C with a primary monoclonal antibody against p-Akt (Ser473) or Akt (CST, Beverly, MA, at a 1:1,000 dilution), followed by horseradish peroxidase (HRP) conjugated secondary antibody (CST, at a 1:5,000 dilution) for 60 min at room temperature after being washed three times with TBST for 10 min each. The membranes were then washed three times with TBST and the immunoreactive bands were visualized using a chemiluminescence (ECL) kit (Biomiga, San Diego, CA). 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 three times. Data were presented as mean ± SD. The results were evaluated by unpaired student's *t*-test or one-way ANOVA using statistical software SPSS 13.0 (SPSS Inc, Chicago, IL). *P* value less than 0.05 was considered to be a statistically significant difference.

RESULTS

HIGH GLUCOSE INHIBITED VIABILITY OF PRIMARY OSTEOBLASTS

In order to determine whether high concentrations of glucose impaired the proliferation of primary rat osteoblasts, cells were exposed to different glucose concentrations (5.5, 15.5, 25.5, and 35.5 mM) for 1 or 2 days, and cell viability was evaluated by the MTT colorimetric assay. High glucose (15.5, 25.5, and 35.5 mM) inhibited cell proliferation in a dose- and time-dependent manner (1 and 2 days). Treatment for 2 days with high glucose (25.5 and 35.5 mM) inhibited osteoblasts growth by 10% and 40% (Fig. 1A).

HIGH GLUCOSE INHIBITED MINERALIZATION AND INCREASED LIPID DROPLET FORMATION OF PRIMARY OSTEOBLASTS

In order to investigate the effect of high glucose on mineralization and lipid droplet formation of primary osteoblasts, cells were fixed and stained with Alizarin red S or ORO, respectively. Alizarin red staining showed that the mineralization of cells was inhibited by high glucose (25.5 mM) on Day 25 (Fig. 1B). While, ORO staining indicated that lipid accumulation significantly increased in high glucose group (25.5 mM) compared to normal glucose group (5.5 mM) (Fig. 1C).

HIGH GLUCOSE-INDUCED ROS INHIBITED OSTEOGENIC DIFFERENTIATION AND INCREASED ADIPOGENIC DIFFERENTIATION IN PRIMARY OSTEOBLASTS

High glucose increased ROS production in primary osteoblasts. In order to evaluate the effect of ROS induced by high glucose on osteoblast differentiation, we measured intracellular ROS by DCFH-DA. The result showed that intracellular ROS level (1.6-fold) in high glucose group (25.5 mM) was significantly higher than that in the

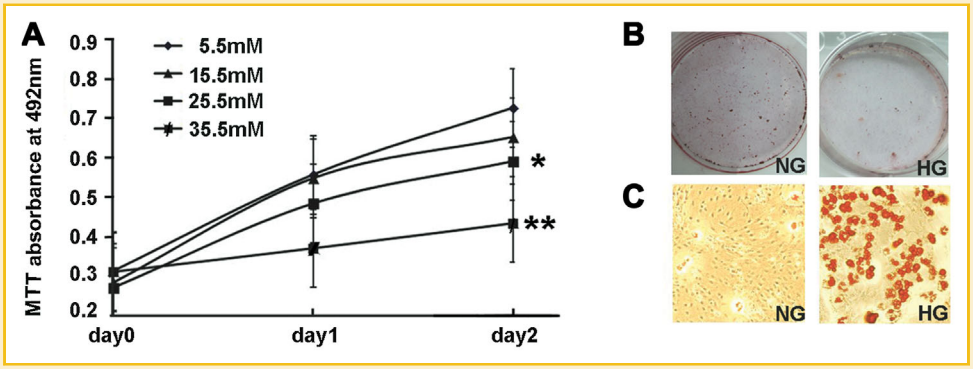

Fig. 1. High glucose inhibited the proliferation and mineralization but increased lipid droplet accumulation of primary osteoblasts. A: High glucose inhibited primary osteoblast proliferation in a time- and concentration-dependent manner. Cells were exposed to normal (5.5 mM) or high glucose (15.5, 25.5, and 35.5 mM) medium for 1 or 2 day, and the cell growth was measured by MTT assay. B: Cells were cultured in inductive medium containing normal glucose (5.5 mM, NG) or high glucose (25.5 mM, HG), alizarin red staining showed that high glucose inhibited mineralized bone nodules. Images were captured with a digital camera. C: Oil red O staining showed that high glucose increased lipid droplets formation. Samples were observed at ×100 magnification. These data represent means ± SD of three independent experiments. $^{*}P < 0.05$, $^{**}P < 0.01$ versus normal glucose.

normal glucose group (5.5 mM) ($P < 0.01$). However, treatment with the ROS scavenger NAC significantly reduced the high glucose-induced ROS production ($P < 0.01$) (Fig. 2A).

High glucose-induced ROS inhibited mineralization and increased lipid droplet accumulation in primary osteoblasts. The quantification of the Alizarin red staining showed that high glucose-induced ROS significantly inhibited formation of mineralized nodules but reversed by NAC (at least $P < 0.01$) (Fig. 2B). In addition, ORO staining indicated that high glucose-induced ROS significantly increased lipid droplet accumulation but reversed by NAC in primary osteoblasts (Fig. 2C).

High glucose-induced ROS increased ALP activity and inhibited collagen I protein level in primary osteoblasts. High glucose (25.5 mM) increased the ALP activity significantly in primary osteoblasts compared to control group (5.5 mM glucose) (Fig. 3A). Consistently, high glucose decreased protein level of collagen I (Fig. 3B). However, treatment with the ROS scavenger, NAC, significantly reduced ALP activity but increased protein level of collagen I under high glucose (Fig. 3A and B).

High glucose-induced ROS inhibited expression of osteogenic genes and increased expression of adipogenic genes in primary osteoblasts. Real-time RT-PCR analysis revealed that the mRNA levels of Runx2, collagen I, and osteocalcin (Fig. 4A), which were markers of osteogenic differentiation, were decreased and the mRNA levels of PPARγ, adipsin, aP2 (Fig. 4B), markers of adiponetic differentiation, were increased in primary osteoblasts under high glucose, but prevented by NAC.

HIGH GLUCOSE-INDUCED ROS INHIBITED OSTEOGENIC DIFFERENTIATION AND INCREASED ADIPOGENIC DIFFERENTIATION VIA ACTIVATED PI3K/AKT IN PRIMARY OSTEOBLASTS

High glucose increased ALP activity and inhibited collagen I protein level via activated PI3K/Akt in primary osteoblasts. In order to provide further mechanistic evidence for a role of PI3K and Akt signaling in high glucose-treated osteoblasts, cells in normal and high glucose culture were treated with or without a PI3K inhibitor, LY294002, for 5 days. The increase of ALP activity and the decrease of collagen I in the culture medium containing high concentration

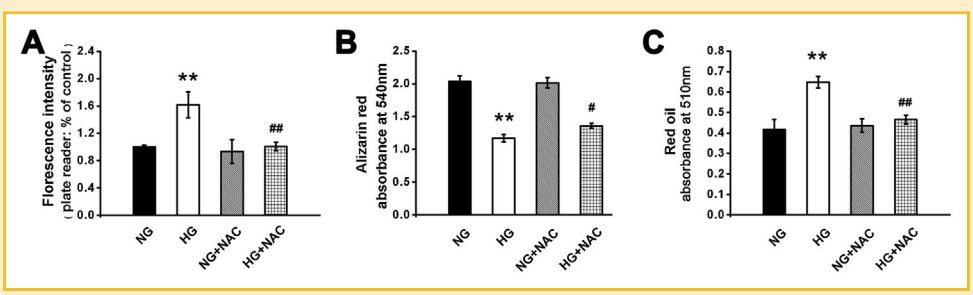

Fig. 2. High glucose-induced ROS inhibited mineralization but increased lipid droplet accumulation of primary osteoblasts. Cells were cultured in the presence of 5.5 mM (normal glucose, NG), 25.5 mM glucose (high glucose, HG) with or without NAC for 2 days and the ROS levels were detected by DCFH-DA staining. A: High glucose increased the production of ROS in primary osteoblasts. B: Alizarin red staining showed that high glucose-induced ROS inhibited mineralization. C: Oil red O staining showed that high glucose-induced ROS promoted lipid droplet formation. These data represent means ± SD of three independent experiments. $^{**}P < 0.01$ versus normal glucose; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ versus high glucose.

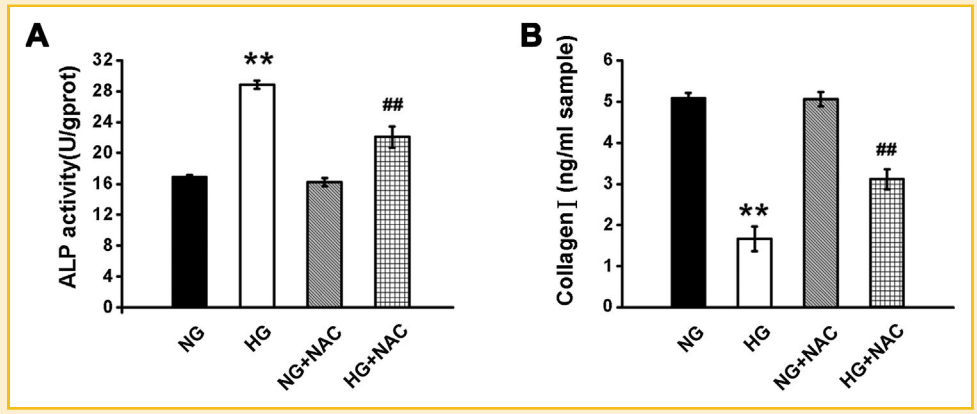

Fig. 3. High glucose-induced oxidative stress increased ALP activity and inhibited collagen I expression in primary osteoblasts. Cells were cultured in the presence of 5.5 mM (normal glucose, NG), 25.5 mM glucose (high glucose, HG) with or without NAC for 5 days, then (A) ALP activity was determined by ALP Kit and (B) collagen I expression was measured by ELISA test. Results are means ± SD, n = 5, $^{**}P < 0.01$ versus normal glucose; $^{\#\#}P < 0.01$ versus high glucose.

glucose (25.5 mM) could be reversed by the addition of LY294002 compared to normal glucose group (5.5 mM) (Fig. 5A and B).

High glucose inhibited expression of osteogenic genes and increased expression of adipogenic genes via activated PI3K/Akt in primary osteoblasts. Besides, high glucose (25.5 mM) inhibited the marker gene expression of osteogenic differentiation (Fig. 6A) and increases expression of adipogenic differentiation (Fig. 6B) remarkably, but reversed by LY294002 treatment.

High glucose-induced ROS stimulated PI3K/Akt in primary osteoblasts. In order to examine whether high glucose-induced ROS stimulated PI3K/Akt, we measured intracellular p-Akt levels with or without LY-294002 and with or without NAC. The results showed that intracellular p-Akt levels increased significantly under high glucose but reversed by addition of LY294002, a PI3K/Akt-specific inhibitor.

Furthermore, high glucose-induced ROS up-regulated the levels of p-Akt remarkably, but restored by NAC (Fig. 7).

DISCUSSION

To our knowledge, we showed for the first time that high glucose-induced ROS production and up-regulated ROS production via PI3K/Akt pathway suppressed osteogenic differentiation, manifested by a

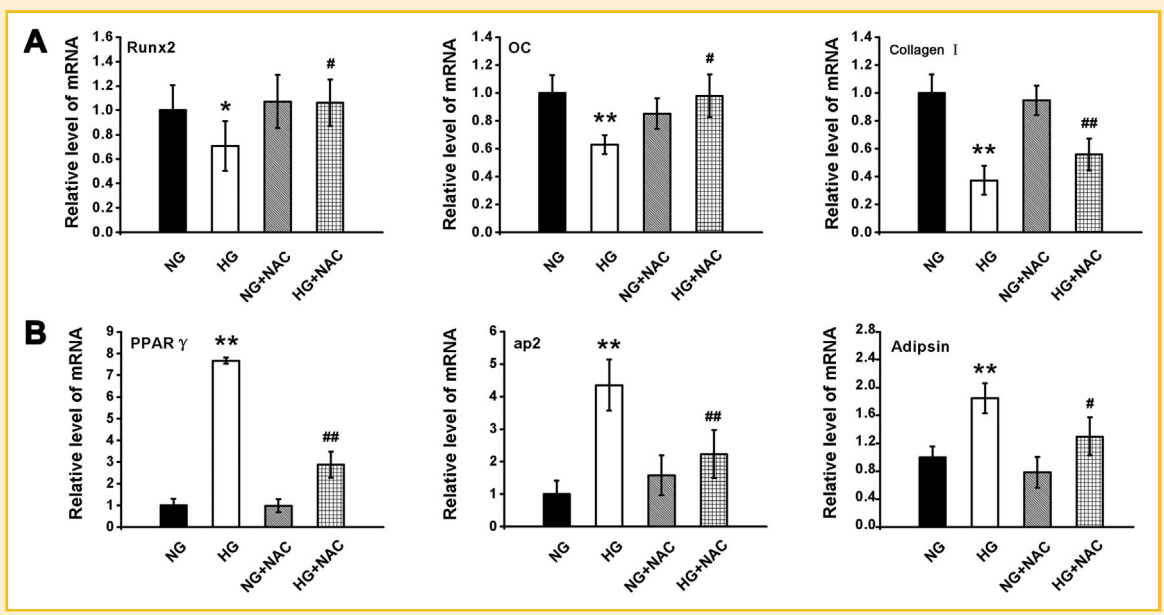

Fig. 4. High glucose-induced oxidative stress inhibited osteogenic genes expression and increased adipogenic genes expression in primary osteoblasts. Cells were cultured in the presence of 5.5 mM (normal glucose, NG), 25.5 mM glucose (high glucose, HG) with or without NAC. Real-time PCR was performed to quantify the expression of osteogenic genes, such as (A) Runx2, osteocalcin (OC), collagen I, and adipogenic genes, such as (B) PPARγ, aP2, adipsin. All expression of target genes was normalized to β-actin gene expression. Results are means ± SD, n = 3, $^{*}P < 0.05$, $^{**}P < 0.01$ versus normal glucose; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ versus high glucose.

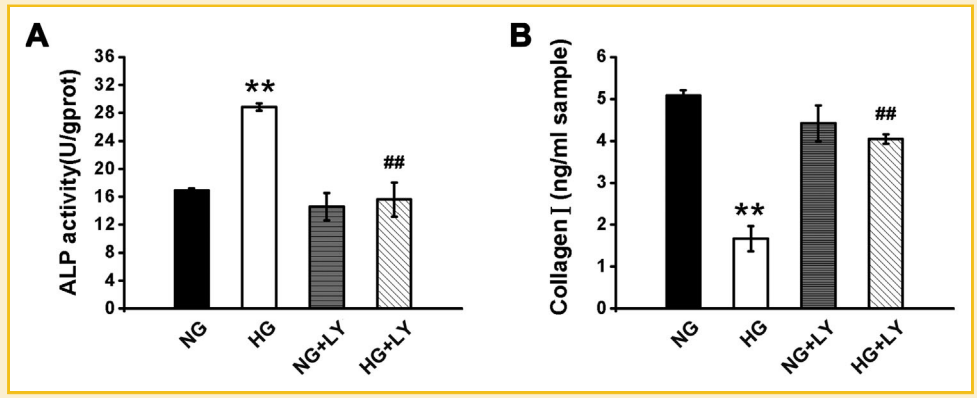

Fig. 5. High glucose increased ALP activity and inhibited collagen I expression via PI3K/Akt pathway in primary osteoblasts. In order to investigate the role of PI3K/Akt signaling, cells were cultured in the presence of 5.5 mM (normal glucose, NG) or 25.5 mM glucose (high glucose, HG) with or without specific PI3K inhibitor LY294002 (LY) for 5 days. A: ALP activity was determined by ALP assay kit. B: Collagen I expression was measured by ELISA test. Results are means ± SD, n = 3, $^{**}P < 0.01$ versus normal glucose; $^{\#\#}P < 0.01$ versus high glucose.

decreased expression of osteogenic markers including Runx2, collagen I, and osteocalcin and the formation of mineralized nodules but increased adipogenic differentiation, with an increased gene expression of adipogenic markers including PPARγ, aP2, adipsin and lipid drop accumulation in rat primary osteoblasts.

Accumulating evidences suggested that hyperglycemia was a potential risk factor contributing to diabetic osteoporosis [Schwartz, 2003; McCabe, 2007]. Bone homeostasis is maintained by a balance between bone resorption by osteoclasts and bone formation by osteoblasts. Decreased mature osteoblasts and increased adipocytes in marrow composition was also a possible mechanism for diabetic bone loss [Botolin et al., 2005; Botolin and McCabe, 2007]. Previous studies from other laboratories have showed that high glucose significantly impaired bone formation by inhibiting cell proliferation, differentiation, and maturation of mouse MSCs or preosteoblastic cell line (MC3T3-E1) [Balint et al., 2001; Gopalakrishnan et al., 2006], but accelerated adipogenesis and accumulation of lipid droplets in mouse MSCs [Chuang et al., 2007]. Lecka-Czernik et al. [1999] found that stable transfection of MSCs with PPARγ, a critical transcription factor in adipogenesis, could suppress Runx2 expression, an important

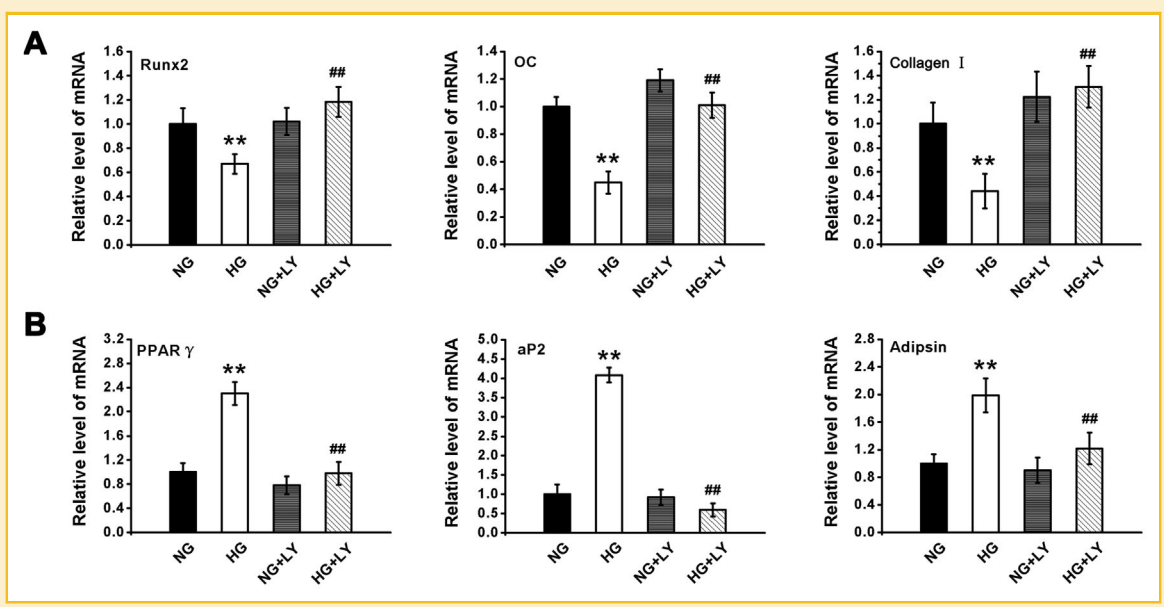

Fig. 6. High glucose inhibited osteogenic genes expression but increased adipogenic genes expression via PI3K/Akt pathway in primary osteoblast. Cells were cultured in the presence of 5.5 mM (normal glucose, NG) or 25.5 mM glucose (high glucose, HG) with or without the specific PI3K inhibitor LY294002. Real-time PCR was performed to quantify the gene expression of osteogenic genes, such as (A) Runx2, OC, collagen I, and adipogenic genes, such as (B) PPARγ, aP2, adipsin. All expression of target genes was normalized to β-actin gene expression. Results are means ± SD, n = 3, $^{**}P < 0.01$ versus normal glucose; $^{\#\#}P < 0.01$ versus high glucose.

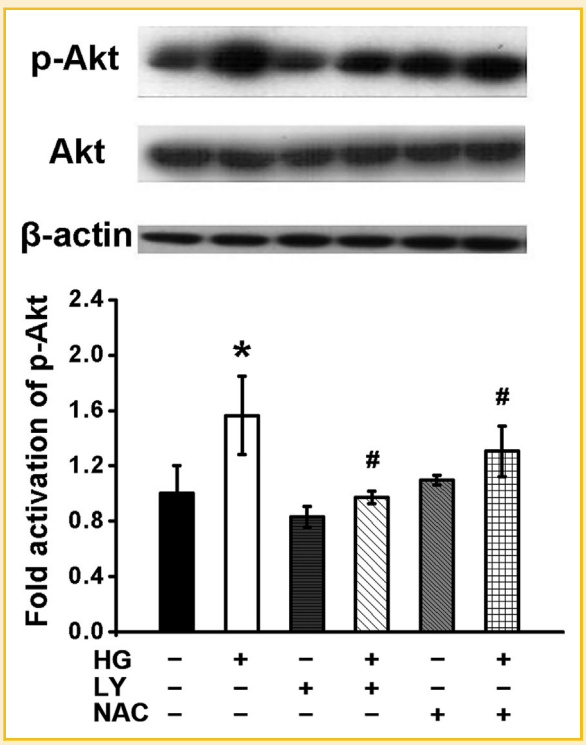

Fig. 7. High glucose-induced ROS stimulated PI3K/Akt in primary osteoblasts. Cells were cultured in normal and high glucose media with or without LY-294002 (LY) and NAC. Phospho-Akt and Akt was detected by immunoblotting using specific antibody and densitometry quantification of activated p-Akt was tested. Results are means ± SD, n = 3, $^{*}P < 0.05$ versus normal glucose; $^{\#}P < 0.05$ versus high glucose.

transcription factor in osteogenesis. Recent observations in our lab have also suggested that high glucose enhanced adipogenic and attenuated osteogenic differentiation in human osteoblastic MG-63 cells through cAMP/PKA/ERK pathway [Wang et al., 2010]. Our present results confirmed that in rat primary osteoblasts, high glucose could also inhibit cell proliferation (Fig. 1A), formation of mineralized nodules (Fig. 1B), as well as osteoblastic differentiation, characterized by decrease of osteogenic mRNA markers (Runx2, osteocalcin, and collagen I) (Fig. 4A) and collagen I protein level (Fig. 3B), but stimulate adipogenic differentiation and accumulation of lipid droplets (Fig. 1C), by increased expression of adipogenic mRNA markers (PPARγ, aP2, and adipsin) (Fig. 4B).

However, the precise mechanisms by which high glucose increased adipogenic differentiation and decreased osteoblastic differentiation remain unclear. Diabetes is associated with increased levels of oxidative stress and an increased risk of fracture [Strotmeyer and Cauley, 2007]. Our experiment also showed that high glucose could induce production of ROS in rat primary osteoblasts, but restored by NAC, a scavenger of ROS (Fig. 2A). Besides, oxidative stress triggered by high glucose inhibited osteoblastic differentiation of MC3T3-E1 [Choi and Kim, 2008]. Furthermore, in our experiment, we demonstrated that in rat primary osteoblasts, oxidative stress induced by high glucose suppressed osteogenic differentiation, characterized by a decrease expression of osteogenic markers including Runx2, collagen I, and osteocalcin and the formation of mineralized nodules. These discovers were consistent with others in vivo experiments that oxidative stress impaired osteoblast formation and function as well as mineralization under the diabetic condition [Hamada et al., 2009b]. However, we found for the first time that oxidative stress induced by high glucose increased adipogenic differentiation in rat primary osteoblasts, by an increase of adipogenic markers including PPARγ, aP2, and adipsin and lipid drop accumulation, but lessened by NAC (Figs. 2–4). Increased adipocyte differentiation may contribute to bone loss in diabetic mice [Botolin et al., 2005; Botolin and McCabe, 2007].

Although the high glucose-induced oxidative stress plays a pivotal role in the pathogenesis of diabetic osteopenia, the signaling molecules involved in inhibiting osteoblastic and increasing adipogenic differentiation under high glucose-induced ROS have not been fully revealed. Our previous experiment suggested that high glucose-accelerated adipogenic and weakened osteogenic differentiation in human osteoblastic MG-63 cells through cAMP/PKA/ERK pathway [Wang et al., 2010]. However, PI3K was required for mouse and human adipocyte differentiation [Aubin et al., 2005] and LY294002, a specific PI3K inhibitor, could block the adipocyte differentiation of 3T3-L1 cells [Tomiyama et al., 1995; Magun et al., 1996; Xia and Serrero, 1999]. Our present research showed that high glucose-induced ROS could enhance p-Akt levels, but restore by NAC (Fig. 7). Moreover, the elevated levels of p-Akt could be restored by specific PI3K inhibitor LY294002 (Fig. 7) under high glucose-induced ROS. In addition, high glucose-induced ROS reduced osteogenic mRNA markers expression (Figs. 4A and 6A), Col I protein level (Figs. 3B and 5B), as well as mineralization (Fig. 2B) but increased adipogenic markers expression (Figs. 4B and 6B) and lipid droplet formation (Fig. 2C) but retrieved by NAC or LY294002. Taken together, we concluded that high glucose-induced ROS production via PI3K/Akt pathway to increase adipogenic differentiation, which contributed to the suppressed osteogenic differentiation. Understanding the mechanisms by which ROS or oxidative stress might modulate intracellular signaling pathways in bone cells is essential for identifying potential molecular targets for manipulation of cellular redox status and therapy for diabetic osteopenia.

REFERENCES

Akune T, Ohba S, Kamekura S, Yamaguchi M, Chung U, Kubota N, Terauchi Y, Harada Y, Azuma Y, Nakamura K, Kadowaki T, Kawaguchi H. 2004. PPARgamma insufficiency enhances osteogenesis through osteoblast formation from bone marrow progenitors. J Clin Investig 113:846–855.

Aubin D, Gagnon A, Sorisky A. 2005. Phosphoinositide 3-kinase is required for human adipocyte differentiation in culture. Int J Obes 29:1006–1009.

Balint E, Szabo P, Marshall C, Sprague S. 2001. Glucose-induced inhibition of in vitro bone mineralization. Bone 28:21–28.

Botolin S, McCabe LR. 2006. Chronic hyperglycemia modulates osteoblast gene expression through osmotic and non-osmotic pathways. J Cell Biochem 99:411–424.

Botolin S, McCabe LR. 2007. Bone loss and increased bone adiposity in spontaneous and pharmacologically induced diabetic mice. Endocrinology 148:198–205.

Botolin S, Faugere MC, Malluche H, Orth M, Meyer R, McCabe LR. 2005. Increased bone adiposity and peroxisomal proliferator-activated receptor-γ2 expression in type I diabetic mice. Endocrinology 146:3622–3631.

Cho SW, Yang JY, Her SJ, Choi HJ, Jung JY, Sun HJ, An JH, Cho HY, Kim SW, Park KS, Kim SY, Baek WY, Kim JE, Yim M, Shin CS. 2011. Osteoblast-targeted overexpression of PPARγ inhibited bone mass gain in male mice and accelerated ovariectomy-induced bone loss in female mice. J Bone Miner Res 26:1939–1952.

Choi EM, Kim YH. 2008. Hesperetin attenuates the highly reducing sugar-triggered inhibition of osteoblast differentiation. Cell Biol Toxicol 24:225–231.

Chuang CC, Yang RS, Tsai KS, Ho FM, Liu SH. 2007. Hyperglycemia enhances adipogenic induction of lipid accumulation: involvement of extracellular signal-regulated protein kinase 1/2, phosphoinositide 3-kinase/Akt, and peroxisome proliferator-activated receptor γ signaling. Endocrinology 148:4267–4275.

García-Hernández A, Arzate H, Gil-Chavarría I, Rojo R, Moreno-Fierros L. 2011. High glucose concentrations alter the biomineralization process in human osteoblastic cells. Bone 50:276–288.

Gopalakrishnan V, Vignesh R, Arunakaran J, Aruldhas M, Srinivasan N. 2006. Effects of glucose and its modulation by insulin and estradiol on BMSC differentiation into osteoblastic lineages. Biochem Cell Biol 84:93–101.

Hamada Y, Fujii H, Fukagawa M. 2009a. Role of oxidative stress in diabetic bone disorder. Bone 45:35–38.

Hamada Y, Fujii H, Kitazawa R, Yodoi J, Kitazawa S, Fukagawa M. 2009b. Thioredoxin-1 overexpression in transgenic mice attenuates streptozotocin-induced diabetic osteopenia: a novel role of oxidative stress and therapeutic implications. Bone 44:936–941.

Hamann C, Goettsch C, Mettelsiefen J, Henkenjohann V, Rauner M, Hempel U, Bernhardt R, Fratzl-Zelman N, Roschger P, Rammelt S, Günther K, Hofbauer LC. 2011. Delayed bone regeneration and low bone mass in a rat model of insulin-resistant type 2 diabetes mellitus is due to impaired osteoblast function. Am J Physiol Endocrinol Metab 301:E1220–E1228.

Hamann C, Rauner M, Höhna Y, Bernhardt R, Mettelsiefen J, Goettsch C, Günther KP, Stolina M, Han CY, Asuncion FJ, Ominsky MS, Hofbauer LC. 2013. Sclerostin antibody treatment improves bone mass, bone strength, and bone defect regeneration in rats with type 2 diabetes mellitus. J Bone Miner Res 28:627–638.

Lecka-Czernik B, Gubrij I, Moerman EJ, Kajkenova O, Lipschitz DA, Manolagas SC, Jilka RL. 1999. Inhibition of Osf2/Cbfa1 expression and terminal osteoblast differentiation by PPARγ2. J Cell Biochem 74:357–371.

Li Y, Liu H, Lv Y, Wang D. 2012. Effect of insulin on prohibitin in the osteogenic differentiation of rat′s mandibular bone marrow stromal cells in high glucose. Shanghai J Stomatol 21:526–530.

Magun R, Burgering B, Coffer P, Pardasani D, Lin Y, Chabot J, Sorisky A. 1996. Expression of a constitutively activated form of protein kinase B (c-Akt) in 3T3-L1 preadipose cells causes spontaneous differentiation. Endocrinology 137:3590–3593.

McCabe LR. 2007. Understanding the pathology and mechanisms of type I diabetic bone loss. J Cell Biochem 102:1343–1357.

Schwartz AV. 2003. Diabetes mellitus: does it affect bone? Calcif Tissue Int 73:515–519.

Strotmeyer ES, Cauley JA. 2007. Diabetes mellitus, bone mineral density, and fracture risk. Curr Opin Endocrinol Diab Obes 14:429–435.

Tomiyama K, Nakata H, Sasa H, Arimura S, Nishio E, Watanabe Y. 1995. Wortmannin. Biochem Biophys Res Commun 212:263–269.

Wang W, Zhang X, Zheng J, Yang J. 2010. High glucose stimulates adipogenic and inhibits osteogenic differentiation in MG-63 cells through cAMP/protein kinase A/extracellular signal-regulated kinase pathway. Mol Cell Biochem 338:115–122.

Xia X, Serrero G. 1999. Inhibition of adipose differentiation by phosphatidylinositol 3-kinase inhibitors. J Cell Physiol 178:9–16.

Zhen D, Chen Y, Tang X. 2010. Metformin reverses the deleterious effects of high glucose on osteoblast function. J Diabetes Complications 24:334–344.